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Venturia inaequalis trapped: molecular quantification of airborne inoculum using volumetric and rotating arm samplers

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Abstract Site-specific high throughput monitoring of airborne ascospores of *Venturia inaequalis*, the causal agent of apple scab, can improve existing warning systems. A new qPCR assay was developed to quantify ascospores collected by a simple rotating-arm spore sampler. The qPCR assay was highly specific and sensitive, with a limit of quantification of 20 ascospores per sample. The new detection system was compared to sampling with a traditional Burkard volumetric spore trap and to microscopic quantification. During controlled ascospore release experiments in a closed environment, strong correlations (ρ: 0.96 to 0.99) were observed between the two types of samplers and the two methods of quantification but significantly larger numbers of spores (log difference: 0.43 to 0.69) were obtained when using the rotating-arm sampler and when using molecular quantification. During comparisons under outdoor conditions over a three-year period, reasonable correlations between the techniques (average ρ = 0.61) were observed. When rotating-arm samplers operate continuously they can get saturated but their counts still correlated better with those from the Burkard sampler than when they only operate during rain and until two hours after. This suggests that ascospores were also captured outside of rain events. Based on these comparisons, molecular quantification of spores captured with the rotating-arm sampler appears to be a sensitive and reliable method to determine airborne ascospores of *V. inaequalis* and holds promise as a tool to guide targeted fungicide applications in commercial orchards as well as to increase our knowledge of the aerobiology of this pathogen.

Keywords qPCR · Rotating-arm spore trap · Burkard spore trap · Aerial spore sampling · Apple scab

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Introduction

Apple (*Malus x domestica* L. Borkh) is a host to many economically important pathogens (Giraud et al. 2010). Apple scab in particular has been a serious problem in temperate climates for over a century. *Venturia inaequalis* (Cooke) G. Winter, the causal agent of apple scab, can be found in almost all areas where apples are produced, but it thrives under cool and moist spring conditions (Bowen et al. 2011; MacHardy 1996).

Sexual fruiting structures, i.e. pseudothecia, are formed in dropped leaves. Within these pseudothecia, the ascospores, the primary inoculum of *V. inaequalis*, develop and mature during winter. Ascospores are

dispersed by rain and wind in spring, causing the first infections of the very susceptible newly formed leaves, flowers and fruitlets (Bowen et al. 2011; MacHardy 1996; MacHardy and Gadoury 1986). Temperature and leaf wetness period determine the ascospore-mediated primary infection levels, as first described by Mills (1944) and later refined by Mills and Laplante (1954) and MacHardy and Gadoury (1989). As the season progresses, additional damage can occur via secondary conidia-mediated infection cycles. After infection, brown necrotic lesions appear on leaves and fruit, ultimately resulting in fruit of reduced size and marketability (MacHardy 1996). The severity of the disease strongly depends on sanitation practices and fungicide applications (Agrios 2005; Holb 2008). Growers who prevent or counteract ascospore-mediated infection can greatly reduce disease severity (Agrios 2005).

Consumers are demanding a reduction or even complete elimination of the use of synthetic fungicides (Ahmed et al. 2011; Avermaete et al. 2017). Integrated Pest Management (IPM), compulsory in Europe since 2014, has already led to a reduction in the use of synthetic fungicides, but a further reduction in the number of treatments is desired, for example by better targeting fungicide applications (Carisse et al. 2010). Weather-based infection forecasting systems (e.g. RIMpro) and the actual airborne ascospore concentrations can give growers early warnings regarding scab (Rosenberger 2016). A standard tool to determine airborne spore concentrations is the "Burkard 7 day volumetric spore trap" (Frenz 1999). Conventionally it is used to trap airborne particles larger than 2 μm, including fungal spores, and quantify them microscopically. However, distinguishing *V. inaequalis* ascospores (5–7 μm wide and 11–15 μm long) from other airborne particles requires a considerable degree of skill. Moreover, airborne spores tend to clump together, which makes quantifying individual ascospores challenging and time-consuming (Irdi et al. 2002; Lacey and West 2006). Also, Burkard traps are costly, and unwieldy in an orchard. For those reasons an alternative sampling tool is desired that would allow accurate and affordable in-field measurement of the aerial ascospore load in individual orchards (Carisse et al. 2005). Rotating-arm samplers such as the Rotorod samplers (IQVIA, Plymouth Meeting, USA) might be appropriate for this purpose as they are relatively inexpensive, small in size, less affected by wind speed and easy to deploy, even at several heights within the rows of an orchard setting (Aylor 1993; Chandelier et al. 2014). The rotating–arm sampler uses two rods covered with either grease or sticky tape to sample airborne particles, including spores. The collection efficiency of the rotating-arm sampler is high for particles equal to or larger than 10 μm (Chandelier et al. 2014; Frenz 1999), which makes it appropriate for the sampling of *V. inaequalis* ascospores. Direct comparison of the Burkard spore trap and the rotating-arm sampler results in different levels of correspondence, mostly dependent on particle size and wind speed (Aylor 1993; Crisp et al. 2013; Irdi et al. 2002). Recently, these spore traps have been combined with quantitative real-time PCR (qPCR)-based methods (Cao et al. 2016; Chandelier et al. 2014; Klosterman et al. 2014). Chandelier et al. (2014) used the combination of a rotating-arm sampler and a qPCR assay to collect and quantify airborne inoculum of *Hymenoscyphus fraxineus*. Spore counts ranging from 20 to more than 10 million could be quantified reliably, making it possible to quantify spores in fields with both low and high inoculum pressure. However, the possibilities for monitoring ascospores of *V. inaequalis* with such rotating-arm samplers have not yet been explored. Meitz-Hopkins et al. (2014) monitored airborne inoculum of *V. inaequalis* with a Burkard volumetric spore trap and a qPCR-based method. They presented a new set of *V. inaequalis*-specific primers, targeting the rDNA internal transcribed spacer region 2 (ITS2). The fungal ITS2 region is a multi-copy and often species-specific region, making it ideal for specific and sensitive molecular detection (San-Blas and Calderone 2008; Turenne et al. 1999). However, validation of specificity was limited for the Meitz-Hopkins et al. (2014) assay. Another qPCR assay targeting *V. inaequalis* was designed by Gusberti et al. (2012), based on the sequence of ATP-binding cassette transporter 2 (ABC2). Fewer specificity issues were expected in their study, but given the single copy nature of the target, the quantification limit in this assay was set at 0.5 pg genomic DNA per PCR reaction. This allows quantification of the pathogen in infected leaves but not detection of small numbers of spores in airborne inoculum.

