Quantification of *Agrobacterium vitis* from grapevine nursery stock and vineyard soil using droplet digital PCR

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Abstract

Current detection methodologies for *Agrobacterium vitis*, causing crown gall of grapevines, are time intensive and lack the ability to quantify pathogen abundance in nursery stock and soil. Information on pathogen abundance is a key component to develop management strategies. The aim of this study was to develop a rapid and sensitive quantification assay for grapevine nursery stock and vineyard soil via droplet digital PCR targeting the *virA* gene. DNA isolated from roots of dormant grapevines originating from nurseries in Germany, California and Ontario were tested for *virA* abundance. Bacterial numbers varied with grapevine origin; plants from California had the highest numbers. In addition, rhizosphere soil from two vineyards in the Okanagan valley in British Columbia was tested over a growing season. Sampling time during the season did not affect *virA* gene abundance. The older vineyard had higher soil *A. vitis* populations than the younger vineyard. The assay developed here has potential for use in national clean plant programs to prevent import of infected grapevine nursery stock and to test vineyard soil for abundance of the pathogen before planting.

Crown gall of grapevines, caused by the gram negative bacterium *Agrobacterium vitis* (*A. viti*s) (Ophel and Kerr 1990), is an economically important disease in grape-growing regions with a continental climate, particularly in those where winter freezing occurs. The pathogen is introduced into the vineyard through contaminated grapevine nursery stock or infects wounded grapevine roots through soil harboring the bacterium (Burr et al. 1998; Sule et al. 1995). Additionally, a recent study has shown wild grapevines as a significant reservoir of inoculum (Orel et al. 2017). Wound sites in trunks and canes caused by winter freezing, mechanical injuries or poor grafting attract *A. vitis* cells and induce gall formation (Burr et al. 1998). Crown gall significantly reduces plant vigor and the disease may cause partial or complete vine death, especially in young vines (Schroth et al. 1988). Economic losses of US$46,500 per 0.4-hectare vineyard over a 6-year period were estimated to be caused by crown gall in Pennsylvania (Stewart and Wenner 2004).

To date, management strategies are mainly based on cultural practices, including planting of less susceptible rootstocks and cultivars, hilling up to cover graft unions with soil to minimize winter injury, training of multiple trunks per vine, or efforts to prevent soil freezing (Burr et al. 1998). However, the most successful strategy is disease prevention by planting healthy grapevine nursery stock into healthy soil (Johnson et al. 2013). A hallmark of *A. vitis* biology is its systemic survival in symptomless vines, resulting in difficulties to produce healthy grapevine stock (Burr et al. 1998). Hot water treatment is applied on some occasions by nurseries before the plants are sold to growers (Burr et al. 1989; Burr et al. 1996). However, this methodology only reduces, but not eliminates *A. vitis* (Burr et al. 1996). To ensure that grapevine stock is pathogen free, it is important

to develop sensitive technologies to detect and quantify *A. vitis* before establishing a new vineyard. Existing indexing methodologies involving collection of bleeding sap or plating on semi-selective medium are time intensive, not sensitive enough or prone to contamination (Bazzi et al. 1987; Bishop et al. 1989; Lehoczky 1971). Newer PCR-based methodologies have improved indexing of grapevine stock (Bini et al. 2008; Eastwell et al. 1995; Peduto et al. 2010; Puławska and Sobiczewski 2005). A recently developed magnetic capture hybridization (MCH) technology showed increased detection sensitivity compared to earlier PCR-based methods because of the removal of PCR inhibitors and the use of quantitative real-time PCR (qPCR) (Johnson et al. 2013). The MCH method was able to detect *A. vitis* in naturally infected grapevines, green shoots, grape buds and on the surface of grape leaves (Johnson et al. 2015). However, MCH includes an enrichment step, where bacteria are grown in a broth before DNA extraction and qPCR are conducted. This enrichment step prevents absolute quantification of *A. vitis.* Information on *A. vitis* abundance in grapevines is needed to increase our understanding of pathogen biology and to determine an *A. vitis* density threshold at which crown gall disease will occur.

Even when healthy grapevines are used for replanting or establishing a new vineyard, *A. vitis* residing in soil can infect healthy plant roots because the bacteria survive on infected, decaying roots in soil for at least two years after removal of diseased grapevines (Bishop et al. 1988; Burr et al. 1987; Burr et al. 1995; Sule et al. 1995). To achieve the goal of planting healthy nursery stock into healthy soil, sensitive soil quantification methodologies are needed in addition to methods for testing of nursery stock.

