Droplet Digital PCR Technology for Detection of *Ilyonectria liriodendri* from Grapevine Environmental Samples

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Abstract

Black-foot disease is one of the most important soilborne diseases affecting planting material in grapevine nurseries and young vineyards. Accurate, early, and specific detection and quantification of black-foot disease causing fungi are essential to alert growers and nurseries to the presence of the pathogens in soil, and to prevent the spread of these pathogens through grapevines using certified pathogen-free planting material and development of resistance. We comparatively assessed the accuracy, efficiency, and specificity of droplet digital PCR (ddPCR) and real-time PCR (qPCR) techniques for the detection

and quantification of *Ilyonectria liriodendri* in bulk and rhizosphere soils, as well as grapevine endorhizosphere. Fungal abundance was not affected by soil-plant fractions. Both techniques showed a high degree of correlation across the samples assessed ($R^2 = 0.95$) with ddPCR being more sensitive to lower target concentrations. Roots of asymptomatic vines were found to be a microbial niche that is inhabited by black-foot disease fungi.

Keywords: fungi, fruit, tree fruits, pathogen detection

Soilborne pathogens can establish a parasitic relationship with their host plants in the rhizosphere. To infect roots, pathogens have to compete with other microbial organisms of the rhizosphere for available microsites and nutrients (Chapelle et al. 2016). To date, the complex grapevine-soilborne pathogen interactions in the rhizosphere are not fully understood. Among diseases caused by soilborne pathogens in grapevine, black-foot has received much attention in recent decades from plant pathologists as it has been implicated in contributing to young grapevine decline syndrome (Gramaje and Armengol 2011). *Cylindrocarpon*-like asexual morphs belonging to the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria* have been associated with black-foot disease (Agustí-Brisach and Armengol 2013; Carlucci et al. 2017; Lombard et al. 2014). The genus *Ilyonectria* represents one of several newly established genera of fungi with *Cylindrocarpon*-like anamorphs (Chaverri et al. 2011), with *Ilyonectria liriodendri* being one of the most prevalent causal agents of black-foot disease (Agustí-Brisach and Armengol 2013).

Internal symptoms of black-foot diseased vines usually range from black, necrotic, sunken lesions on roots to reddish brown discoloration in the base of the rootstock (Halleen et al. 2006). Foliar symptoms associated with black-foot disease are practically indistinguishable from those observed in Petri disease affected vines and include delayed bud break, chlorotic foliage with necrotic margins,

overall stunting, and wilting of leaves or entire shoots (Agustí-Brisach and Armengol 2013). These symptoms may also resemble those associated with abiotic disorders such as spring frost, winter damage, nutrient deficiency, and/or water stress (Gramaje et al. 2018).

Black-foot disease is particularly important in grapevine nurseries and new plantations. *Cylindrocarpon*-like asexual morphs produce conidia, and some species also produce chlamydospores in culture, which indicates that those propagules are likely to be produced on stem bases of infected vines and the diseased roots. The conidia are spread in soil water and the chlamydospores can allow these pathogens to survive in the soil for extended periods of time (Petit et al. 2011). Infection can occur through the small wounds made when roots break off during the planting process, through the incomplete callusing of the lower trunk, or through wounds made in the grapevine propagation process, such as disbudding wounds, from which the infection progresses downward to the base of the trunk (Halleen et al. 2006).

Traditionally, detection and identification of black-foot disease fungi in grapevine has been performed by morphological approaches (Chaverri et al. 2011) or by multiplex PCR system (Alaniz et al. 2009). Although reliable for a preliminary identification and classification, these techniques are not practical to detect low levels of black-foot pathogens that are anticipated during early stages of infection. Recently, real-time PCR (qPCR) has become a useful technique for increasing the sensitivity and specificity for detecting and quantifying *Cylindrocarpon*-like asexual morphs (Agustí-Brisach et al. 2014; Langenhoven et al. 2018; Tewoldemedhin et al. 2011). The digital PCR (ddPCR) has only recently been adapted to detect plant pathogens in agricultural systems from biomedical disciplines where it showed to be a more sensitive technology compared with qPCR (Bahder et al. 2016, 2018; Dreo et al. 2014; Miotke et al. 2014; Rački et al. 2014).

The objectives of this study were therefore: i) to design a ddPCR protocol that is capable to detect and quantify *I. liriodendri* in soil and roots, ii) to evaluate the overall sensitivity of ddPCR for detection of *I. liriodendri* compared with qPCR, and iii) to compare the abundance of *I. liriodendri* in different habitats inside and outside of grapevine roots.

