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

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Abstract

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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.

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 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; Tewoldemehdin 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 more sensitive technology compared with qPCR (Bahder et al. 2016, 2018; Dreo et al. 2014; Miotke et al. 2014; Racki 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. *Ilyonectria liriodendri* isolate BV-0596 was obtained from the culture collection of the 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 25ºC 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, USA) with 4 steel beads of 2.38 mm and 2 of 3 mm diameter (Qiagen, Hilden, Germany) using a FastPrep-24TM5G (MP Biomedicals, California, USA) at 5 m/s for 20 s twice. DNA integrity and quality were assessed by gel electrophoresis visualizing the samples previously stained with RedSafe (iNtRON Biotechnology, Lynnwood, WA, USA). DNA samples were quantified using the Invitrogen Qubit 4 Fluorometer with Qubit dsDNA HS (High Sensitivity) Kit (Thermo Fisher Scientific, Waltham, MA, USA). 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 PrimerQuest®Design Tool (Integrated DNA Technologies, Inc. Coralville, IA, USA) and labeled at the 5' end with Hetrachloro-6-carboxyfluorescin (HEX) and a double-quencher (internal ZEN with 3' Iowa Black FQ). The probe sequence is 5'-/HEX/TCCGAGCGT/ZEN/CATTTCAACCCTCAA/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 *Cylindrocarpon*-like asexual morphs associated

with black-foot disease, in particular those belonging to the genera *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria.* Each reaction contained 1x Supermix for Probes (Bio-Rad Laboratories, Hercules, CA, USA), 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). 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 1x 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 to 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 (endorhizosphere). 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 solution (9 g/L NaCl). They were vortexed for 5 min to detach the soil particles and immediately centrifuged at 1,503 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 (Cq) (qPCR) values obtained for each specific isolate DNA dilution were plotted against the logarithm of the concentration (fg μl^{-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, QuantaSoftTM (Bio-Rad) for ddPCR and CFX Maestro (Bio-Rad) for qPCR.

177 Signal threshold levels were set automatically by the instrument software and the LOD was
178 identified by the last dilution in which successful amplification of all DNA replicates occurred.
179
180 **Quantification of black-foot pathogens in environmental samples.** To compare both
181 quantification techniques in environmental samples, the 60 grapevine samples collected at 5
182 young vineyards in La Rioja were analyzed by both ddPCR and qPCR technologies. All samples,
183 along with a non-template control (NTC) reaction (water), two positive controls containing DNA
184 of a soil sample tested positive to *I. liriodendri* by ITS high-throughput amplicon sequencing,
185 and DNA extracted from a pure culture of *I. liriodendri* isolate BV-0596, were analyzed by
186 qPCR and ddPCR in triplicate. In addition, negative controls (serial dilutions of DNA from
187 grapevine and soil lacking target fungi) were used alone or spiked with the same amount of
188 target DNA in order to assess inhibition in the assays. The mean DNA concentration and the
189 standard deviation were determined from five replicates per dilution. For the qPCR results, copy
190 number was calculated with the following formula: (DNA amount (g) * 6.022 x 10^{23} (copy/mol)
191 / (DNA length (bp) * 660 (g/mol/bp)) (Lee et al. 2006; Lee et al. 2016), where DNA amount was
192 the concentration of DNA (g) and DNA length was the length of *I. liriodendri* BV-0596 genome,
193 60 Mbp (unpublished data). The efficiency of both ddPCR and qPCR technologies to quantify *I.*
194 *liriodendri* from environmental samples was compared. Values from the *I. liriodendri* DNA
195 concentration obtained with each technique were transformed by log (n/N ∗ 1000 + 1). Where n
196 was the DNA concentration detected on each sample and N was the total DNA concentration
197 detected. An analysis of correlation between both transformed datasets was performed in R
198 version 3.5 (R Core Team 2017) using the corrr package. DNA concentration values using both
199 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).

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 59ºC. 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 fractions 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. 3), 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. 2).