In this study we have designed and validated a qPCR assay for specific and sensitive detection and quantification of *V. inaequalis* ascospores. Second, we

compared the microscopic versus molecular quantification of ascospores captured using an inexpensive rotating arm trap versus the standard Burkard volumetric spore trap under controlled and semi-field conditions.

Materials and methods

Spore traps

Seven-day volumetric Burkard spore traps (Burkard Manufacturing, Rickmansworth, UK and Burkard Scientific, Uxbridge, UK) were used to sample air at 10 l/min, catching the spores on Melinex tape coated with a thin layer of Vaseline (VWR International, Oud-Heverlee, Belgium). The tape was cut into segments, with one segment representing one day. The segments intended for molecular quantification were placed in individual microcentrifuge tubes and stored at −20 °C until DNA extraction. Segments intended for microscopic quantification were mounted on glass slides using glycerine jelly and ascospores were counted microscopically using a Nikon Eclipse TE 300 at 250x magnification for the controlled ascospore release experiments and a Zeiss CBP 3293 at 200x magnification for the outdoor experiments.

The rotating-arm spore traps, based on the design of Chandelier et al. (2014), contained a 12 V electromotor (2100 to 3150 RPM) powering a 4-cm radius arm, which held two replaceable acrylic sampling rods (40x2x2 mm). The spore captures of rotating arm-samplers rotating at 2100, 2300, 2600, 2900 or 3150 RPM (two samplers at each speed) were compared after placing them together on top of leaves releasing ascospores in a controlled environment. During each of the four experiments, no significant differences (p values of 0.20, 0.91, 0.32 and 0.78) and no trends were observed in the numbers of captured spores over the range of rotational speeds. The average CV per experiment was 30.1% (data not shown). Rods intended for molecular spore quantification were covered with 60 mm^2 (30 × 2 mm) double-sided adhesive tape (Tesa®, Hamburg, Germany). After sampling, both rods were placed in a single microcentrifuge tube and stored at −20 °C until DNA extraction. Rods intended for microscopic quantification were covered with a thin layer of Vaseline (60 mm^2). After sampling, rods were placed into an adaptor for support, a microscope cover glass was placed on top and ascospores were counted using an Olympus BX40F4 microscope at 300x magnification for the controlled ascospore release experiments and a Zeiss CBP 3293 at 200x magnification for the outdoor experiments.

DNA extraction from ascospores on sampling rods or tape

Sample pre-processing and DNA extraction was based on De Backer (2012) with further optimization of homogenization time and speed as well as DNA extraction method. The pre-processing started with the addition of 200 µl 0.1% IGEPAL® CA-630 and 200 mg of 0.5 mm Zirconium/Silica beads (BioSpec Products, Bartlesville, USA) to the microcentrifuge tubes containing the rods or tape. The samples were homogenized twice for 1 min at 5000 rpm (Bertin Technologies, Precellys 24, Montigny le Bretonneux, France). The tubes were then incubated at 65 °C for 15 min in a shaking heat block (neoLab, thermomixer, Heidelberg, Germany) at 1400 rpm. DNA was extracted with the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) using the CTAB lysis buffer. To obtain consistent results, tubes were vortexed for a few seconds after the addition of the lysis buffer and then centrifuged at 5500 rpm (Witeg, CF-5, Wertheim, Germany) for 10 s, followed by a 30 min incubation at 65 °C. The remaining steps were conducted according to the manufacturer's instructions including final elution in 50 µl elution buffer.

