Quantification of *Cadophora luteo-olivacea* From Grapevine Nursery Stock and Vineyard Soil Using Droplet Digital PCR

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Abstract

Cadophora luteo-olivacea is the most prevalent *Cadophora* species associated with Petri disease and esca of grapevine. Accurate, early, and specific detection and quantification of *C. luteo-olivacea* are essential to alert growers and nurseries to the presence of the pathogens in soil and to prevent the spread of this pathogen through grapevine planting material. The aim of this study was to develop molecular tools to detect and quantify *C. luteo-olivacea* inoculum from environmental samples. Species specific primers based on the β -tubulin gene and a TaqMan probe for droplet digital PCR (ddPCR) and quantitative PCR (qPCR) were first developed to detect and quantify purified DNA of the target fungus. Specificity tests showed that the primers were able to amplify the *C. luteo-olivacea* DNA (20 isolates) while none of the 29 nontarget fungal species (58 isolates) tested were amplified. The ddPCR was

shown to be more sensitive compared with qPCR in the detection and quantification of *C. luteo-olivacea* at very low concentrations and was further selected to accurately detect and quantify the fungus from environmental samples. Twenty-five of the 94 grafting plants (26.6%) analyzed by ddPCR tested positive to *C. luteo-olivacea* DNA (>3 copies/ μ l). *C. luteo-olivacea* was barely detected from vineyard soils. The procedure employed in this study revealed the presence of the pathogen in symptomless vines, which makes implementation of this technique suitable for certification schemes of *C. luteo-olivacea*-free grapevine planting material.

Keywords: esca, droplet digital PCR, Petri disease, quantitative PCR, *Vitis vinifera*

The genus *Cadophora* was established by Lagerberg et al. (1927), with *Cadophora fastigiata* as the type species. Since then, a total of 14 species of *Cadophora* have been described (Crous et al. 2015, 2017, 2019; Gams 2000; Harrington and McNew 2003; Linnakoski et al. 2018; Travadon et al. 2015; Walsh et al. 2018). The known *Cadophora* species occur in several habitats such as soil (Aislabie et al. 2001; Arenz et al. 2006; Hujšlová et al. 2010; Kerry 1990), decaying wood (Arenz et al. 2006; Blanchette et al. 2004; Held et al. 2005; Morrell and Zabel 1985; Nilsson 1973), or as pathogens (Di Marco et al. 2004; Spadaro et al. 2011; Travadon et al. 2015) in plants. The most prominent diseases in which *Cadophora* spp. are involved are Petri disease and esca, which occur on young and mature grapevines, respectively, and are caused by a complex of fungi, often including several species of *Phaeoacremonium* and *Phaeomoniella chlamydospora* (Gramaje et al. 2018). Both diseases are characterized by the initial blockage of xylem vessels by fungal colonization or plant response to colonization (Edwards et al. 2007), leading to gradual dieback of the trunk and arms, and the eventual death and decline of the entire plants (Gramaje et al. 2018). *Cadophora* species isolated from grapevine include *C. luteo-olivacea*, *C. malorum*, *C. melinii*, *C. novi-eboraci*, *C. orientoamericana*, *C. spadices*, and *C. viticola*, with *C. luteo-olivacea* being the most prevalent species associated with diseased vines in California (Rooney-Latham 2005), Canada (Úrbez-Torres et al. 2014), Uruguay

(Navarrete et al. 2011), South Africa (Halleen et al. 2007), New Zealand (Manning and Mundy 2009), and Spain (Gramaje et al. 2011).

The biology and epidemiology of *C. luteo-olivacea* affecting grapevines remain poorly understood. This fungus has been isolated from both asymptomatic (Eichmeier et al. 2018; Halleen et al. 2007; Kraus et al. 2019) and symptomatic (Gramaje et al. 2009, 2011; Úrbez-Torres et al. 2014) grapevine wood in nursery planting material and young vineyards. In addition, this species was collected from symptomless vascular tissues of weeds collected in commercial vineyards (Agustí-Brisach et al. 2011), and from grapevine nursery fields using bait plants (Agustí-Brisach et al. 2013). In addition, several stages of the grapevine propagation process have been shown to be potential inoculum sources of this fungus (Gramaje et al. 2011).