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

246 experiments since our input DNA concentration was 10 ng/20 µL reaction. DNA shearing is
247 recommended for input DNA concentrations >66 ng/20 µL reaction using the Bio-Rad
248 QX100/200 system (Hindson et al. 2011). We found that both ddPCR and qPCR showed the
249 potential of being efficient techniques to detect and measure *I. liriodendri* DNA associated with
250 black-foot disease, with a strong correlation between them. These findings are in agreement with
251 those obtained by Kim et al. (2014), who found a high quantitative agreement between DNA
252 quantity measured with ddPCR and qPCR while examining population dynamics of bacteria in
253 soil.

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

267 The ddPCR showed to be more sensitive as compared with qPCR in the detection and
268 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 (Reis et al. 2013; Agustí-Brisach et al. 2014), even with the absence of grapevine (Cardoso et al. 2013; Berlanas et al. 2017). Black-foot disease fungi can survive in soil for multiple years in the absence of 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 only 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

USA (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 (Langenhoven et al. 2018; Berlanas et al. 2019) 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.

Literature Cited

Agustí-Brisach, C., and Armengol, J. 2013. Black-foot disease of grapevine: An update on taxonomy, epidemiology and management strategies. Phytopathol. Mediterr. 52:245–261.

Agustí-Brisach, C., Gramaje, D., León, M., García-Jiménez, J., and Armengol, J. 2011. Evaluation of vineyard weeds as potential hosts of black-foot and Petri disease pathogens. Plant Dis. 95:803–810.

Agustí-Brisach, C., Mostert, L., and Armengol, J. 2014. Detection and quantification of

Ilyonectria spp. associated with black-foot disease of grapevine in nursery soils using multiplex nested PCR and quantitative PCR. Plant Pathol. 63:316–322.

Alaniz, S., Armengol, J., León, M., García-Jiménez, J., and Abad-Campos, P. 2009. Analysis of genetic and virulence diversity of *Cylindrocarpon liriodendri* and *C. macrodidymum* associated with black foot disease of grapevine. Mycol. Res. 113:16–23.

Bahder, B. W., Helmick, E. E., Mou, D. F., Harrison, N. A., and Davis, R. 2018. Digital PCR technology for detection of palm-infecting phytoplasmas belonging to group 16SrIV that occur in Florida. Plant Dis. 102:1008–1014.

Bahder, B. W., Soto, N., Komondy, L., Mou, D-F., Humphries, A. R., and Helmick, E. E. 2019. Detection and quantification of the 16SrIV-D phytoplasma in leaf tissue of common ornamental palm species in Florida using qPCR and dPCR. Plant Dis. http://doi.org/10.1094/PDIS-01-19-0010-RE.

Bahder, B. W., Zalom, F. G., Jayanth, M., and Sudarshana, M. R. 2016. Phylogeny of geminivirus coat protein sequences and digital PCR aid in identifying *Spissistilus festinus* as a vector of grapevine red blotch-associated virus. Phytopathology 106:1123–1230.

Berlanas, C., López-Manzanares, B., and Gramaje, D. 2017. Estimation of viable propagules of black-foot disease pathogens in grapevine cultivated soils and their relation to production systems and soil properties. Plant Soil 417:467-4799.

Berlanas, C., Ojeda, S., López-Manzanares, B., Andrés-Sodupe, M., Bujanda, R., Martínez-Diz, M. P., Díaz-Losada, E., and Gramaje, D. 2019. Occurrence and diversity of black-foot disease fungi in symptomless grapevine nursery stock in Spain. Plant Dis. DOI: 10.1094/PDIS-03-19-0484-RE.

Blaya, J., Lloret, E., Santísima-Trinidad, A. B., Ros, M., and Pascual, J. A. 2016. Molecular

methods (digital PCR and real-time PCR) for the quantification of low copy DNA of *Phytophthora nicotianae* in environmental samples. Pest Manag Sci. 72:747–753.

Bonito, G., Reynolds, H., Robeson, M. S., Nelson, J., Hodkinson, B. P., Tuskan, G., Schadt, C. W., and Vilgalys, R. 2014. Plant host and soil origin influence fungal and bacterial assemblages in the roots of woody plants. Mol. Ecol. 23:3356-3370.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. 2009. The MIQE guidelines:Minimum Information for publication of quantitative real-time PCR experiments. Clin. Chem. 55:611–622. doi:10.1373/clinchem.2008.112797.