qPCR assay

Sequences of the rDNA ITS regions of 30 accessions from 21 *Venturia* species, including seven accessions of *V. inaequalis* (Online resource 1), were aligned using MEGA6 (Tamura et al. 2013). Based on the location of species-specific nucleotides, primer and probe characteristics and inter-primer distance, a *V. inaequalis*-specific forward primer (VinaeqF2: 5'-GCGCATCC CCACCCTC-3′), a non-specific reverse primer (VinaeqR1: 5'-AGATCCGTTGTTGAAAGTTT TGTTTT-3′) and a specific TaqMan probe (VinaeqP: 5′-6-FAM-CGTTTGCGAGGGGCCCCG-BHQ-3′) were manually designed. All reactions were conducted in a reaction volume of 25 µl, containing 5 µl of DNA template, 500 nM of each primer, 200 nM VinaeqP and the Maxima™ Probe qPCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA). qPCR reactions were

performed in a 7900HT qPCR system (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA). Amplification conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 1 min at 60 °C. Primers VinaF2 and VinaR (Meitz-Hopkins et al. 2014) at 800 nM and the SYBR Green Master Mix (Thermo Fisher Scientific, Massachusetts, USA) were used to benchmark the newly designed primers. Two technical replicates were used per sample. All qPCR runs contained a no-template control and a 10-fold dilution series of cloned DNA target (10^6 to 10^3 target copies per tube). The copy number was established as described by Whelan et al. (2003). To obtain the cloned target, the rDNA ITS region from *V. inaequalis* was PCR-amplified using primers ITS1 and ITS4 (White et al. 1990) and ligated into the PCRII-TOPO vector according to Van Poucke et al. (2012). Based on the linear regression of the quantification cycle (Cq) versus the log of the number of target copies, the Cq value of each sample could be translated into the number of target copies; these were comparable between qPCR runs.

Isolates used to test the specificity and sensitivity of the qPCR assay are listed in Table 1. They were chosen based on genetic relation to the target or potential presence in or near an apple orchard. Genomic DNA (gDNA) was extracted from 100 mg fresh weight of mycelia using the 30 min CTAB lysis method from the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany). DNA concentrations were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and diluted to 100 pg per PCR reaction for the specificity tests.

Assay sensitivity and standard curves were determined using *V. inaequalis* gDNA extracted from mycelium (10-fold serial dilutions from 1 ng per PCR reaction to 1 fg DNA per PCR reaction from four separate conidial isolates) as well as from ascospores (two 10-fold serial dilutions from 5000 to 5 spores spiked on either Burkard tape or sampling rods), together with a cloned target dilution series. Ascospores were harvested from wetted infected leaves placed on top of a grid. Spores were guided into a microcentrifuge tube using a funnel and a vacuum pump (Welch, WOB-L pump 2522, Germany). The spores were suspended in water and their concentration was determined using a Bürker counting chamber. Using these standard curves, the Cq of any sample could eventually be translated into an equivalent number of ascospores.

Comparison of quantification methods and sampler types under controlled ascospore release conditions

Leaves were collected from heavily infected orchards during leaf drop and stored outdoors in mesh bags to allow natural development and maturation of pseudothecia. After three months the bags were placed at 20 ± 5 °C for three weeks to complete and speed up the ripening process of the developed asci. Before the start of the experiment, the maturity of the ascospores was examined via the squash mount technique (Gadoury and MacHardy 1982). Leaves were then spread evenly on the floor of a transparent plastic tent (approximately 3 × 4 × 2 m) placed in a greenhouse at 20 ± 5 °C. Four Burkard spore traps and four rotating-arm samplers were placed above the leaves with the rotating-arm sampler rods and the air intake of the Burkard samplers at similar heights (30 ± 2 cm). Before the start of every experiment, leaves were redistributed to ensure a homogeneous sampling. Ascospore release was induced by spraying the leaves with tap water using a water hose until they were moist. Leaves were moistened twice: at the start and halfway through each 3-h trapping experiment. For each type of sampler, two samples were analysed microscopically and two were analysed molecularly as described above. Four such trapping experiments were conducted during two consecutive days at similar inoculum levels (= one period). Leaves were then collected in the mesh bags and kept dry at 20 ± 5 °C for several weeks, after which the inoculum levels in the leaves had dropped and the trapping experiments were repeated. This procedure was conducted three times in total (= three periods) and allowed trapping over a range of 3 to 4 log levels of the inoculum.