Effective management of Petri disease and esca of grapevine demands the implementation of an integrated disease management (IDM) strategy that combines both preplanting and postplanting control measures to lead recovery from the diseases and to reduce the risk of pathogen spread to new vines (Gramaje et al. 2018). Use of *C. luteo-olivacea*-free grapevine graftlings to establish new vineyards in pathogen-free soils is a key factor of the IDM strategy (Gramaje et al. 2018). Therefore, accurate and early in planta detection and quantification of *C. luteo-olivacea* that ensure healthy propagation of grapevine planting material are crucial to avoid pathogen-infected vines from being planted and to facilitate the management of Petri disease and esca (Gramaje et al. 2018). Accurate in planta quantification of the fungal pathogen biomass would also facilitate the correct characterization of tolerance or resistance to Petri disease and esca in commercial grapevine germplasm collections.

In recent years, molecular techniques have been reported to be useful for detecting pathogens from different environmental samples because they are more sensitive and rapid than conventional techniques. In the case of *C. luteo-olivacea*, specific primers circumscribed within the internal transcribed spacer (ITS) region were developed for a rapid detection of *C. luteo-olivacea* from kiwi and pome fruits (Spadaro et al. 2011), as well as artificially inoculated stems of grapevine nursery planting stock (Navarrete et al. 2011).

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However, these primers were not specifically designed for quantitative polymerase chain reaction (qPCR). In addition, since 2011, several *Cadophora* species, some of them phylogenetically close to *C. luteo-olivacea* (e.g., *C. antarctica*, *C. malorum*, *C. meredithiae*), have been described based on a multilocus sequence analysis using the ITS, β -tubulin (BT), and translation elongation factor 1- α (EF) genes due to the ineffectiveness of the ITS region alone in resolving *Cadophora* species (Crous et al. 2015; Linnakoski et al. 2018; Travadon et al. 2015; Walsh et al. 2018).

Although qPCR is still one of the most powerful and sensitive gene analysis techniques and is used for a broad range of applications, droplet digital PCR (ddPCR) has recently emerged as the next generation of PCR technology overcoming several disadvantages of qPCR such as the dependence of the use of a DNA standard curve and the lower tolerance to some PCR inhibitors, different types of DNA templates, sequence variations, master mixes, and PCR assays (Cao et al. 2013; Hindson et al. 2011; Pavšič et al. 2016; Rački et al. 2014). Increased sensitivity of ddPCR over qPCR has been also recently reported in several studies (Bahder et al. 2018; Cavé et al. 2016; Kim et al. 2014; Martínez-Diz et al. 2020; Porcellato et al. 2016).

The first objective of this study was to design successful ddPCR and qPCR assays based on TaqMan technology that are capable of detecting *C. luteo-olivacea*. The second objective was to evaluate the overall sensitivity of ddPCR for detection of the fungus as compared with qPCR. The third objective was to quantify *C. luteo-olivacea* in grapevine nursery stock and vineyard soil using the most efficient molecular technique. The reliability and accuracy of the detection and quantification protocols are essential for their use in the certification of *C. luteo-olivacea*-free planting material and to improve the knowledge about the biology of the pathogen in the vineyard.

Materials and Methods

PCR primers and probe development. Sequences of the ITS noncoding DNA region of fungal ribosomal DNA (rDNA) and the BT gene were retrieved from GenBank (<https://www.ncbi.nlm.nih.gov/>) for seven *Cadophora* spp. known to infect grapevines, five *Cadophora* spp. isolated from other hosts (Supplementary Table S1), and 20 fungal species associated with grapevine trunk diseases (Supplementary Table S2). The 45 reference sequences for *Cadophora* spp. included *C. antarctica* (1), *C. fastigiata* (1), *C. finlandica* (1), *C. helianthi* (3), *C. luteo-olivacea* (12), *C. malorum* (5), *C. melinii* (3), *C. meredithiae* (6), *C. novi-eboraci* (4), *C. orientoamericana* (2), *C. spadicis* (4), and *C. viticola* (3) (Supplementary Table S1). All sequences were aligned with ClustalW using MEGA 7 (Kumar et al. 2016) to identify potential binding sites for *C. luteo-olivacea*. The designed primers were tested for their theoretical specificity to the target fungal species using Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>) and MEGA 7 prior to in vitro testing.