Cao, Y., Raith, M. R., and Griffith, J. F. 2015. Droplet digital PCR for simultaneous quantification of general and human-associated fecal indicators for water quality assessment. Water Res. 70:337–349.

Cardoso, M., Diniz, I., Cabral, A., Rego, C., and Oliveira, H. 2013. Unveiling inoculum sources of black foot pathogens in a commercial grapevine nursery. Phytopatol. Mediterr. 52:298–312.

Carlucci, A., Lops, F., Mostert, L., Halleen, F., and Raimondo, M. L. 2017. Occurrence fungi causing black foot on young grapevines and nursery rootstock plants in Italy. Phytopathol. Mediterr. 56:10–39.

Cavé, L., Brothier, E., Abrouk, D., Bouda, P.S., Hien, E., and Nazaret, S. 2016. Efficiency and sensitivity of the digital droplet PCR for the quantification of antibiotic resistance genes in soils and organic residues. Appl Microbiol Biotechnol. 100:10597–10608.

Chapelle, E., Mendes, R., Bakker, P. A. H., and Raaijmakers, J. M. 2016. Fungal invasion of the rhizosphere microbiome. ISME J. 10:265–268.

361 Chaverri, P., Salgado, C., Hirooka, Y., Rossman, A.Y., and Samuels, G.J. 2011. Delimitation of
362 *Neonectria* and *Cylindrocarpon* (Nectriaceae, Hypocreales, Ascomycota) and related genera
363 with *Cylindrocarpon*-like anamorphs. Stud. Mycol. 68:57–78.
364 Cherif, H., Marasco, R., Rolli, E., Ferjani, R., Fusi, M., Soussi, A., Mapelli, F., Blilou, I., Borin,
365 S., Boudabous, A., and Cherif. A. 2015. Oasis desert farming selects environment specific
366 date palm root endophytic communities and cultivable bacteria that promote resistance to
367 drought. Environ. Microbiol. Rep. 7:668–78.
368 Dreo, T., Pirc, M., Ramšak, Ž., Pavšič, J., Milavec, M., Žel, J., and Gruden, K. 2014. Optimising
369 droplet digital PCR analysis approaches for detection and quantification of bacteria: a case
370 study of fire blight and potato brown rot. Anal. Bioanal. Chem. 406:6513–6528.
371 Dubrovsky, S., and Fabritius, A. L. 2007. Occurrence of *Cylindrocarpon* spp. in nursery
372 grapevines in California. Phytopathol. Mediterr. 46:84–86.
373 Gramaje, D., and Armengol, J. 2011. Fungal trunk pathogens in the grapevine propagation
374 process: potential inoculum sources, detection, identification and management strategies.
375 Plant Dis. 95:1040–1055.
376 Gramaje, D., Berlanas., Díaz-Losada., E., Martínez-Diz, M. P., Antonielli, L., Gorfer, M., and
377 Compant, S. 2019. Comparative genomics of Dactylonectria torresensis strains from
378 grapevine soil and weeds highlight potential mechanisms in pathogenicity and lifestyle.
379 Page 95 in: Proc. 11th Int. Workshop Grapevine Trunk Dis., Penticton, Canada.
380 Gramaje, D., Úrbez-Torres, J. R., and Sosnowski, M. R. 2018. Managing grapevine trunk
381 diseases with respects to etiology and epidemiology: current strategies and future prospects.
382 Plant Dis. 102:12–39.
383 Halleen, F., Fourie, P. H., and Crous, P. W. 2006. A review of black foot disease of grapevine.