Droplet digital PCR (ddPCR) is a recently developed technology for absolute nucleic acid quantification (Hindson et al. 2011). The nucleic acids in a sample are partitioned into ~ 20,000 droplets that support PCR amplification of single template molecules. The DNA binding dye included in the super-mix is used to distinguish positive droplets (amplification of target molecule) from negative droplets (no target molecule present) via automated droplet flow-cytometry. The number of target molecules in the sample can be calculated using Poisson statistics (Hindson et al. 2011). Advantages over other quantitative technologies such as qPCR are the possibility for absolute quantification without the need of standards, the insignificance of primer efficiencies and the increased assay sensitivity due to dilution of PCR inhibitors in the droplets. Accordingly, the objective of this study was to develop a rapid and sensitive ddPCR assay to quantify *A. vitis* populations in grapevine nursery stock and vineyard soil. The assay was used to determine *A. vitis* abundance in i) planting material originating from nurseries in Germany, California (United States) and Ontario (Canada) and in ii) soils of two vineyards in the Okanagan Valley, British Columbia (Canada) over a growing season.

Material and Methods

Droplet digital PCR. Droplet digital PCR was performed on a BioRad QX200 system using an EvaGreen assay (BioRad, Hercules, CA, USA). The PCR reaction contained 11 µl QX200 super-mix (BioRad), 1 µl of each 2.2 µM primer solution (final concentration 100 nM for each primer) and 2 µl of template in a final volume of 22 µl. The PCR reactions were mixed, centrifuged briefly, and 20 µl transferred into the

sample well of a DG8™ cartridge (BioRad). After adding 70 µl of QX200™ droplet generation oil (BioRad) into the oil wells, the cartridge was covered using a DG8™ gasket, and droplets generated using the QX200™ droplet generator (BioRad). Droplets were carefully transferred into PCR plates using a multi-channel pipette and the plate sealed using PCR plate heat seal foil and the PX1™ PCR plate sealer (BioRad). PCR was performed in a C1000 touch thermal cycler (BioRad) using the following PCR program: 95°C for 5 min; 40 cycles of: 95°C for 30 sec, optimal annealing temperature (°C) for 1.20 min; 4°C for 5 min; 90°C for 5 min and hold at 4°C; ramp rate was 2.5°C/sec. PCR plates were transferred into a QX200™ droplet reader (BioRad) and reads analyzed using QuantaSoft™ software (BioRad).

Droplet digital PCR assay development. A thermal gradient PCR experiment was conducted to establish the optimal annealing temperature for each primer pair *virA*, *pehA* (Eastwell et al. 1995) and *virD2* (Johnson et al. 2013). *Agrobacterium vitis* strain CG47 (Eastwell et al. 1995) was grown on Luria-Bertani (LB) (Bertani 1951) plates at 28°C for 2 days. DNA was extracted using the E.Z.N.A.® bacterial DNA kit (Omega Bio-tek, Norcross, GA, USA) and 2 µl of a 1:1000 dilution used as template. Temperatures tested ranged from 53.5-60°C. Primer pairs used were *virAfor*/*virArev*, *pehAfor*/*pehArev* (Eastwell et al. 1995) and *virD2.For1*/*virD2.Rev1* (Johnson et al. 2013).

Detection limit. *Agrobacterium vitis* strain CG47 DNA (Eastwell et al. 1995) was extracted as above, the concentration determined by Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA) and a 10-fold serial dilution of 1 - 0.00001 ng/µl prepared. One µl of each dilution was used as template for ddPCR and the experiment repeated 3 times.

To assess the detection limit in grapevines, *A. vitis* strain CG47 (Eastwell et al. 1995) was grown on LB plates at 28°C for 2 days. Bacteria were adjusted to 10^8 CFU/ml in 1 x sterile phosphate-buffered saline (PBS), pH 6.8. Roots of grapevine seedlings cv. Riesling were cut into 2 mm pieces using pruning shears and 0.3 g spiked with 100 µl of a serial dilution of *A. vitis* in 1 x PBS (10^7-10^1 CFU/ml) or 1 x PBS only as negative control. DNA was isolated using the MoBio Powersoil® DNA extraction kit, according to the manufacturer's instructions (MoBio, Carlsbad, CA, USA). DNA was eluted in 100 µl of elution buffer provided with the kit. Concentrations and absorbance ratios were determined by Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA). Aliquots of nucleic acids were stored at -20°C until use. Two µl were used as template for ddPCR and the experiment repeated 3 times.

Because *A. vitis* is host specific to grapes, cherry orchard soil was used to assess the detection limit. Soil was sterilized by microwaving for 3 min, mixed and microwaved again for 3 min. Soil was stored overnight at 4°C and the microwaving repeated as above. Soil (0.25 g) was spiked with 100 µl of a serial dilution of *A. vitis* in 1 x PBS (10^7-10^1 CFU/ml) or 1 x PBS only as negative control. DNA was extracted and processed as above, and the experiment repeated 3 times.