Comparison of quantification methods and sampler types under semi-field conditions

The samplers and quantification techniques were also compared in an outdoor environment. To establish a sufficiently high and homogeneous ascospore source, the samplers were not placed in a regular orchard but on top of collected infected leaves in a nearby field. We designated this as “semi-field conditions”. During three consecutive years, leaves infected with *V. inaequalis* were collected after leaf drop and stored in mesh bags in the field to allow natural maturation of pseudothecia during winter. In February of the subsequent year (i.e.,

Table 1 Specificity of the qPCR assay when using the target (*Venturia inaequalis*) and 18 non target fungal species that are either genetically closely related to the target or that are frequently found in or nearby apple orchards. All samples were tested at 100 pg per reaction volume. Mean quantification cycle (± stdev) of two technical replicates of the assay with the VinaeqF2-VinaeqR1 primers and the VinaeqP probe

Pathogen	Isolate code	Origin	Cq
Venturia inaequalis	4 s	*Malus* x *domestica*	22.6 ± 0.4
Venturia asperata	INRA	NK	NA
Venturia cerasi	CBS 444.54	*Prunus cerasus*	NA
Venturia chlorospora	CBS 253.57	*Salix americana*	NA
Venturia pyrina	KUL1	*Pyrus communis*	NA
Venturia saliciperda	CBS 214.27	NK	NA
Alternaria infectoria	185	*Pyrus communis*	NA
Backusella recurva	CBS 318.52	*Fragaria sp.*	NA
Botrytis cinerea	250	*Malus* x *domestica*	NA
Epicoccum nigrum	75 K	NK	NA
Colletotrichum acutatum	KULA59	*Malus* x *domestica*	NA
Fusarium graminearum	1745	NK	NA
Fusarium oxysporum	151 K	NK	NA
Monilinia sp.	378	*Malus* x *domestica*	NA
Neonectria ditissima	171	*Malus* x *domestica*	NA
Neofabraea sp.	KUL2	*Malus* x *domestica*	NA
Nigrospora sp.	KUL3	*Malus* x *domestica*	NA
Penicillium expansum	226	air	NA
Rhizopus sexualis	CBS 336.39	*Fragaria sp.*	NA

NA = no amplification

NK = not known

February of 2016, 2017, 2018), the leaves were placed under netting on the bottom of two shallow pits (pits placed 25 m apart, pit diameter = 3 m). Samples were taken from April 7–June 8, 2016; April 5–July 4, 2017; and April 20–June 8, 2018. In all years, a single Burkard trap was positioned in the centre of each pit on top of the leaves. In 2016, molecular and microscopic analysis switched between the two Burkard traps, while in 2017 and 2018 the type of analysis remained consistent per Burkard trap. Rotating-arm samplers were also positioned on top of the leaves (30 ± 2 cm above the leaves), but due to limitations in terms of space and number of samples that could be processed, different numbers of samplers and different timing of sampling (continuous sampling versus associated with rain events) were used in the different years (Table 2). In 2017, three rotating-arm samplers were also placed in each pit: one was assigned to microscopic quantification, and two to molecular quantification. One of the latter sampled only during daylight during rain events and until 2 h after the rain was no longer detected by the sensor. The other sampled continuously, from the day after a rain event until the day after the next rain event. Daily counts of Burkard samples were merged for comparison with the sampling periods of the rotating arm samplers. In 2018, two rotating-arm samplers, sampling only in daylight during rain and until two hours after a rain event, were placed in one of the pits. During this experiment, for each rotating-arm sampler one rod was assigned to microscopic quantification and the other rod to molecular quantification to allow direct comparisons of quantification method within the same sampler. Counts were doubled to allow comparison with normal captures (two rods per sample). In 2018, owing to several overloaded samples, microscopic quantification was only possible for three sampling periods (24 April - 27 April, 27 April - 30 April and 30 April - 4 May). Burkard samples were taken daily, although only samples from days with rain events were quantified microscopically. Rainfall (in mm) was collected by a rain

Table 2 Distribution of samplers (Burkard versus rotating-arm) for the different quantification methods (molecular versus microscopic) and sampling triggers (rain sensor versus continuous) in the three years of the semi-field trials

Year of leaf collection	Year of ascospore capture experiment	Number of Burkard spore traps (and pit location)		Number of rotating-arm samplers (and pit location)			
		Molecular	Microscopic	Molecular		Microscopic	
		Continuous	Continuous	Continuous	Rain sensor	rain sensor	
2015	2016	1 (switching between pit 1 and pit 2)	1 (switching between pit 1 and pit 2)	0	0	0	
2016	2017	1 (pit 1)	1 (pit 2)	2 (1 in each pit)	2 (1 in each pit)	2 (1 in each pit)	
2017	2018	1 (pit 1)	1 (pit 2)	0	2 (1 rod per sampler in pit 2)	2 (1 rod per sampler in pit 2)	

gauge and wind speed data were recorded with a weather station (Priva, De Lier, Netherlands).

Data analyses

Data obtained during the controlled ascospore release experiments were not normally distributed following the Shapiro-Wilk test. After log transformation, the distribution of differences was symmetrical (Shapiro-Wilk test), allowing the use of the Wilcoxon signed-rank test to compare the different quantification methods. The differences between the spore counts obtained by the different quantification techniques of the semi-field experiments were analysed using the non-parametric sign test. All correlations and their statistical significance (p value) were calculated using the Spearman rank correlation test. Statistical analyses were performed using Jump Pro 13 (SAS Institute) with a significance level $\alpha = 0.05$.