Fungal isolates. Fifty-three isolates representing nine *Cadophora* spp. were used to test the specificity of the designed primers to *C. luteo-olivacea* by conventional PCR (Supplementary Table S3). These isolates were previously identified to species by DNA sequencing of the ITS region, and partial regions of the BT and EF genes (Crous et al. 2019; Eichmeier et al. 2020; Gramaje et al. 2011; Travadon et al. 2015; Úrbez-Torres et al. 2014). In addition, 35 fungal isolates of 21 other nontarget species collected from grapevine or weed were also used in this study (Supplementary Table S4). The identities of these nontarget fungal species were confirmed by DNA sequencing of the ITS region and partial region of the BT gene.

DNA extraction from pure cultures. Fungal mycelium of all target and nontarget species was homogenized with four steel beads of 1.4 mm diameter (Qiagen, Hilden, Germany) using a FastPrep-24TM5G (MP Biomedicals, Irvine, CA, U.S.A.) at 5 m/s for 20 s twice. Total DNA was then extracted using the E.Z.N.A. Plant Miniprep kit (Omega Bio-Tek, Doraville, GA, U.S.A.) following the manufacturer's instructions. DNA integrity and quality were assessed by 1% agarose gel (agarose D1 Low EEO, Conda, Madrid, Spain)

electrophoresis visualizing the samples previously stained with RedSafe Nucleic Acid Staining Solution (20,000 $\times$) (iNtRON Biotechnology, Lynnwood, WA, U.S.A.). DNA samples were quantified using the Invitrogen Qubit 4 Fluorometer with Qubit dsDNA HS (High Sensitivity) Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Primer optimization and specificity test by conventional PCR.

A gradient PCR was conducted on a BioRad T100 Thermal Cycler (BioRad, Mississauga, ON, Canada) to obtain the optimum annealing temperature with the following thermal cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s annealing (gradient run of 50 to 60°C), 30 s at 72°C, and a final extension of 72°C for 7 min. The 25 μ l PCR reactions contained 0.4 μ M of each primer, 12.5 μ l of 2 $\times$ FastGene Taq ReadyMix PCRKit, and 20 ng of extracted DNA. PCR products were separated by electrophoresis in a 1% agarose gel (Conda) stained with RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology). Bands were observed under UV light using the Molecular Imager Chemi-Doc XRS (Bio-Rad Laboratories, Pty, Ltd., Hercules, CA, U.S.A.).

Isolate selection and DNA serial dilutions. *C. luteo-olivacea* isolate BV-0577 was obtained from the culture collection of the Instituto de Ciencias de la Vid y del Vino (ICVV). Total fungal DNA was extracted and quantified as described before. Seven-fold dilution series were prepared, ranging from 1,000 to 1 fg μ l⁻¹ of DNA, to generate DNA standard curve by ddPCR and qPCR. Three independent DNA standard curves were obtained using separate pathogen DNA sources that were treated as independent experiments.

TaqMan assay design and ddPCR parameters. ddPCR was performed on a Bio-Rad QX200 system using a TaqMan assay. A probe was designed using the PrimerQuestDesign Tool (IDT) and labeled at the 5' end with Hexachloro-6-carboxyfluorescein (HEX) and a double-quencher (internal ZEN with 3' Iowa Black FQ). Each reaction (20 μ l) contained 1 $\times$ Supermix for Probes (Bio-Rad), 750 nM of each primer, 250 nM of the probe, and 2 μ l of DNA template. The PCR reactions were mixed, centrifuged briefly, and 20 μ l transferred into the sample well of a DG8 cartridge (Bio-Rad). After adding 70 μ l of QX200 droplet generation oil (Bio-Rad) into the oil wells, the cartridge was covered using a DG8 gasket, and droplets generated using the QX200 droplet generator (Bio-Rad). Droplets were carefully transferred into PCR plates using a multichannel pipette and the plate sealed using PCR plate heat seal foil and the PX1 PCR plate sealer (Bio-Rad). A thermal-gradient PCR experiment was conducted to establish the optimal annealing temperature for the primers. Temperatures tested were 52.9, 55.0, 56.7, 59.4, 61.7, 63.3, and 64°C. PCR was performed in a C1000 touch thermal cycler (Bio-Rad) using the following PCR thermal cycling conditions: initial denaturation stage of 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, optimal annealing temperature (55°C) for 60 s, and a final incubation of 10 min at 98°C. PCR plates were transferred into a QX200 droplet reader (Bio-Rad) and reads were analyzed using QuantaSoft software (Bio-Rad). DNA of *C. luteo-olivacea* isolate BV-0577 was used as a template.