Nursery plant material, experimental vineyards and DNA extraction. Ready-to-plant dormant rooted grapevines from commercial nurseries were donated by local wineries in the Okanagan region in Canada in spring 2016 and stored in a cold room at 4°C until use. Information on origin, scion and rootstock cultivars of dormant vines is listed in Table 1. A total of 45 grapevines (5 plants per cultivar from each nursery) were evaluated. For each grapevine, four primary roots were separated from the plant and

cut at the top, middle and end into 2-mm pieces using pruning shears that were cleaned with 70% ethanol between samples. Cherry tree roots were used as negative control. DNA was isolated from 0.3 g of root tissue per sample using the MoBio Powersoil® DNA extraction kit as described above. Dormant rooted grapevine stock from the same shipments as the plants used for ddPCR analysis, were planted in spring 2016 by the respective winery. In spring 2017, four vineyards where the vines were not used as replants or where the tested nursery stock had the highest concentrations of *A. vitis* were monitored for crown gall symptoms by visually rating 90 plants.

Both vineyards used for sampling of soil throughout the 2017 growing season are located in Kelowna, British Columbia (Canada) and are owned and managed by the same winery. Details of the vineyards are listed in Table 2. Vineyard soil was sampled five times over the growing season according to growth stages of the grapevines: 02-May (bud break), 20-June (bloom), 06-July (pea size berries), 28-August (veraison), 10-October (after harvest). Six vines per site with visible crown gall symptoms were randomly chosen and the same vines were sampled each time. Soil was sampled from underneath the dripline 20 cm away from the trunk using a 30 cm soil probe. One soil core from the right and left side of the trunk were combined. Three samples taken from adjacent grassland were collected as controls. The intact soil cores were transported to the laboratory and grapevine roots with attached rhizosphere soil removed with tweezers. After air-drying, rhizosphere soil was separated from roots and 0.25 g used for DNA extraction using the MoBio Powersoil® DNA extraction kit as described above. The leftover soil was sieved through a 20-mm sieve and sent to the BC Ministry of Environment, Analytical Chemistry Laboratory, Victoria for determination of pH (1:1 in

H_2O), total carbon and nitrogen (C/N ratio), and particle size using standard protocols of the facility.

Statistical analysis. All data were tested for normality using the Shapiro-Wilk test, log transformed and analyzed by ANOVA using R. Tukey's HSD and Welch's t-test were used for mean separation of nursery stock and soil data, respectively.

Results

Droplet digital PCR, detection and quantification limit. Specificity of the *virA*, *pehA* and *virD2* primers for *A. vitis* has been shown in earlier studies (Eastwell et al. 1995, Johnson et al. 2013). The optimal annealing temperature for primer pair *virA* using pure culture *A. vitis* CG47 DNA in ddPCR was established at 59.6°C, which resulted in the best separation between positive and negative droplets and in the highest number of positive droplets (Figure 1). *PehA* primers (Eastwell et al. 1995) generated a product at annealing temperatures ranging from 56-60°C (Figure 2). Primers targeting the *virD2* gene (Johnson et al. 2013) also produced positive droplets, however, rain caused difficulties in efficiently separating positive from negative droplets in ddPCR, indicating the need for further optimization if targeting of the *virD2* gene is required (data not shown).

The detection limit of pure culture *A. vitis* strain CG47 DNA was 0.1 pg/µl with a detection frequency of 100% (Figure 3). There were no positive droplets (no detection) in the no template control. The quantification limit for *A. vitis* in spiked grapevine roots and soil was determined by plotting the log value of ddPCR data against bacterial numbers from the dilution series (Figure 4). The lower limit of the linear range of the

assay determined the quantification limit, which was 1000 *A. vitis* cells (R^2 = 0.99) for both. The limit of detection for both was 100 cells, which were detected with a frequency of 66% for soil and 33% for root samples.

Quantification of *A. vitis* in nursery stock. Nurseries had a significant effect on *A. vitis* abundance in roots (p < 0.001, Table 1). Plants originating from Ontario nurseries had fewer *virA* copies (305-932 copies / 0.3 g roots) compared to plants from the three Californian nurseries (over 17,000 copies / 0.3 g roots). There was considerable copy number variation among replicates, indicated by the high standard deviation among samples from the same nursery. A Riesling sample from Germany had 10,278 copies / 0.3 g root and a different clone from the same nursery had a 3.6 times higher concentration. Cherry root samples served as a negative control and showed no positive droplets. Grapevine nursery stock from the same shipments as the test samples used for ddPCR was planted in spring 2016 in the Okanagan Valley (BC). The following winter was cold, with minimum air temperatures frequently reaching less than -20°C during December 2016 and January 2017, conducive for disease development. In spring 2017, one year after planting, four vineyards were monitored for gall development (Table 1). Sample ID 8 had the highest *A. vitis* abundance, and 30 out of 90 rated plants in the vineyard from the same shipment had visible galls.