Results

Assay sensitivity, specificity and standard curves

Ten-fold serial dilutions from 1 ng per PCR reaction to 1 fg DNA per PCR reaction allowed an evaluation of the sensitivity of the qPCR analysis. As little as 10 fg gDNA of *V. inaequalis* and 10 plasmid DNA copies per PCR reaction could be detected reliably at a Cq value (± stdev) of 35.3 (± 0.4) and 34.4 (± 0.5), respectively. Smaller amounts (1 fg gDNA per PCR reaction) were occasionally still detected. When using gDNA as well as plasmid DNA (pDNA), standard curves showed a clear linear relationship over a dynamic range of 6 and 7 log levels, respectively (Fig. 1).

Specificity was determined using gDNA from 19 fungal species, including six *Venturia* species. Only DNA of the target organism was amplified (Table 1), indicating that the qPCR assay was specific for *V. inaequalis*. The probeless qPCR assay developed by Meitz-Hopkins et al. (2014) was more sensitive than the new assay but a non-specific amplification was observed with *Venturia saliciperda* (Cq = 35.9 ± 0.3). Therefore, we chose to develop our own, more specific Taqman assay (Heid et al. 1996).

Additional standard curves were established to translate the number of target copies to the number of spore equivalents for each substrate of the spore capture

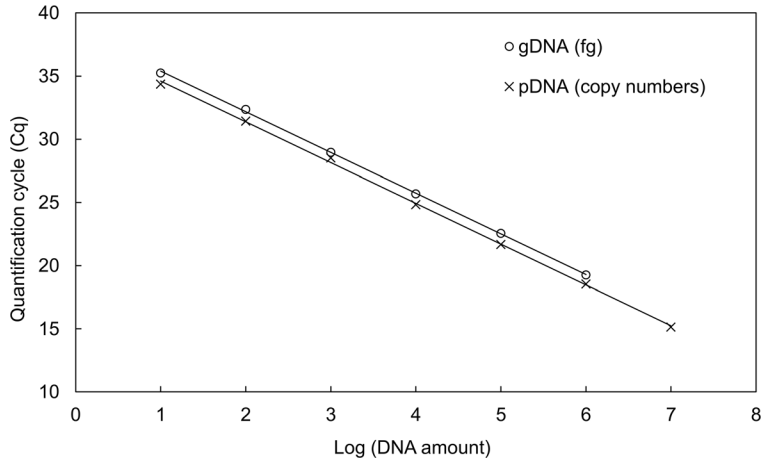

Fig. 1 Linear relationship between gDNA (in fg) and pDNA (in target copy number) derived from the qPCR analysis with the VinaeqF2-R1 primers and the VinaeqP probe. The regression curves for gDNA ($Cq = -3.22 \times \log(gDNA) + 38.62$; $R^2 > 0.99$) and pDNA ($Cq = -3.23 \times \log(pDNA) + 37.84$; $R^2 > 0.99$) were derived from four and two 10-fold dilution series, respectively, with two technical replicates each time. Standard deviations were always <0.25 for gDNA and < 0.2 for pDNA and were therefore omitted from the graph

devices (rods versus Burkard tape) and the DNA extraction method used. For the rotating-arm sampler this resulted in the standard curve: log (spore equivalents) = 0.936 x log (plasmid copy number) - 0.073 ($R^2 > 0.99$). For the Burkard sampler this was: log (spore equivalents) = 0.793 log (plasmid copy number) + 0.436 ($R^2 >$ 0.99). The limit of quantification (LOQ) was set at 20 spores per substrate. For the rods this was obtained at an average (± stdev) Cq value of 33.2 (± 0.5) and for the Burkard tape at an average (± stdev) Cq value of 32.8 (± 0.5). Below this level detection was only qualitative. For example, 5 spores were still detected at an average Cq value of 36.2 (± 0.4), but were not detected in one of the two technical replicates.

Comparison of quantification methods and samplers under controlled ascospore release conditions

The numbers of ascospores detected using the two sampling devices (Burkard sampler versus rotating arm sampler) and the two quantification methods (microscopic versus molecular) are presented in Fig. 2, using the Burkard sampler with microscopic quantification as the reference technique. During each trapping experiment, the difference between the two replicate spore samplers of each technique was only 0.23 ± 0.16 log units (average ± stdev). The comparison of the spore trap systems and methods of quantification is shown in Table 3. All techniques were strongly correlated (Spearman's rank correlation coefficient ranging from 0.96 to 0.99) but yielded a significantly different number of spores according to the Wilcoxon signed-rank tests. The rotating arm samplers captured significantly more spores than the Burkard samplers for each quantification method (Table 3). The molecular quantification yielded a significantly larger number of spores than the microscopic quantification for each capture device (Table 3).