Real-time PCR assay parameters. Real-time PCR assays were performed on a Bio-Rad CFX384 system (Bio-Rad) using the same primers and TaqMan probe as described above. Each reaction contained 2 μ l of DNA template, 5 μ l of 1 $\times$ Supermix for Probes (Bio-Rad), 500 nM of probe, and 750 nM of each primer. The reaction mix was adjusted to a final volume of 10 μ l with sterile distilled water. Thermal cycling conditions included 10 min of initial denaturation at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C, and annealing at 55°C for 60 s. Both ddPCR and qPCR were performed at BIODONOSTIA Health Research Institute (San Sebastián, Spain).

Standard curves and limit of detection determination. DNA of *C. luteo-olivacea* was quantified using a standard curve constructed with DNA dilution series of the isolate BV-0577. Analyses were performed as previously described and the standard curve was generated following the MIQE guidelines (Bustin et al. 2009). The quantified target copy number (copies μ l⁻¹) (ddPCR) values obtained for each specific isolate DNA dilution were plotted against

the concentration ($\text{fg } \mu\text{L}^{-1}$) of each isolate DNA dilution. Regarding qPCR, the quantification cycle (Cq) values were plotted against the logarithm of the concentration ($\text{fg } \mu\text{L}^{-1}$). The standard deviation and the mean DNA concentration were determined from 12 replicates per dilution.

Sensitivity of ddPCR and qPCR was assessed by estimating the limit of detection (LOD) of both techniques, using the resulting standards curves to determine the minimum DNA concentration that can be detected in three consecutive assays. The coefficient of determination (R^2) of the standard curve were obtained using the specific software of each system, QuantaSoft (Bio-Rad) for ddPCR and CFX Maestro (Bio-Rad) for qPCR. The levels of signal threshold were set automatically by the analyzer software and the LOD was determined by the last dilution in which successful amplification of all DNA replicates occurred.

Estimator of tolerance to inhibitors. The tolerance of ddPCR and qPCR to soil and wood DNA inhibitors was estimated with reactions containing different inhibitor concentrations. DNA of a soil sample that tested negative to *C. luteo-olivacea* by ITS high-throughput amplicon sequencing was used (Berlanas et al. 2019). DNA of a wood sample was extracted from asymptomatic xylem tissue of a grapevine rootstock cutting. Soil and wood DNA stock samples were obtained as mentioned below and adjusted to 12 and 2 $\text{ng}/\mu\text{L}$, respectively. Inhibitors were spiked into the same amount of *C. luteo-olivacea* DNA at appropriate volumes such that the inhibitor concentrations were 12 (stock), 6 (1/2 dilution), 3 (1/4 dilution), 1.2 (1/10 dilution), 0.12 (1/100 dilution), and 0.012 (1/1,000 dilution) $\text{ng}/\mu\text{L}$ for soil DNA, and 2 (stock), 1 (1/2 dilution), 0.5 (1/4 dilution), 0.2 (1/10 dilution), 0.02 (1/100 dilution), and 0.002 (1/1,000 dilution) $\text{ng}/\mu\text{L}$ for wood DNA. The percentage of inhibition for each sample was calculated as the ratio of the target concentration in the presence of different amounts of inhibitors. Thus, 100% inhibition represented total inhibition (no positive signal) and 0% represented no inhibition. Double-distilled water plus *C. luteo-olivacea* DNA was used as a control. Four replicates were used in the experiment. For the qPCR results, copy number was calculated with the following formula: $(\text{DNA amount (g)} \times 6.022 \times 10^{23} \text{ (copy/mol)}) / (\text{DNA length (bp)} \times 660 \text{ (g/mol/bp)})$ (Lee et al. 2016), where DNA amount was the concentration of DNA (g) and DNA length was the length of *C. luteo-olivacea* BV-0577 genome, 55 Mbp (unpublished data).