384 Phytopathol. Mediterr. 45:S55–S67.
385 Hindson, B. J., Ness, K. D., Masquelier, D. A., Belgrader, P., Heredia, N. J., Makarewicz, A. J.,
386 Bright, I. J., Lucero, M. Y., Hiddessen, A. L., Legler, T. C., Kitano, T. K., Hodel, M.
387 R.,Petersen, J. F., Wyatt, P. W., Steenblock, E. R., Shah, P. H., Bousse, L. J., Troup, C. B.,
388 Mellen, J. C., Wittmann, D. K., Erndt, N. G., Cauley, T. H., Koehler, R. T., So, A. P., Dube,
389 S., Rose, K. A., Montesclaros, L., Wang, S. L., Stumbo, D. P., Hodges, S. P., Romine, S.,
390 Milanovich, F. P., White, H. E., Regan, J. F., Karlin-Neumann, G. A., Hindson, C. M.,
391 Saxonov, S., Colston, B. W., 2011. High-throughput droplet digital PCR system for
392 absolute quantitation of DNA copy number. Anal. Chem. 83:8604–8610.
393 Hoshino, T., and Inagaki, F. 2012. Molecular quantification of environmental DNA using
394 microfluidics and digital PCR. Syst. Appl. Microbiol. 35:390–395.
395 Hussain, M., Fantuzzo, R. Mercorelli, S., and Cullen, C. 2016. A direct droplet digital PCR
396 method for quantification of residual DNA in protein drugs produced in yeast cells. J.
397 Pharm. Biomed. Anal. 123:128–131.
398 Kim, T. G., Jeong, S-Y., and Cho, K-S. 2014. Comparison of droplet digital PCR and
399 quantitative real-time PCR for examining population dynamics of bacteria in soil. Appl.
400 Microbiol. Biotechnol. 98: 6105–6113.
401 Langenhoven, S. D., Halleen, F., Spies, C. F. J., Stempien, E., and Mostert, L. 2018. Detection
402 and quantification of black foot and crown and root rot pathogens in grapevine nursery soils
403 in the Western Cape of South Africa. Phytopathol. Mediterr. 57:519–537.
404 Lee, C., Kim, J., Shin, S. G., and Hwang, S. 2006. Absolute and relative QPCR quantification of
405 plasmid copy number in *Escherichia coli*. J. Biotechnol. 123:273–280.
406 Lee, J., Foong, Y. H., Musaitif, I., Tong, T., and Jefcoate, C. 2016. Analysis of specific RNA in

cultured cells through quantitative integration of q-PCR and N-SIM single cell FISH images: Application to hormonal stimulation of StAR transcription. Mol. Cell. Endocrinol. 429:93–105.

Lombard, L., Van der Merwe, N. A., Groenewald, J. Z., and Crous, P. W. 2014. Lineages in Nectriaceae: re-evaluating the generic status of Ilyonectria and allied genera. Phytopathol. Mediterr. 53:515–532.

Malapi-Wight, M., Salgado-Salazar, C., Demers, J., Veltri, D., and Crouch, J.A. 2015. Draft genome sequence of *Dactylonectria macrodidyma*, a plant-pathogenic fungus in the Nectriaceae. Genome Announc 3:e00278-15. doi:10.1128/genomeA.00278-15.

Manici, L. M., Kelderer, M., Caputo, F., Saccà, M. L., Nicoletti, F., Topp, A. R., and Mazzola, M. 2018. Involvement of *Dactylonectria* and *Ilyonectria* spp. in tree decline affecting multi-generation apple orchards. Plant Soil 425:217-230.

Mendiburu, F. 2015. agricolae: Statistical Procedures for Agricultural Research. R package Version 1.2-3. [http://CRAN.R-project.org/package=agricolae].

Miotke, L., Lau, B. T., Rumma, R. T., and Ji, H. P. 2014. High sensitivity detection and quantification of DNA copy number and single nucleotide variants with single copy droplet digital PCR. Anal. Chem. 86:2618–2624.

Morisset, D., Štebih, D., Milavec, M., Gruden, K., and Žel, J. 2013. Quantitative analysis of food and feed samples with droplet digital PCR. PLoS ONE 8:e62583.

Palumbo, J. D., O'Keeffe, T. L., and Fidelibus, M. W. 2016. Characterization of *Aspergillus* section *Nigri* species populations in vineyard soil using droplet digital PCR. Let. App. Microbiol. 63:458–465.

Petit, E., Barriault, E., Baumgartner, E., Wilcox, W. F., and Rolshausen, P. E. 2011.