Materials and Methods

Fungal isolate selection and DNA serial dilutions. *I. liriodendri* isolate BV-0596 was obtained from the culture collection of the

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

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Instituto de Ciencias de la Vid y del Vino (ICVV) (Spain). Fungal mycelium and conidia from pure cultures grown on potato dextrose agar for 2 to 3 weeks at 25°C in the dark were scraped and homogenized in 2-ml tubes with 600 µl of P1 buffer of the kit E.Z.N.A.

Plant Miniprep kit (Omega Bio-tek, Norcross, GA, U.S.A.) with 4 steel beads of 2.38 mm and 2 of 3 mm diameter (Qiagen, Hilden, Germany) using a FastPrep-24TM5G (MP Biomedicals, California, U.S.A.) at 5 m/s for 20 s twice. DNA integrity and quality were

Fig. 1. Standard curves obtained using *Ilyonectria lirioidendri* isolate BV-0596 DNA dilutions ranging from 10,000 to 1 $\text{fg } \mu\text{l}^{-1}$. The logarithm of the concentration of each isolate DNA dilution was plotted against: **A**, Target DNA concentration (copy number μl^{-1}), in order to construct the ddPCR standard curve; **B**, Quantification cycle (Cq), in order to construct the qPCR standard curve. Data points represent amplification results of five replicates. The reaction efficiency was 0.83 and 0.97% for ddPCR and qPCR analysis, respectively.

assessed by gel electrophoresis visualizing the samples previously stained with RedSafe (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.). Serial dilutions ranging from 10,000 to 1 fg μL^{-1} of the DNA were prepared for quantification purposes 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. Digital Droplet PCR (ddPCR) was performed on a Bio-Rad QX200 system using a TaqMan assay. A probe was designed using the PrimerQuestDesign Tool (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.) and labeled at the 5' end with Hexachloro-6-carboxyfluorescein (HEX) and a double-quencher (internal ZEN with 3' Iowa Black FQ). The probe sequence is 5'-HEX/TCCGAGCGT/ZEN/CATTT CAACCCTCAA/3IABKFQ/-3'. Primers YT2F (Tewoldemedhin et al. 2011) and Cyl-R (Dubrovsky and Fabritius 2007) were used in the experiment. These primers amplify the main *Cylindrocarp*-like asexual morphs associated with black-foot disease, in particular those belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria*. Each reaction contained 1× Supermix for Probes (Bio-Rad Laboratories, Hercules, CA, U.S.A.), 20 μM of each forward and reverse primer solution (final concentration 750 nM for each primer), 10 μM of the probe, and 2 μL of DNA template resulting in a final volume of 20 μL . The PCR reactions were mixed, centrifuged briefly, and 20 μL transferred into the sample well of a DG8TM cartridge (Bio-Rad). After adding 70 μL of QX200TM droplet generation oil (Bio-Rad Laboratories) into the oil wells, the cartridge was covered using a DG8TM gasket, and droplets generated using the QX200TM droplet generator (Bio-Rad Laboratories). Droplets were carefully transferred into PCR plates using a multi-channel pipette, and the plate was sealed using PCR plate heat seal foil and the PX1TM PCR plate sealer (Bio-Rad Laboratories).

Table 1. Droplet digital PCR and Quantification Cycle (Cq) obtained in real-time PCR average data for the serial dilutions of *Ilyonectria liriodendri* isolate BV-0596 DNA ($n = 5$). Values represent the mean $\pm$ SE.

DNA concentration (fg μL^{-1})	ddPCR (copies μL^{-1})	qPCR (Cq)
10,000	41.3 $\pm$ 0.98	26.0 $\pm$ 0.10
1,000	30.3 $\pm$ 0.32	29.3 $\pm$ 0.07
100	17.3 $\pm$ 0.36	32.6 $\pm$ 0.20
50	14.0 $\pm$ 0.84	33.9 $\pm$ 0.13
10	4.5 $\pm$ 0.45	36.3 $\pm$ 0.29
5	1.2 $\pm$ 0.16	N/A
1	N/A	N/A

PCR was performed in a C1000 touch thermal cycler (Bio-Rad Laboratories) using the following thermal cycling conditions: initial denaturation stage of 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s and annealing temperature (59°C) for 60 s, and a final extension of 10 min at 98°C. PCR plates were transferred into a QX200TM droplet reader (Bio-Rad Laboratories) and reads analyzed using QuantaSoftTM software (Bio-Rad Laboratories). A thermal-gradient PCR experiment was conducted to establish the optimal annealing temperature for the primers using DNA of *I. liriodendri* isolate BV-0596 as a template.