Quantification of *A. vitis* in vineyard soil. *Agrobacterium vitis* abundance in vineyard 1 ranged from 1,244 to 64,224 copies / g soil and in vineyard 2 from 85 to 5,827 copies / g soil (Figure 5). Grapevine growth stage did not affect *A. vitis* abundance when data were combined (p = 0.059). Vineyard had a significant effect; *A. vitis* abundance was on average 6 times higher at vineyard 1 compared to vineyard 2 (p

< 0.001). Average soil pH was 7.4 for vineyard 1, which was higher than the average soil pH of 6.9 for vineyard 2 ($p < 0.001$), but pH did not affect *A. vitis* abundance ($p = 0.27$). On the other hand, the C/N ratio significantly affected bacterial abundance ($p < 0.001$) and was higher in vineyard 2 (C/N = 14.87) compared to vineyard 1 (C/N = 12; $p < 0.001$).

Discussion

A ddPCR assay to allow for sensitive absolute quantification of the plant pathogen *A. vitis* population in roots of grapevine nursery stock and vineyard soil was developed. For the purpose of this study, targeting of the *virA* gene was chosen because the VirA/VirG two-component regulatory system is located on the tumor-inducing plasmid and is essential for virulence (Stachel and Zambryski 1986, Wiese and Binns 2016). However, the *virA* primers do not amplify a product from a subset of tumorigenic *A. vitis* strains that carry vitopine-type plasmids (Burr and Otten 1999; Otten et al. 1996). Therefore, other primers, such as primers targeting *pehA*, were successfully tested to allow for adaptation of the assay. *PehA* primers are specific for *A. vitis* and target a gene coding for polygalacturonase located on the bacterial chromosome (Eastwell et al. 1995). However, the results of this study indicate that success of a ddPCR assay may be gene/primer specific and require optimization steps for each new target gene, because the assay did not work well when primer pair *virD2* was used.

Detection limits were compared to the study conducted by Johnson et al. (2013). The *virA* ddPCR assay was 10-fold more sensitive compared to the *virD2* qPCR MCH

assay when purified *A. vitis* DNA was used. However, MCH detected as few as 10 cells when grape tissue was inoculated, while the *virA* ddPCR assay was able to detect 100 cells when roots were spiked with *A. vitis* cells. Because PCR-inhibitory substances that might have been co-isolated from grapevine roots are diluted with ddPCR, they should only play a minor role and not affect the detection limit greatly. However, in qPCR, inhibitory substances may lead to a reduced assay efficiency, which in turn can lead to a quantification bias (Kim et al. 2014). Therefore, if the goal of a future study is to detect very few *A. vitis* cells, MCH is favorable. If quantification is desired, the ddPCR assay is the method of choice, but both methods will require more evaluation with naturally contaminated plants to determine their applicability under different scenarios. Increased sensitivity of ddPCR assays over qPCR assays has been reported in other studies where environmental DNA samples were used (Kim et al. 2014; Cavé et al. 2016), but others report comparable sensitivities (Dreo et al. 2014; Blaya et al. 2015). A major advantage of ddPCR over qPCR is the ability for absolute quantification, without the need of an external standard. This greatly facilitates comparability of data and experimental setup where standards are not readily available. However, ddPCR requires more time to set up, compared to qPCR. This is because droplets need to be generated and read, in addition to running the PCR cycles. Cost per well is about 3.9 times higher for ddPCR compared to qPCR. However, in qPCR each sample has to be run in triplicates and the cost of the standards needs to be included in the calculation. This makes ddPCR generally more cost-effective, but exact numbers depend on the individual set-up of the reactions (numbers of samples, points on the standard curve, number of replicates).

Because of the unequal distribution of *A.vitis* in dormant canes (Johnson et al. 2015), cuttings from the same plant in an infected nursery mother block will likely have varying bacterial numbers, as indicated by the variation of *virA* copy numbers in the replicate test samples. Because of this and the uncertainty of *A. vitis* location within roots, it is important to test a sufficient number of samples and to follow a root sampling procedure similar to the one developed in this study.

Although this study mainly describes a novel methodology and only a limited number of nurseries was tested via the *virA* assay, results indicate that the plants with the highest numbers of *A. vitis* were originating from Californian nurseries. The same cultivar Chardonnay on the same rootstock 3309 harbored 300 times more *A. vitis* cells when originating from California compared to Ontario. Conditions in California are not conducive to crown gall development because of a warmer climate with no winter freezing. Plants in grapevine mother blocks may be systemically infected with *A. vitis*, but do not develop disease. In addition, other economically important grapevine pathogens such as viruses (*Grapevine leafroll associated viruses* or Grapevine red blotch virus) or bacteria (*Xylella fastidiosa*) are of greater importance in California, leading to different testing foci. Because *A. vitis* is a non-regulated plant pathogen, no testing is required before shipment. However, plants imported to Canada or to other cool climate grape-growing regions in the United States, such as New York and Washington State, are at risk to develop crown gall disease upon planting. Thus, it is important to produce crown gall-free nursery stock and test purchased material with the newly developed sensitive methodologies, such as MCH and ddPCR, before planting. It is also important to emphasize that results cannot be generalized as only six nurseries

were compared in this study. A future study should increase the number of nurseries tested in varying regions worldwide in order to draw a potential significant correlation between nursery material origin and *A. vitis* abundance.