Comparison of quantification methods and sampler types under semi-field conditions

In 2017, the Burkard spore traps and the rotating-arm samplers were placed in pits at two locations (25 m apart). No significant difference was observed between the two pits based on the sign test and using the most sensitive detection method (continuously rotating rotating-arm sampler with molecular quantification: $p = 0.82$). Hence, the mean values of the two pits were used to evaluate the rotating-arm samplers. In 2018 the two rotating-arm samplers were compared in the same pit only.

Over the course of the ascospore release season in 2016, 2017 and 2018, the number of spores (or spore equivalents) varied between type of sampler and/or quantification technique (Fig. 3a–c). Table 4 shows the correlation coefficient and the p value according to the sign test of the spore trap systems and methods of quantification under semi-field conditions. In 2016 (Fig. 3a), a correlation was observed between the molecular and microscopic quantification of the Burkard

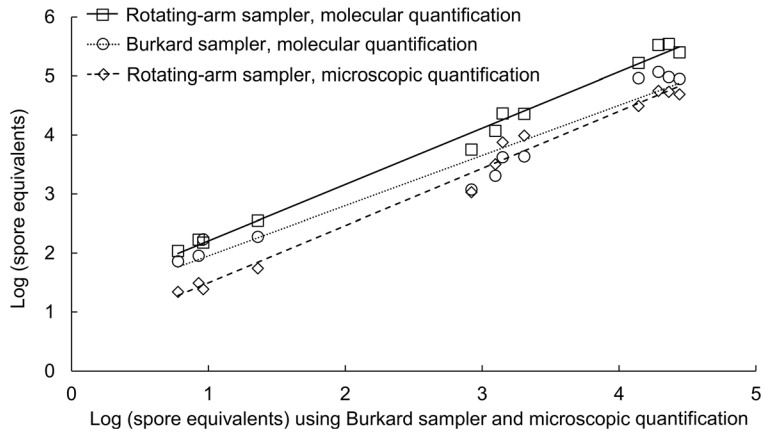

Fig. 2 Correlation between microscopic quantification of spores collected using the Burkard spore trap and (1) spores trapped by a rotating-arm sampler and quantified by qPCR, (2) spores trapped by the Burkard spore trap and quantified by qPCR and (3) spores trapped by rotating-arm samplers and quantified microscopically. Each data point is the average of two measurements with an average difference of 0.23 ± 0.16 log units between the two replicate spore samplers (not shown). The line equations are $y = 0.955 \times x + 1.250$, $y = 0.851 \times x + 1.101$, and $y = 0.970 \times x + 0.525$ with x being the log of the number of spore equivalents of the microscopically quantified Burkard samples and y being the spore equivalents registered according to (1), (2) and (3), respectively

spore trap samples but the molecularly quantified samples were significantly higher. A weak correlation was observed between rainfall and spore release from 22 March until 8 June (Burkard molecular quantification: $\rho = 0.34$, p value = 0.01, Burkard microscopic quantification: $\rho = 0.47$, p value = 0.0004) but a strong correlation was found from 5 April until 25 May (Burkard molecular quantification: $\rho = 0.71$, p value <0.0001, Burkard microscopic quantification: $\rho = 0.77$, p value <0.0014).

In 2017 (Fig. 3b) a correlation ($\rho = 0.62$) was again found between the microscopically and molecularly quantified Burkard spore trap samples with the number of detected spores from the molecularly quantified samples also being significantly larger (Δ spore equivalents = 4448 ± 7248; p value <0.0001). The method of quantification was not significantly different for the samples from the rotating arm samplers, however. The data from the continuously sampling rotating-arm samplers when quantified molecularly correlated with those from both molecularly and microscopically evaluated Burkard samples (Table 4). The molecularly quantified samples from the rotating arm samplers were not significantly different from the molecularly quantified Burkard samples. A moderate correlation ($\rho = 0.51$) was found between the data from the rotating-arm sampler with the rain sensor and the Burkard spore traps. The samples from the continuously rotating versus rain sensor-based rotating arm samplers were well correlated ($\rho = 0.68$, $p = 0.0008$) but significantly more spores were detected when sampling continuously. Overall, the Burkard sampler quantified by microscope yielded the lowest number of spores (2619 ± 4506, excluding zero detections). In 2017 a clear correlation was present

Table 3 Average difference between the means of each pair of techniques during the tests under controlled ascospore release conditions. *P* value is based on the Wilcoxon signed-rank test

technique	to technique	Δ log (spore equivalents)	*p* value
Burkard / molecular	Burkard / microscopic	0.68	<0.0005
Burkard / molecular	Rotating-arm / microscopic	0.27	0.04
Rotating-arm / microscopic	Burkard / microscopic	0.43	<0.0005
Rotating-arm / molecular	Burkard / molecular	0.43	0.001
Rotating-arm / molecular	Burkard / microscopic	1.12	<0.0005
Rotating-arm / molecular	Rotating-arm / microscopic	0.69	<0.0005