430 Cylindrocarpon species associated with black-foot of grapevine in northeastern United
431 States and southeastern Canada. Am. J. Enol. Viticult. 62:177–183.
432 Porcellato, D., Narvhus, J., and Skeie, S. B. 2016. Detection and quantification of *Bacillus*
433 *cereus* group in milk by droplet digital PCR. J. Microbiol. Methods 127:1–6.
434 R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for
435 Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.
436 Racki, N., Dreo, T., Gutierrez-Aguirre, I., Blejec, A., and Ravnikar, M. 2014. Reverse
437 transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil
438 and water samples. Plant Meth. 10:42.
439 Reis, P., Cabral, A., Nascimento, T., Oliveira, H., and Rego, C. 2013. Diversity of *Ilyonectria*
440 species in a young vineyard affected by black foot diseases. Phytopathol. Mediterr. 52:335-
441 346.
442 Tewoldemedhin, Y. T., Mazzola, M., Mostert, L., and McLeod, A. 2011. *Cylindrocarpon* species
443 associated with apple tree roots in South Africa and their quantification using real-time
444 PCR. Eur. J. Plant Pathol. 129:637–651.
445 Voegel, T., and Nelson, L. M. 2018. Quantification of *Agrobacterium vitis* from Grapevine
446 Nursery Stock and Vineyard Soil using Droplet Digital PCR. Plant Dis. 102:2136–2141.
447 Xu, X., Passey, T., Wei, F., Saville, R., and Harrison, R. J. 2015. Amplicon-based metagenomics
448 identified candidate organisms in soils that caused yield decline in strawberry. Horticulture
449 Research 2: 15022. doi: 10.1038/hortres.2015.1022
450 Yang, R., Paparini, A., Monis, P., and Ryan, U. 2014. Comparison of next-generation droplet
451 digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium*
452 oocysts in faecal samples. Int. J. Parasitol. 44:1105–1113.

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Figure captions

Fig. 1. Standard curves obtained using *Ilyonectria liriodendri* isolate BV-0596 DNA dilutions ranging from 10,000 to 1 fg μl^{-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.

Fig. 2. The distribution of DNA concentration of *I. 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.

Fig. 3. Droplet digital PCR amplitude plot showing all accepted droplets with a clear distinction between positives (green) and negatives (grey) in each soil-plant fraction (bulk soil, rhizosphere

475 and roots), positive control (*I. liriodendri* isolate BV-0596 DNA) and non-template control
476 (NTC), confirming the assay optimization.

477

478 ## Tables

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

482

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

484 **Table 2**. Droplet profile and digital PCR quantitation data from DNA extracts of the environmental samples from five vineyards (n=4). Values
485 represent the mean±SE from four replicates.
486

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

487
488 [1]DNA of a pure culture of *I. liriodendri* isolate BV-0596 (n=1)
489 [2]DNA of soil sample tested positive to *I. liriodendri* (n=1)
490 [3]DNA from grapevine (n=2)
491 [4]Water (n=2)
492

493

494

495

Supplementary Table S1. Soil physicochemical properties and management practices of the five vineyards examined in this study. Values represent the mean±SE.

	Vineyard-1	Vineyard-2	Vineyard-3	Vineyard-4	Vineyard-5
Coordinates	42,583560º, -2,853296º	42,588604º, -2,868726º	42,539882º, -2,766227º	42,499781º, -2,781054º	42,593068º, -2,851397º
Location	Haro	Haro	Briones	Briones	Haro
Year of plantation	2013	2013	2014	2012	2013
Extension (ha)	0.93	8.32	3.00	5.11	1.96
Altitude (m)	478	482	501	497	499
Physicochemical properties					
pH	8.1[a]±0.02	8.1±0.02	8.2	8.2	8.2±0.02
P mg/100g	3.39±0.25	2.6±0.2	2.4±0.3	3.1±0.3	3.2±0.2
K mg/100g	17.2±0.7	17.1±0.4	16.4±0.5	19.1±0.6	20.5±0.5
S mg/100g	4.3±0.5	3.9±0.4	4.1±0.3	4.1±0.4	4.2±0.4
Mg mg/100g	24.3±0.3	27.1±0.3	25.7±0.5	21.4±0.4	26.7±0.5
Mn mg/100g	3.5±0.8	2.9±0.7	2.8±0.7	2.8±0.7	2.8±0.8
Fe mg/100g	8.6±0.4	8.7±0.2	10.6±0.2	7.7±0.4	8.7±0.5
Ca mg/100g	3979.6±220.8	4346.9±120.4	4347.5±109.8	3731.3±176.4	3503.0±126.7
Na mg/100g	2.4±0.2	2.83±0.1	3.1±0.2	3.0±0.2	3.4±0.2
SOM%	0.95±0.03	1.05±0.2	1.05±0.03	0.95±0.05	1.07±0.08
Clay%	18.5±0.2	22.2±0.3	25.3±0.3	21.9±0.6	22.9±0.4
Sand%	39.9±0.3	33.8±0.4	34.3±0.5	35.1±0.6	34.3±0.9
Silt%	41.6±0.6	44.0±1.1	40.4±0.8	43.0±0.7	42.8±0.5
CO_3Ca	14.15±0.02	15.67±0.23	13.9±0.1	14.5±0.4	14.9±0.6
CEC mekv/100g	8.9±0.3	10.4±0.1	11.2±0.2	9.6±0.2	9.1±0.3
EC mS/cm	0.14	0.16	0.16	0.15	0.16
Assim. Ca mekv/100g	15.1±0.2	17.4±0.2	16.5±0.2	15.5±0.3	14.8±0.4
Assim. Mg mekv/100g	1.57±0.05	1.81±0.11	1.75±0.12	1.70±0.15	1.63±0.21
Soil temperature (ºC) (July)	20.9	21.0	21.6	21.3	20.9
Soil management practices					
Irrigation system	Drip irrigation				
Fertilization	2 applications per year				
Pest management practices	5 spray treatments against powdery and downy mildew per year				
Herbicide treatment	Yes				