Real-time PCR assay parameters. Real-time PCR assays were performed on a CFX384 real time PCR system (Bio-Rad Laboratories) using the same primers and TaqMan probe as described above. Each reaction contained 2 μL of DNA template, 5 μL of 1× Supermix for Probes (Bio-Rad), containing 500 nM of probe and 750 nM of each primer. The reaction mix was adjusted to a final volume of 10 μL with sterile distilled water. Thermal cycling conditions were as follows: 10 min of initial denaturation at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C and annealing at 62°C for 60 s. Both ddPCR and qPCR were performed at BIODONOSTIA Health Research Institute (San Sebastián, Spain).

Environmental sample collection. Grapevine samples were collected at five young vineyards of Tempranillo cultivar grafted onto 110 Richter rootstock maintained in La Rioja (Spain) located between 2.2 and 14.9 km distance from each other (Supplementary Table S1). These vineyards were under similar soil, climatic, and management conditions. In each vineyard, three different sample types were studied in June 2017 (flowering): bulk soil, soil surrounding roots (rhizosphere), and roots (endorrhizosphere). Four plants per vineyard were chosen to represent the same aspect of the plant and position within the vineyard, and four samples were randomly collected from each soil-plant fraction (bulk, rhizosphere, and endorhizosphere). Sampled vines did not show any symptom of disease or nutrient deficiency, and root tissue did not have any rot or necrosis that could be associated to black-foot or other diseases caused by soilborne pathogens. A total of 60 samples were collected.

Bulk soil samples were collected with a sterile spade 1 m from each stem at depths of 40 to 50 cm. Homogenized dry soil was then passed through a 1-mm-pore size sieve and divided into two subsamples, each one for *I. liriodendri* detection and quantification, and soil chemistry analyses, respectively. Roots and rhizosphere soil samples were collected with a sterile spade close to the stem at depths of 40 to 50 cm, where the root system was denser. All samples were stored on dry ice in sterile bags at the time of sampling and brought to the laboratory for further processing within 24 h from the time of sampling. A total of 5 g of the sampled roots with rhizosphere soil particles attached were placed in sterile tubes containing 9 ml of physiological

Table 2. Droplet profile and digital PCR quantitation data from DNA extracts of the environmental samples from five vineyards ($n = 4$). Values represent the mean $\pm$ SE from four replicates.

	Bulk soil			Rhizosphere soil			Endorhizosphere		
	Accepted droplets	(+) Droplets	Copies μL^{-1}	Accepted droplets	(+) Droplets	Copies μL^{-1}	Accepted droplets	(+) Droplets	Copies μL^{-1}
Vineyard 1	17,400 $\pm$ 930	417 $\pm$ 143	293 $\pm$ 103	16,400 $\pm$ 1,420	1,250 $\pm$ 723	889 $\pm$ 495	16,200 $\pm$ 971	482 $\pm$ 75	370 $\pm$ 79
Vineyard 2	12,400 $\pm$ 1,570	194 $\pm$ 44.0	183 $\pm$ 28	11,800 $\pm$ 1,480	442 $\pm$ 147	441 $\pm$ 147	11,500 $\pm$ 1,230	285 $\pm$ 123	321 $\pm$ 166
Vineyard 3	18,100 $\pm$ 606	2,910 $\pm$ 784	2,090 $\pm$ 610	18,500 $\pm$ 751	2,270 $\pm$ 1,210	1,820 $\pm$ 1,150	18,500 $\pm$ 670	3,410 $\pm$ 2,360	2,700 $\pm$ 2,000
Vineyard 4	16,600 $\pm$ 653	3,320 $\pm$ 1,820	3,190 $\pm$ 2,100	18,200 $\pm$ 282	1,830 $\pm$ 649	1,260 $\pm$ 453	16,700 $\pm$ 604	2,770 $\pm$ 1,000	2,300 $\pm$ 894
Vineyard 5	14,900 $\pm$ 1,160	833 $\pm$ 445	624 $\pm$ 298	16,000 $\pm$ 756	925 $\pm$ 561	722 $\pm$ 428	16,400 $\pm$ 755	640 $\pm$ 126	468 $\pm$ 96
(+) control ^a	19,200 $\pm$ 0	20 $\pm$ 0	12 $\pm$ 0	19,200 $\pm$ 0	20 $\pm$ 0	12 $\pm$ 0	19,200 $\pm$ 0	20 $\pm$ 0	12 $\pm$ 0
(+) control ^b	16,400 $\pm$ 0	411 $\pm$ 0	299 $\pm$ 0	16,400 $\pm$ 0	411 $\pm$ 0	299 $\pm$ 0	16,400 $\pm$ 0	411 $\pm$ 0	299 $\pm$ 0
(-) control ^c	16,100 $\pm$ 436	0	N/A	16,100 $\pm$ 436	0	N/A	16,100 $\pm$ 436	0	N/A
(-) control ^d	18,600 $\pm$ 1,660	0	N/A	18,600 $\pm$ 1,660	0	N/A	18,600 $\pm$ 1,660	0	N/A