Indexing for presence or absence of the pathogen does not predict whether disease will occur in the vineyard. Disease development depends on pathogen density in the plant and environmental conditions that can lead to vine injuries, such as freezing temperatures (Burr et al. 1998). Monitoring for galls in the year following planting indicated that a high *A. vitis* concentration may be required to cause the development of galls. In addition, plants from the same shipment as sample ID 8 did develop galls in the vineyard, but plants from sample ID 9 did not, although the *virA* copy numbers found in the nursery stock were high. Sample ID 8 vineyard was located at a site with a higher elevation, but distant from Okanagan Lake, which moderates temperature. Sample ID 9 vineyard was located on a hill beside the lake, which allowed for better soil drainage and likely reduced freeze injuries. Therefore, many other factors beside the *A. vitis* concentration of nursery stock, such as site characteristics, management practices and environmental conditions, need to be taken into account.

Because bacteria on decaying necrotic grapevine roots in soil represent a second major source of *A. vitis* inoculum for replanted grapevines (Burr et al. 1998; Sule et al. 1995), the ddPCR assay was expanded to quantify the *A. vitis* soil population. Few reports on *A. vitis* abundance in soil exist (Bishop et al. 1988; Burr et al. 1987; Burr et al. 1995; Pu and Goodman 1993). Bacteria are present in higher numbers in soil associated with grapevine roots than with non-host rhizospheres (Bishop et al. 1988) and population density has been estimated by dilution plating to be 9000 CFU / g soil

(Pu and Goodman 1993) and between 10^3-10^7 CFU / g soil (Burr et al. 1987) in vineyards with crown gall symptoms. We found numbers of up to 64,000 *virA* copies / g soil, with higher numbers consistently present in the older vineyard. Because both vineyards are planted with cv. Chardonnay from the same nursery, grown on land with no previous grapevines and undergoing similar management strategies, we hypothesize that nursery material was infected with *A. vitis* before planting and that abundance of *A. vitis* in rhizosphere soil depends on age of the vineyard. In an older vineyard, more roots may decay and release bacteria into soil. In order to draw a significant correlation between vineyard age and *A. vitis* population in soil, and to recommend removal of grapevines at the onset of gall symptoms to prevent soil contamination, further studies are required, as the current one only tested a five and a three-year-old vineyard.

Grapevine growth stage did not have an effect on *A. vitis* abundance in soil. This is in contrast to the *A. vitis* population within grapevines, where a seasonal effect has been reported (Pu and Goodman 1993). The study reported detection of *A.vitis* in 32 and 25% of plants, 2 and 3 years after planting, respectively, when sampling occurred in April. Sampling in fall (Oct, Nov or Dec) did not result in *A. vitis* recovery. Soil likely serves as primary inoculum for roots of healthy grapevines, but roots of mature grapevines may serve as a reservoir of bacteria, allowing translocation of *A. vitis* through xylem sap pressure into trunk and canes in spring, as previously proposed (Pu and Goodman 1993).

Although the results of this study are based on a one-year survey, an interesting observation was that the *A. vitis* abundance in soil was correlated with the soil C/N ratio, with a high C/N ratio resulting in fewer bacteria. A promising future management

strategy may include application of soil amendments with a high C/N ratio, such as bark wood mulch and compost, but further studies are needed.

A potential application of the ddPCR assay lies within a national clean plant program to ensure that only healthy planting material is imported. In Canada, high numbers of plants are imported for the fast-growing wine industry from certified nurseries in France, Germany and the United States. However, plants are not tested for crown gall because *A. vitis* is a non-regulated grape pathogen and sensitive methodologies for crown gall testing were lacking until recently. The ddPCR assay developed here can reduce the time needed to screen grapevine stock for *A. vitis* abundance as the assay allows for quantification within 1-2 days, making efficient testing of high numbers of samples possible. Other methodologies, such as callusing of cuttings followed by plating on semi selective media, take 6 weeks for completion (Johnson et al. 2013). Pruning an excessive grapevine root system before planting is a standard procedure done by growers; and thus, the ddPCR assay can be regarded as non-destructive to the plant. However, a negative test result does not necessarily indicate that no bacteria are present in the rootstock and/or scion. Another application of the ddPCR assay could be for testing plants and soil in varying stages of the propagation process in grapevine nurseries. This could lead to the identification of the initial source of *A. vitis* and/or the stage of propagation where the infection occurs. Based on the results, nurseries could improve sanitation management within the propagation process.