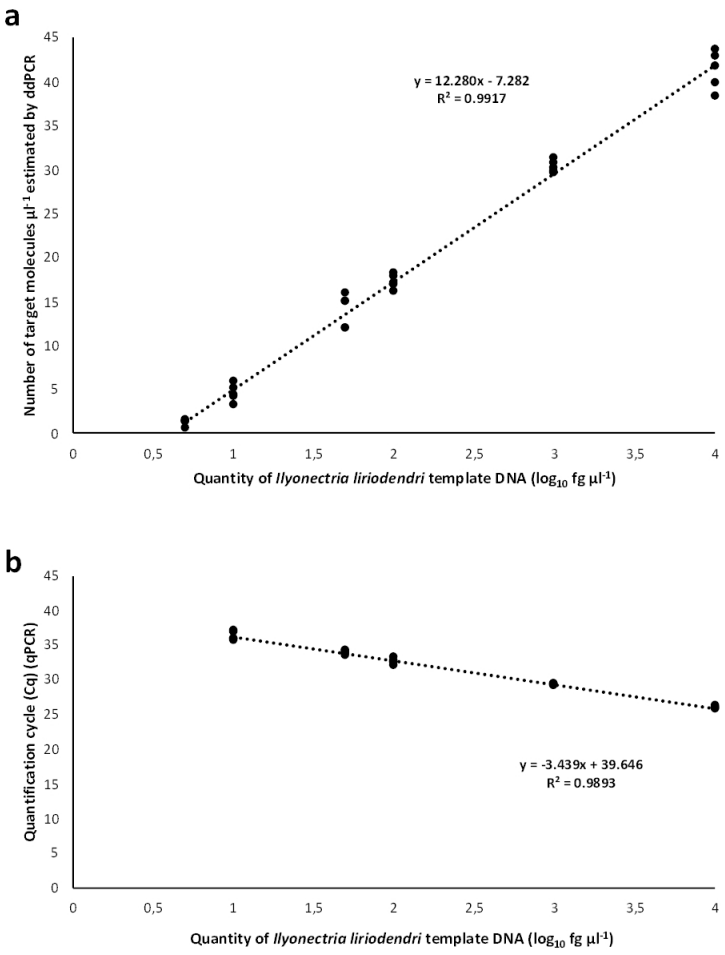

Figure 1

190x254mm (150 x 150 DPI)