^a DNA of a pure culture of *I. liriodendri* isolate BV-0596 ($n = 1$).

^b DNA of soil sample tested positive to *I. liriodendri* ($n = 1$).

^c DNA from grapevine ($n = 2$).

^d Water ($n = 2$).

solution (9 g/liter NaCl). They were vortexed for 5 min to detach the soil particles and immediately centrifuged at $1,503 \times g$ for 5 min. The supernatant was discarded and the remaining soil fraction was used to represent the rhizosphere fraction. The roots devoid of soil particles were placed in a new tube and surface sterilized according to Cherif et al. (2015).

DNA extraction. The bulk soil and rhizosphere DNA were extracted from 0.5-g sample using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). For endorhizosphere DNA, before DNA extraction, roots were sequentially washed in 70% ethanol and distilled water. Upon this treatment, bark was carefully peeled out and the DNA was extracted from 0.5 g tissue using the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, South Korea).

Standard curves determination. A standard curve was constructed with DNA dilution series of *I. liriodendri* isolate BV-0596. Analyses were performed as previously described and the standard curve was generated following the MIQE guidelines (Bustin et al. 2009). The estimated number of target molecules per μl (ddPCR) and the quantification cycle (C_q) (qPCR) values obtained for each specific isolate DNA dilution were plotted against the logarithm of the concentration ($\text{fg } \mu\text{l}^{-1}$) of each isolate DNA dilution.

Sensitivity of ddPCR and qPCR was assessed 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 amplification efficiency (E) and 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. Signal threshold levels were set automatically by the instrument software, and the LOD was identified by the last dilution in which successful amplification of all DNA replicates occurred.

Quantification of black-foot pathogens in environmental samples.

To compare both quantification techniques in environmental samples, the 60 grapevine samples collected at 5 young vineyards in La Rioja were analyzed by both ddPCR and qPCR technologies. All samples, along with a nontemplate control (NTC) reaction (water), two positive controls containing DNA of a soil sample tested positive to *I. liriodendri* by ITS high-throughput amplicon sequencing, and DNA extracted from a pure culture of *I. liriodendri* isolate BV-0596, were analyzed by qPCR and ddPCR in triplicate. In addition, negative controls (serial dilutions of DNA from grapevine and soil lacking target fungi) were used alone or spiked with the same amount of target DNA in order to assess inhibition in the assays. The mean DNA concentration and the standard deviation were determined from five replicates per dilution. 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. 2006, 2016), where DNA amount was the concentration of DNA (g) and DNA length was the length of *I. liriodendri* BV-0596 genome, 60 Mbp (unpublished data). The efficiency of both ddPCR and qPCR technologies to quantify *I. liriodendri* from environmental samples was compared. Values from the *I. liriodendri* DNA concentration obtained with each technique were transformed by $\log(n/N \times 1,000 + 1)$. Where n was the DNA concentration detected on each sample and N was the total DNA concentration detected. An analysis of correlation between both transformed datasets was performed in R version 3.5 (R Core Team 2017) using the corr package. DNA concentration values using both quantification methods were calculated for each fraction and vineyard. Significance levels for mean values were determined by the Kruskal-Wallis one-way analysis of variance on ranks and mean separation was conducted at $P < 0.05$. The analysis was performed using R package agricolae (Mendiburu 2015).