Environmental sample collection. A total of 94 one-year-old dormant grapevine grafted plants of ‘Tempranillo’/110 Richter (110 R) combination ready to be sold to growers with uniform root distribution of length 8 cm were obtained from a nursery in Navarra (northern Spain) and used in this experiment. The basal, mid, and apical parts of the rootstock were examined. Segments of 3 cm long were cut, washed under running tap water, surface sterilized in 33% sodium hypochlorite (commercial 40 g Cl/liter) for 1 min, and rinsed twice with sterile distilled water. Upon this treatment, bark was carefully peeled, and approximately 10 mg of small pieces of necrotic xylem tissues for each plant part were collected and placed into 2-ml tubes for DNA extraction. DNA was extracted from the 10 mg using the i-genomic Plant DNA Extraction Mini Kit (Intron Biotechnology, South Korea).

Grapevine soil samples were collected from 11 young vineyards in La Rioja (Spain) and four young vineyards in Galicia (Spain) (Supplementary Table S5). La Rioja vineyards were grown with ‘Tempranillo’ grafted onto 110 R rootstock. In Galicia vineyards, the cultivars were ‘Treixadura’ (soils 12, 14, and 15) or ‘Brancellao’ (soil 13) grafted onto 110 R rootstock. In each vineyard, two different sample types were studied at flowering (June 2017): soil surrounding roots (rhizosphere) and bulk soil. Three plants per vineyard were randomly selected and three samples were collected from each soil-plant fraction (bulk soil and rhizosphere). Sampled vines did not show any symptom of nutrient deficiency or disease. A total of 90 samples were collected.

For bulk soil samples, a sterile spade at 1 m from each stem (depths of 40 to 50 cm) was used. Soil was homogenized and passed through a 1 mm pore size sieve and split into two subsamples, each one for *C. luteo-olivacea* detection and quantification, and soil

chemistry analyses, respectively. Soil samples from the rhizosphere were collected with a sterile spade close to the stem at depths of 40 to 50 cm. All samples were then stored on dry ice in sterile bags at the sampling time and brought to the laboratory for further processing within 24 h after the sampling time. Sampled roots (5 g) with rhizosphere soil particles attached were put in sterile tubes with 9 ml of physiological solution (9 g/liter NaCl). They were vortexed for 5 min to detach the soil particles and immediately centrifuged at 4,000 rpm for 5 min. The supernatant was removed, and the remaining fraction of soil was employed to represent the rhizosphere compartment. The rhizosphere and bulk soil DNA was extracted from 0.5 g sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany).

Quantification of *C. luteo-olivacea* on environmental samples. The 94 grapevine samples collected from a nursery and the 90 soil samples collected at 15 young vineyards in La Rioja and Galicia were analyzed by the most efficient quantification technology according to the limit of detection and the tolerance to inhibitors. All samples along with a nontemplate control (NTC) reaction (water) and three positive controls containing DNA of a soil and a grapevine wood sample tested positive to *C. luteo-olivacea* by ITS high-throughput amplicon sequencing and DNA extracted from a pure culture of *C. luteo-olivacea* isolate BV-0577 were run by ddPCR in triplicate. Wells with less than three positive droplets were considered negative according to BIORAD recommendation (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf). DNA template from environmental samples was standardized to 10 $\text{ng } \mu\text{L}^{-1}$.