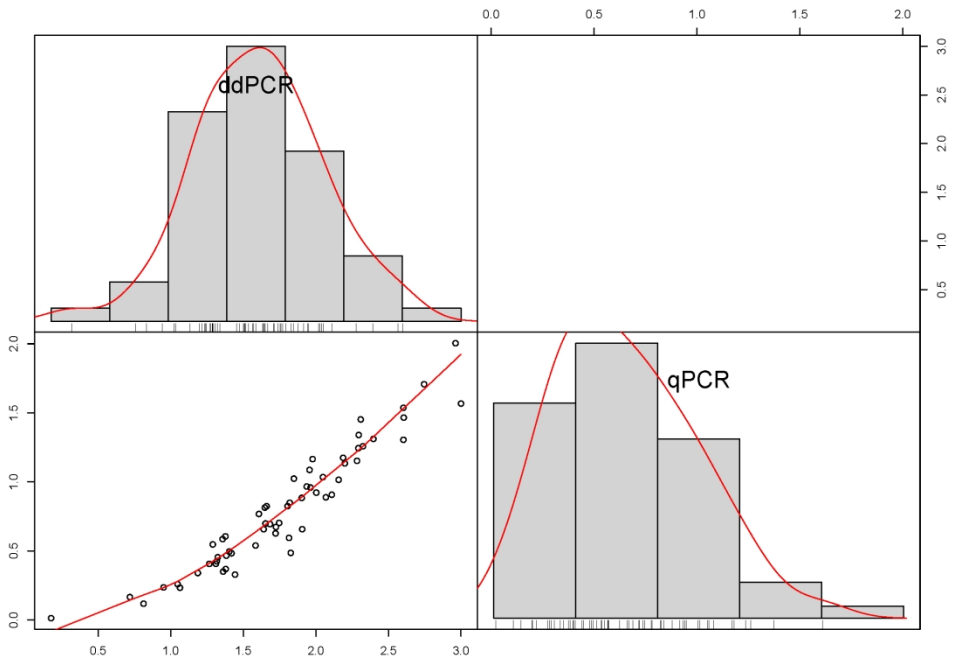

Figure 2

296x209mm (200 x 200 DPI)

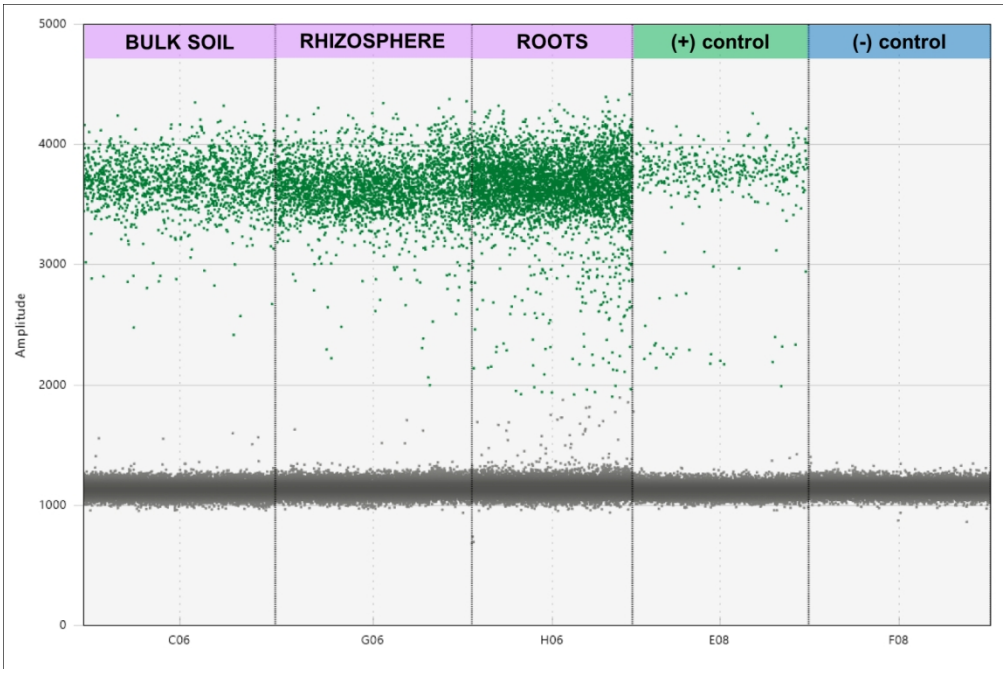
BULK SOIL
RHIZOSPHERE
ROOTS
(+) control
(-) control
Amplitude
5000
4000
3000
2000
1000
0
C06
G06
H06
E08
F08

Figure 3