Testing of nursery stock and vineyard soil via ddPCR prior to vineyard establishment can ensure that only uncontaminated grapevine stock is planted into

healthy soil. Other continuing studies should include the determination of nursery stock and vineyard soil *A.vitis* abundance threshold, in order to predict disease occurrence and to facilitate disease management.

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Table 1: Origin, cultivar and ddPCR results of tested grapevine nursery stock

Sample ID	Origin[a]	Nursery #	Cultivar	Rootstock	*virA* copies/0.3 g roots (± SD)[b]	Tukey grouping[c]	Gall occurence in %[d]
1	Canada	a	Merlot	3309	305 (± 364)	d	na[e]
2	Canada	a	Pinot Noir	3309	306 (± 144)	cd	0
3	Canada	a	Pinot Gris	3309	932 (± 456)	cd	na
4	United States	b	Cabernet Sauvignon	3309	17,208 (± 11,366)	b	1
5	Canada	c	Chardonnay	3309	885 (± 824)	cd	na
6	Germany	d	Riesling	3309	9,430 (± 18,967)	c	na
7	Canada	a	Pinot Noir	SO4	463 (± 466)	cd	na
8	United States	e	Chardonnay	3309	259,656 (± 71,434)	a	33
9	United States	f	Syrah	101-14	100,427 (± 19,516)	a	0

[a]Nursery stock originating from Canada and the United States were received from Ontario and California, respectively
[b]Numbers are means (n=5) followed by the standard deviation
[c]Different letters indicate significant differences between samples by Tukey's studentized range (HSD) test ($p < 0.05$)
[d]Vineyards with planted nursery stock from the same shipment as samples used for ddPCR were visited one year after planting and 90 plants monitored for gall development
[e]not applicable – vineyards were not monitored for gall development

Table 2: Vineyards used in the study

	Vineyard 1	Vineyard 2
Cultivar	Chardonnay	Chardonnay
Rootstock	101-14	3309c
Nursery	Nursery e	Nursery e
Year planted	2012	2014

Age at time of sampling	5	3
% sand/silt/clay	26/43/31	67/30/3
Previous land use	apple orchard	grassland

Table 3: *VirA* copy numbers[a] / 0.25 g soil in vineyard 1 and 2 over the 2017 growing season

	Bud Break	Bloom	Berries	Veraison	Harvest
Vineyard 1	8,803 (± 7,165)	12,115 (± 7,000)	7,095 (± 7,128)	22,068 (± 24,915)	8,731 (± 11,826)
Vineyard 2	1,047 (± 673)	614 (± (423)	745 (± 533)	2,328 (± 1,590)	1,922 (± 655)

[a] numbers are means (n=6) followed by the standard deviation

Figure legends

Figure 1: A gradient ddPCR assay was used to determine the optimal annealing temperature for the *virA* primer pair using pure culture *A. vitis* strain CG47 DNA. A temperature of 59.6°C resulted in the best separation between positive (higher amplitude) and negative droplets (lower amplitude baseline). NTC: negative template control

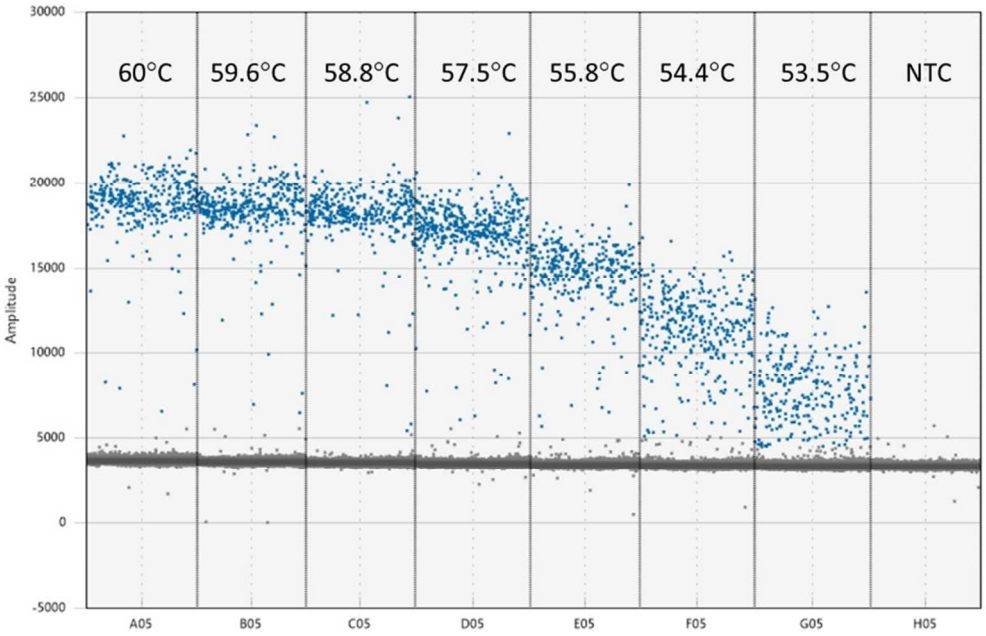
Figure 2: A gradient ddPCR assay was used to determine the optimal annealing temperature for the *pehA* primer pair using pure culture *A. vitis* strain CG47 DNA . Separation between positive (higher amplitude) and negative droplets (lower amplitude baseline) was equal at all the temperatures tested. NTC: negative template control