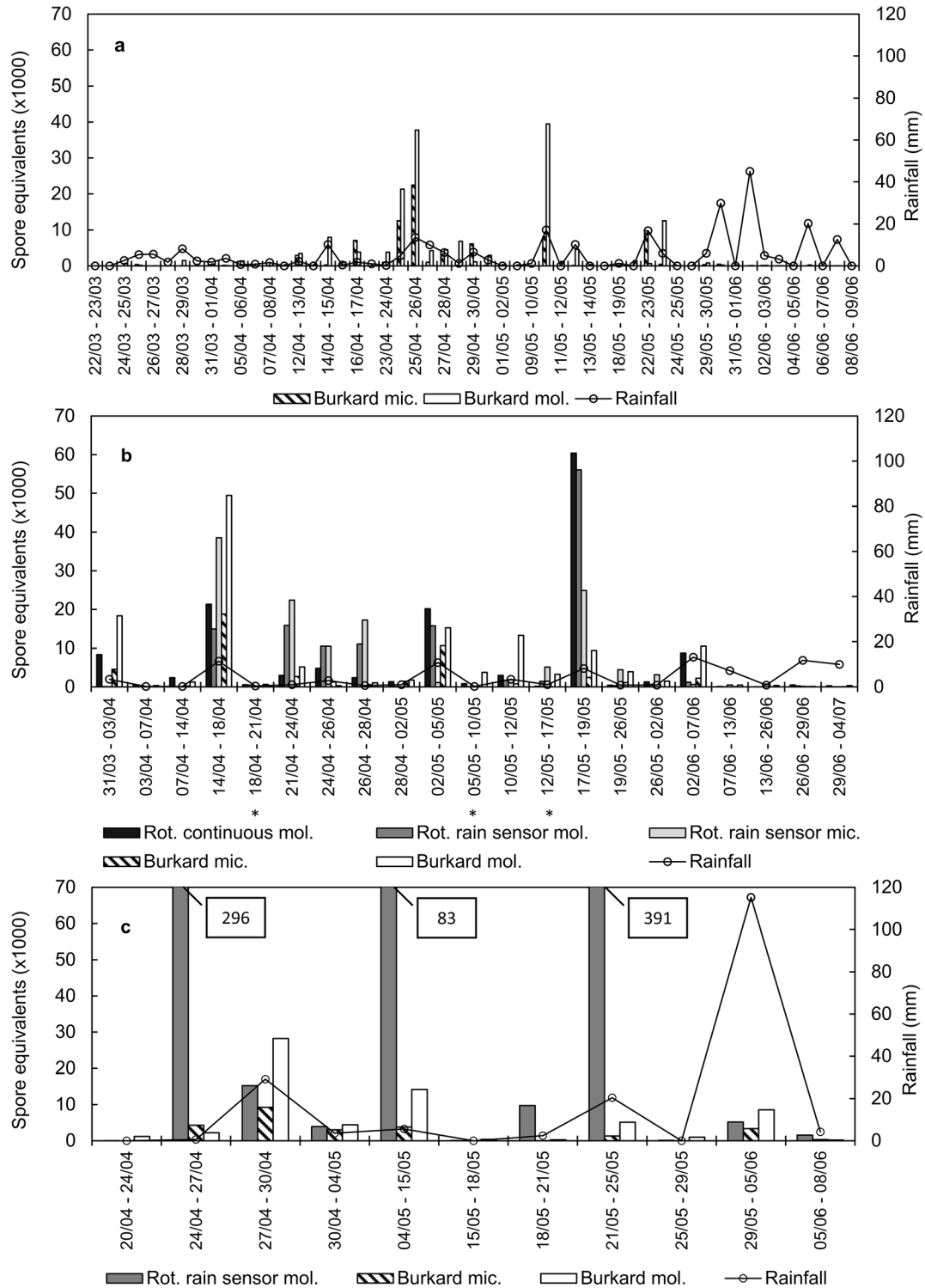

Fig. 3 Number of spores detected in the semi-field trial of 2016 (**a**), 2017 (**b**) and 2018 (**c**) during which spore traps were positioned above heavily infected leaf material in the field. In 2018, the dominant wind direction was suboptimal during the following periods: 27 Apr - 30 Apr, 29 May - 05 Jun, 05 Jun - 08 Jun. Burkard = Burkard sampler, Rot = rotating-arm sampler, mol. = molecular quantification, mic. = microscopic quantification. There are 53, 21 and 11 sampling points in 2016, 2017 and 2018, respectively. Sampling points during which the Burkard samples were not quantified microscopically in 2017 are indicated with *