Results

Primer development and validation. The design of the primers for *C. luteo-olivacea* was based on the alignment of the BT gene sequences. In the case of the ITS region, no binding sites suitable for qPCR/ddPCR primers were identified. The primers CloBT-F (5'-GAATATATGCAGACTCATTCAC-3') and CloBT-R (5'-AATATTAGGATTTTGTTCATGT-3') were designed to anneal to the BT gene region for all target *C. luteo-olivacea* isolates. Conventional PCR showed that the optimal annealing temperature for the combination of both primers was 51°C, obtaining an amplicon product of 153 bp when all *C. luteo-olivacea* DNA was tested (Supplementary Table S3). No PCR products were observed with the DNA of the 29 nontarget species (Supplementary Tables S3 and S4) and the NTC (Supplementary Fig. S1).

Detection and quantification limit by ddPCR and qPCR. A TaqMan probe was developed for its use in both molecular techniques (HEX-5'-CC AACTCGA/ZEN/GCGCCTGAACGT/3IABkFQ/-3'). The optimal annealing temperature for primers using pure culture *C. luteo-olivacea* BV-0577 DNA in ddPCR (Fig. 1) and qPCR was established at 55°C. Both methods showed good linearity within the quantification range with high coefficient of determination (R^2) of 0.9235 and 0.9190. The minimum target concentration detectable was 25 $\text{fg}/\mu\text{L}$ for ddPCR and 125 $\text{fg}/\mu\text{L}$ for qPCR (Fig. 2); thus, the limit of detection (LOD) was established at these concentrations for each technique. The NTC showed no positive amplification.

Influence of inhibitors on the ddPCR assay. The resilience of ddPCR and qPCR assays to DNA samples is shown in Figure 3. In the soil DNA test, the suppression curve of ddPCR was beneath that of qPCR. At the lowest dilution, the percentages of inhibition were -7 and 20.1% for ddPCR and qPCR, respectively. The soil DNA stock sample (12 $\text{ng}/\mu\text{L}$) reached 32 and 73% inhibition in ddPCR and qPCR, respectively. No inhibition was found in wood DNA samples. No amplification was found in control samples (double-distilled water plus *C. luteo-olivacea* DNA).

Quantification of *C. luteo-olivacea* from environmental samples. DNA of *C. luteo-olivacea* was detected in 76 of the 94 grafting plants (80.8%) analyzed by ddPCR (Supplementary Table S6). Of those, 25 grafted plants (26.6%) had at least three positive droplets and were called a sample positive (Supplementary Table S7). Fungal concentrations in these positive plants ranged from 3.05 to 116.5 copies/ μL (Supplementary Table S6). Only one rhizosphere soil sample tested positive to *C. luteo-olivacea* DNA (8 copies/ μL). DNA concentration and purity values of each soil and rhizosphere sample

are shown in Supplementary Table S8. Positive controls containing DNA from soil or grapevine wood were positive to *C. luteo-olivacea*. No positive droplets occurred using NTC.

Discussion

The present study represents the first approach to assess ddPCR and qPCR as reliable tools to detect and quantify the fungal pathogen *C. luteo-olivacea*. Specificity tests demonstrated that the primers developed in this study were able to amplify the *C. luteo-olivacea* DNA, while no PCR products were observed with the DNA of all the other nontarget fungal species tested, including other fungal trunk pathogens of grapevine. For this study, we selected the BT gene for the experiments because it occurs as a single-copy gene in the fungal genome. In contrast to the ITS, this number is not likely to vary between different isolates within a species (Herrera et al. 2009; Longo et al. 2013). The use of BT gene for the design of the *C. luteo-olivacea*-specific primer set is suitable because of the high inter-specific variability of the *Cadophora* genus for this gene (Travadon et al. 2015).

The ddPCR showed to be more sensitive compared with qPCR for the detection and quantification of this fungus at very low concentrations. Therefore, ddPCR was selected to accurately detect and quantify *C. luteo-olivacea* from environmental samples. The better performance of ddPCR over qPCR is in accordance with existing reports (Bahder et al. 2018; Cavé et al. 2016; Kim et al. 2014; Martínez-Diz et al. 2020; Porcellato et al. 2016). ddPCR is the latest DNA quantification technology being used widely in several scientific fields (Hussain et al. 2016; Morisset et al. 2013; Palumbo et al.