Figure 3: The detection limit of the *virA* ddPCR assay was determined using a 10-fold serial dilution of *A. vitis* strain CG47 DNA. A concentration of 0.1 pg/µl was detected

with a frequency of 100%. The experiment was repeated three times. NTC: negative template control

Figure 4: Quantification limit of the *virA* ddPCR assay in roots and vineyard soil spiked with serial dilutions of *A. vitis* CG47 cells. Data points represent the mean of three separate experiments. The lower limit of the linear range of the assay determined the quantification limit (1000 cells for roots and soil).

Figure 5: Log *virA* copy numbers per gram of rhizosphere soil. 1: vineyard 1, 2: vineyard 2. Soil was sampled at five grapevine growth stages in 2017; bud break: 02-May, bloom: 20-June, berries: 06-July, veraison: 28-August, harvest 10-October. Bars indicate standard error of the mean (n=6). Different lower case letters within one growth stage indicate differences of least squares means using the Welch's t-test at $p < 0.05$.



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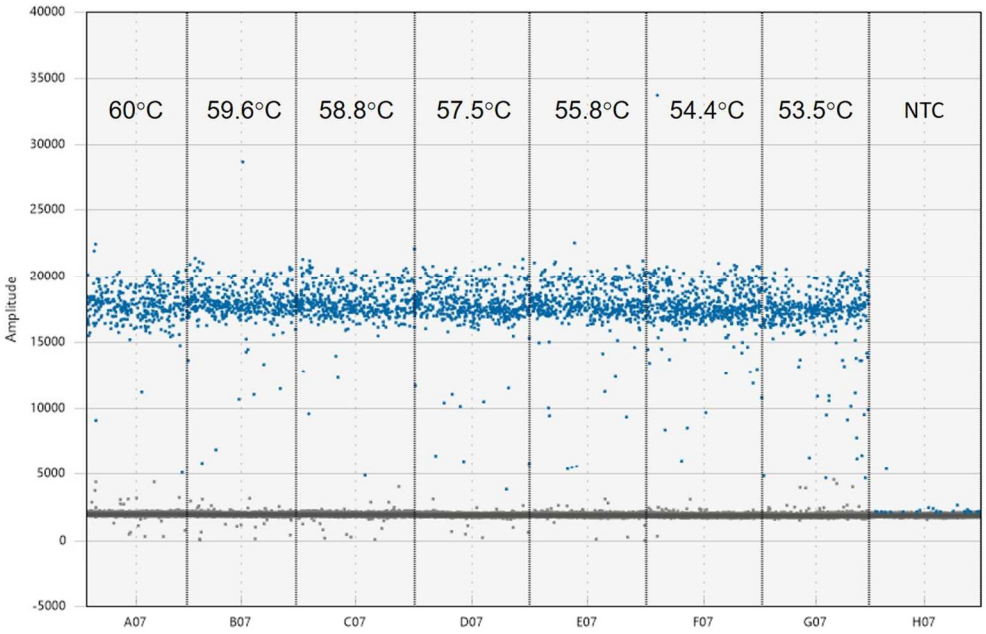

Figure 2: A gradient ddPCR assay was used to determine the optimal annealing temperature for the *pehA* primer pair using pure culture *A. vitis* strain CG47 DNA. Separation between positive (higher amplitude) and negative droplets (lower amplitude baseline) was equal at all the temperatures tested. NTC: negative template control