Fig. 2. Droplet digital PCR amplitude plot showing all accepted droplets with a clear distinction between positives (mostly between 3,000 and 4,000) and negatives (mostly around 1,000) in each soil-plant fraction (bulk soil, rhizosphere, and roots), positive control (*Ilyonectria liriodendri* isolate BV-0596 DNA), and nontemplate control (NTC), confirming the assay optimization.

Results

Detection and quantification limit of genomic DNA of cultured *I. liriodendri* by ddPCR and qPCR. The optimal annealing temperature for primers using pure culture *I. liriodendri* BV-0596 DNA in ddPCR was established at 59°C. Both methods showed good linearity within the quantification range with a high coefficient of determination (R^2) of 0.9917 and 0.9893 and a reaction efficiency of 0.83 and 0.97 for ddPCR and qPCR, respectively (Fig. 1). The minimum target concentration detectable was the 5 fg μL^{-1} dilution for ddPCR and the 10 fg μL^{-1} dilution for qPCR (Table 1); thus, the LOD was established at these concentrations for each technique. The NTC showed no positive amplification.

Quantification of black-foot pathogens from environmental samples. DNA of *I. liriodendri* was detected in all soil-plant fraction samples in the five vineyards assessed. Significant differences in the abundance of *I. liriodendri* were detected among vineyards with both techniques ($P < 0.01$). Concentrations ranged from 1.79 to 20.98 pg μL^{-1} in vineyard 1, 0.77 to 8.73 pg μL^{-1} in vineyard 2, 1.99 to 53.8 pg μL^{-1} in vineyard 3, 0.03 to 38.58 pg μL^{-1} in vineyard 4, and 0.34 to 29.43 pg μL^{-1} in vineyard 5 by qPCR (data not shown). In ddPCR, concentrations ranged from 96 to 2,350 copies μL^{-1} in vineyard 1, 75 to 860 copies μL^{-1} in vineyard 2, 190 to 8,680 copies μL^{-1} in vineyard 3, 4.7 to 9,470 copies μL^{-1} in vineyard 4, and 40 to 1,920 copies μL^{-1} in vineyard 5 (data not shown). Average number of copies in each vineyard per soil-plant fraction obtained by ddPCR are shown in Table 2. In each vineyard, no significant differences in the abundance of *I. liriodendri* were detected among soil-plant fractions with both techniques ($P > 0.05$). Overall, concentrations ranged from 0.5 to 38.68 pg μL^{-1} in bulk soil, 0.03 to 53.8 pg μL^{-1} in rhizosphere, and 0.78 to 107.73 pg μL^{-1} in roots fraction by qPCR. Average concentrations in each soil-plant fraction were 10.07 pg μL^{-1} (bulk soil), 10.49 pg μL^{-1} (rhizosphere), and 11.53 fg μL^{-1} (endorhizosphere). In

ddPCR, concentrations ranged from 40 to 9,470 copies μL^{-1} in bulk soil, 4.7 to 5,270 copies μL^{-1} in rhizosphere, and 75 to 8,680 copies μL^{-1} in roots fraction. Average number of copies in each soil-plant fraction were 1,275 copies μL^{-1} (bulk soil), 1,028 copies μL^{-1} (rhizosphere), and 1,233 copies μL^{-1} (endorhizosphere). No PCR inhibition or positive droplets noticed using negative controls by qPCR or ddPCR (Fig. 2), respectively. The correlation analysis showed a high and positive significant correlation between *I. liriodendri* DNA quantified using both the ddPCR and qPCR techniques ($R^2 = 0.95$) (Fig. 3).

Discussion

Digital PCR is the latest DNA quantification technology that can be broadly used in several scientific fields (Cao et al. 2015; 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; Voegel and Nelson 2018). The present study represents the first approach to assess the ddPCR as a reliable tool to detect and quantify pathogenic fungi associated with grapevine trunk diseases. In particular, black-foot disease is one of the main soilborne diseases affecting planting material and young vineyards worldwide (Gramaje et al. 2018). DNA was not sheared prior to the experiments since our input DNA concentration was 10 ng/20 μL reaction. DNA shearing is recommended for input DNA concentrations >66 ng/20 μL reaction using the Bio-Rad QX100/200 system (Hindson et al. 2011). We found that both ddPCR and qPCR showed the potential of being efficient techniques to detect and measure *I. liriodendri* DNA associated with black-foot disease, with a strong correlation between them. These findings are in agreement with those obtained by Kim et al. (2014), who found a high quantitative agreement between DNA quantity measured with ddPCR and qPCR while examining population dynamics of bacteria in soil.