2016; Porcellato et al. 2016; Yang et al. 2014), including plant pathology (Bahder et al. 2018, 2019; Maheshwari et al. 2017; Martínez-Diz et al. 2020; Pandey et al. 2020; Selvaraj et al. 2019; Voegel and Nelson 2018). Developing a robust ddPCR assay with increased sensitivity over qPCR would be beneficial to diagnostic laboratories and researchers by identifying early infections in soil and grapevine planting material. ddPCR has additional benefits, such as the absolute quantitative target detection without the need for standard curve construction (Yang et al. 2014), obviating the preparation of reference DNA templates (Kim et al. 2014), and that the inhibitory substances had little effect on DNA quantification using this technique (Hoshino and Inagaki 2012). These features and the results obtained in the present study make ddPCR an attractive tool for measuring environmental samples allowing a better understanding and monitoring of *C. luteo-olivacea* in the future.

The ddPCR assay developed in this study was successful in detecting *C. luteo-olivacea* DNA from necrotic xylem tissues of externally healthy grapevine grafted plants ready to be sold to growers. This finding highlights the urgent need to implement early, accurate, and specific in planta detection and quantification of *C. luteo-olivacea* to prevent the spread of Petri disease in grapevine propagation material. *C. luteo-olivacea* was barely detected from vineyard soils. This finding is in agreement with previous research performed in Spain (Agustí-Brisach et al. 2011, 2013). Three strains of this fungus were previously isolated from symptomless vascular tissues of two weeds collected in commercial vineyards (Agustí-Brisach et al. 2011), and only four strains were collected from grapevine nursery fields using bait plants (Agustí-Brisach et al. 2013). The presence of *Cadophora*

Fig. 1. A gradient droplet digital PCR (ddPCR) assay was used to determine the optimal annealing temperature for the primer pair using pure culture *Cadophora luteo-olivacea* isolate BV-0577 DNA. The bold horizontal line represents the threshold, defining positive droplets above (ddPCR amplification) and negative droplets below (lack of amplification). A temperature of 55°C resulted in the best separation between positive and negative droplets. NTC = negative template control.

Fig. 2. Standard curves obtained of *Cadophora luteo-olivacea* isolate BV-0577 DNA dilutions ranging from 1,000 to 25 fg μL^{-1} . The concentration of each isolate DNA dilution was plotted against target DNA concentration (copy number μL^{-1}) in order to construct the ddPCR standard curve (top). For the qPCR standard curve (bottom), the logarithm of the concentration of each isolate DNA dilution was plotted against the quantification cycle (Cq). Data points represent amplification results of 12 replicates.

Fig. 3. Influence of samples spiked with serial dilutions of two inhibitors on quantification of *Cadophora luteo-olivacea* isolate BV-0577 by ddPCR and qPCR. A 100% inhibition represents a completely suppressed reaction (no positive signal) and a 0% inhibition indicates no suppression. The measurements were carried out in duplicate.

spp. in soils has been recently confirmed using ITS high-throughput amplicon sequencing approach (Martínez-Diz et al. 2019); however, the lack of resolution of the ITS for *Cadophora* spp. made difficult to accurately assign operational taxonomic units to species level.

At high soil DNA concentrations, ddPCR showed greater tolerance of soil DNA inhibitors compared with qPCR. This finding makes ddPCR highly applicable and promising in challenging and complex scenarios such as soil-borne pathogen analysis, where high fungal microbiome diversity leads to potentially high inhibitor concentrations. By diluting the soil DNA stock, quantified *C. luteo-olivacea* copy number from qPCR and ddPCR became similar, indicating that dilution was a useful way to remedy PCR inhibition. Both ddPCR and qPCR showed high tolerance against wood DNA inhibitors and were not inhibited when 2 ng of DNA extracted from vine wood was used as a template. We therefore conclude that our sampling and DNA extraction method allows absolute quantification of *C. luteo-olivacea* DNA within total DNA extracts from grapevine wood samples.

In conclusion, we developed for the first time a robust ddPCR protocol targeting the BT gene for the specific detection and quantification of *C. luteo-olivacea* from grapevine nursery stock and vineyard soil. The procedure used in this study revealed the presence of the pathogen in symptomless plants, which makes implementation of this technique suitable for certification schemes of *C. luteo-olivacea*-free planting material.

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