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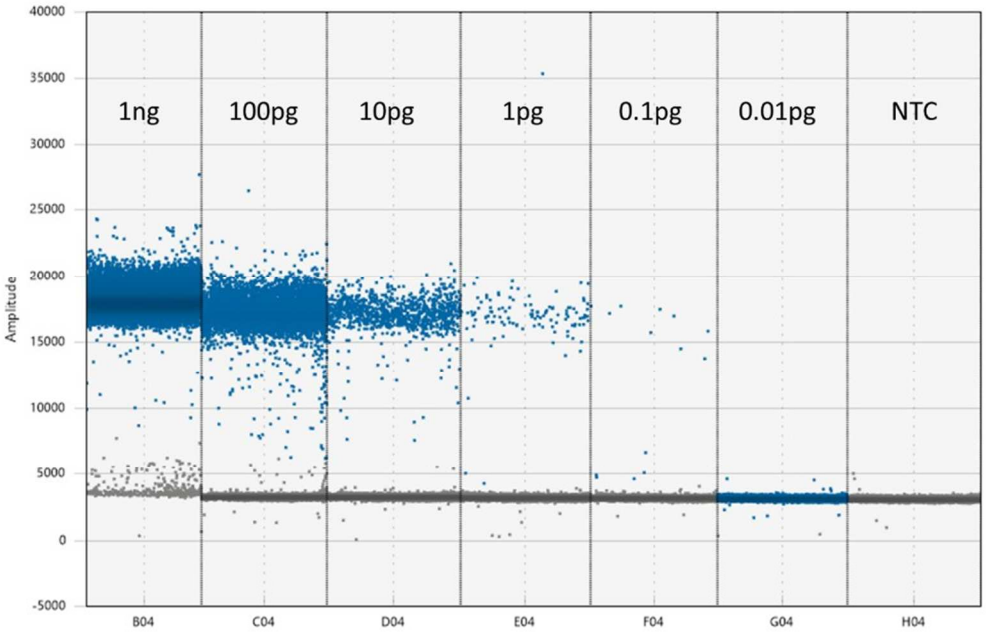

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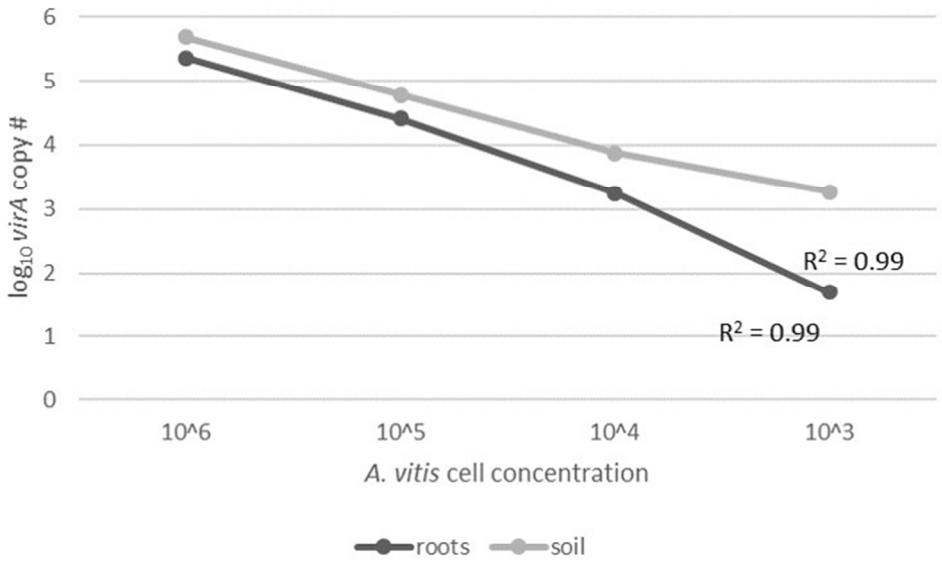

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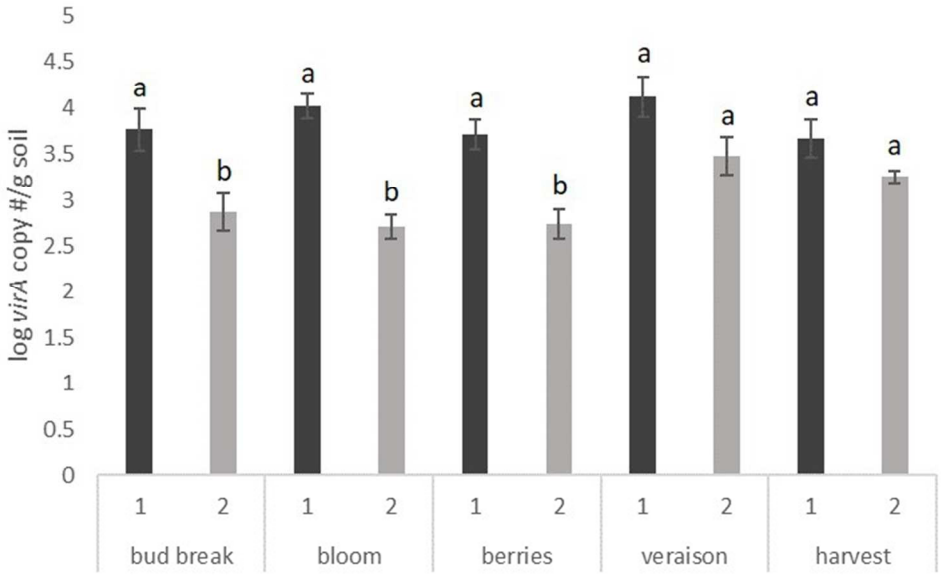

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127x76mm (150 x 150 DPI)