Fig. 3. The distribution of DNA concentration of *Ilyonectria liriodendri* values is shown on the diagonal. The bivariate scatter plot with a fitted line is displayed on the bottom of the diagonal and the Spearman correlation value ($P < 0.05$) is indicated on the top of the diagonal.

Quantification of copy number from complex samples containing multiple target species may be inaccurate. However, our attempt to design specific primers for black-foot disease genera (*Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria*) from available gene sequences in the GenBank database (internal transcribed spacer region, histone H3, translation elongation factor 1- α , and β -tubulin genes) were unsuccessful, due to lack of highly conserved gene regions among these closely related phylogenetic genera. In this study, *I. liriodendri* BV-0596 genome size was used in the calculation of copy number across samples, and the DNA of this isolate was also used for the standard curve determination and to establish the optimal annealing temperature for the primers. A limitation of this approach that needs to be stated is that bias in the calculation of the copy number may be introduced due to different genome sizes from the fungal species associated with black-foot disease: 58 Mbp in *D. macrodidyma* isolate JAC15-245 (Malapi-Wight et al. 2015) and 64 Mbp in *D. torresensis* isolate BV-0666 (Gramaje et al. 2019).

The ddPCR was more sensitive compared with qPCR in the detection and quantification of this fungal pathogen at very low concentrations. Increased sensitivity of digital PCR over qPCR has been reported in other studies (Bahder et al. 2018; Cavé et al. 2016; Kim et al. 2014; Porcellato et al. 2016), and similar sensitivity was highlighted by others (Blaya et al. 2016; Dreo et al. 2014) when comparing both techniques. Developing a robust ddPCR assay with increased sensitivity of ddPCR over qPCR would be beneficial to researchers and diagnostic laboratories by identifying early infections in grapevines and soil. Additional benefits were reported in several recent studies for ddPCR, such as it obviates the preparation of reference DNA templates (Kim et al. 2014), the absolute quantitative target detection without the need of standard curves construction (Yang et al. 2014), and that the inhibitory substances had a 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 alternative for measuring environmental samples allowing a better understanding and monitoring of fungal pathogens associated with grapevine trunk diseases in the future.

The abundance of *I. liriodendri* was not affected by soil or plant as source of DNA. Recent studies have shown that black-foot inoculum pressure in vineyard soils is frequently high (Agustí-Brisach et al. 2014; Reis et al. 2013), even with the absence of grapevine (Berlanas et al. 2017; Cardoso et al. 2013). Black-foot disease fungi can survive in soil for multiple years in the absence of a suitable host due to the production of chlamydospores after infected vines have been removed (Petit et al. 2011). These pathogens have also been frequently associated with the roots of herbaceous plants (Langenhoven et al. 2018) and weeds (Agustí-Brisach et al. 2011). It is also important to note that *Cylindrocarpon*-like asexual morphs seem to be quite frequent in roots and the endorhizosphere of diverse plants, not just in grapevines. Several studies show that they are indeed among the dominant fungi found in soils with strawberry (Xu et al. 2015) and forest trees (Bonito et al. 2014), and in roots of apple in South Africa (Tewoldemedhin et al. 2011) and the United States (Manici et al. 2018). This frequent and high occurrence of *Cylindrocarpon*-like asexual morphs in different environments may explain the lack of specialization of these fungi to specific plant-associated ecological niches (rhizosphere and endorhizosphere).

Both the qPCR and ddPCR methods revealed that healthy grapevine plants harbor *Cylindrocarpon*-like asexual morphs that are causal agents of black-foot disease, demonstrating that these fungi can act as endophytic and/or latent pathogenic microorganisms in grapevine. In the scientific literature, observations of black-foot disease fungi as endophytes colonizing asymptomatic vines (Berlanas et al. 2019; Langenhoven et al. 2018) or other plant species (Agustí-Brisach et al. 2011; Langenhoven et al. 2018) have been documented. Many of these asymptomatic plants are cereals and brassicaceous crops, used in crop rotations in grapevine nurseries (Langenhoven et al. 2018), and weeds, which may be present in field nurseries and established vineyards along with cultivated crops (Agustí-Brisach et al. 2011; Langenhoven et al. 2018). The

occurrence of black-foot disease pathogens in asymptomatic vines highlights the urgent need to implement early, accurate, and specific in planta detection and quantification of these fungi to prevent the spread of black-foot disease in grapevine propagation material.

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