Table 4 Spearman's correlation coefficients and the *p* value based on the sign test during the semi-field trials of 2016, 2017 and 2018

technique	to technique	Spearman's ρ	*p* value of sign test
2016			
Burkard / microscopic	Burkard / molecular	0.69*	<0.0001
2017			
Burkard / microscopic	Burkard / molecular	0.62*	<0.0001
Burkard / microscopic	Rotating-arm / continuous / molecular	0.78*	0.0002
Burkard / microscopic	Rotating-arm / rain sensor / molecular	0.52*	0.07
Burkard / microscopic	Rotating-arm / rain sensor / microscopic	0.40	0.19
Burkard / molecular	Rotating-arm / continuous / molecular	0.66*	0.19
Burkard / molecular	Rotating-arm / rain sensor / molecular	0.51*	0.08
Burkard / molecular	Rotating-arm / rain sensor / microscopic	0.37	>0.99
Rotating-arm / rain sensor / microscopic	Rotating-arm / continuous / molecular	0.40	0.66
Rotating-arm / rain sensor / microscopic	Rotating-arm / rain sensor / molecular	0.83*	>0.99
Rotating-arm / rain sensor / molecular	Rotating-arm / continuous / molecular	0.68*	0.03
2018			
Burkard / microscopic	Burkard / molecular	0.74*	0.065
Rotating-arm / rain sensor / molecular	Burkard / molecular	0.55	>0.99
Rotating-arm / rain sensor / molecular	Burkard / microscopic	0.75*	0.001

p values <0.05 of Spearman's ρ are indicated with *

between rainfall and spore release from 31 March to 2 June (ρ ranged from 0.54 to 0.79, *p* value <0.05).

Similarly to 2016 and 2017, in 2018 (Fig. 3c) a strong correlation was again seen between the Burkard samples quantified molecularly and microscopically ($\rho = 0.74$, $p = 0.009$). Again, more spores were detected in the molecularly quantified samples but due to a larger variance and non-symmetry of the data, this difference was not significant. A significant difference and a correlation was found between the rotating-arm sampler quantified molecularly and the Burkard spore trap quantified microscopically during this year as well. No significant difference nor correlation was found between the rotating-arm sampler and the Burkard sampler quantified molecularly. On three occasions in 2018 (24 April - 27 April, 27 April - 30 April and 30 April −04 April) one rod of a rotating-arm sampler was evaluated microscopically and one molecularly. These data correspond clearly with 254,932 versus 296,873 spores, 8108 versus 15,241 spores, and 416 versus 3918 spores for the microscopic versus molecular quantification during those three sampling periods, respectively. Rainfall correlated with all sampling techniques and spore traps (ρ ranging from 0.61 to 0.71 and *p* value ranging from 0.01 to 0.048). Wind speed (data not shown) had no significant effect in any of the years (ρ ranging from −0.19 to 0.37 and *p* value ranged from 0.053 to 0.8).

Discussion

To improve the warning systems for apple scab based on the measurement of the local ascospore pressure, we have developed a system with potential for sensitive, accurate and fast detection of ascospores. It is also affordable and applicable to a considerable number of samples (orchards). We tested a rotating-arm-based sampling device and coupled it to a novel specific and sensitive qPCR assay.

The new qPCR assay has improved sensitivity and/or specificity over existing assays (Daniëls et al. 2012; Gusberti et al. 2012; Meitz-Hopkins et al. 2014). We obtained a limit of quantification (LOQ) of 10 fg genomic DNA, which together with optimized DNA extraction techniques resulted in the reliable quantification of 20 ascospores when captured by the rotating-arm sampler, similar to the limit of detection reported for other fungi by Billones-Baaijens et al. (2018); Cao et al. (2016); Chandelier et al. (2014). Both in silico and in vitro analyses indicate complete specificity of the assay, and no high end Cq values were observed in absence of target DNA.

The first comparison of the rotating-arm sampler and the Burkard sampler, using microscopic as well as molecular spore quantification, was made during controlled

ascospore release events in a closed environment. This eliminated variation due to wind, rain and provided a homogeneous inoculum source. Spore counts were higher when using the rotating arm sampler and/or when using molecular quantification, making the novel technique (rotating arm sampler coupled to molecular quantification) the most sensitive. The lower spore numbers detected with the microscopic quantification may be due to ascospore loss while mounting the Burkard slides or rods, due to difficulties distinguishing ascospores that were clumped together, or due to difficulties to discriminate *V. inaequalis* spores from other airborne particles (Irdi et al. 2002; Tormo-molina et al. 1996). Studies comparing rotating-arm samplers to the Burkard spore trap in a controlled environment such as a greenhouse are limited. Heffer et al. (2005) compared different air samplers including the Burkard personal volumetric air sampler, the Rotorod sampler, the Air-O-Cell™ and a filter cassette in an Environmental Exposure Unit (EEU) with predetermined pollen levels. The Rotorod sampler, Air-O-Cell™ and filter cassette captured similar numbers of spores when corrected for sampling efficiency while the Burkard spore trap captured significantly more spores. Heffer et al. (2005) stated this was an overestimation compared to the other traps and deemed the results obtained by the Rotorod spore trap consistent, reliable and likely close to the absolute value. Aylor (1993) compared the Rotorod and the standard volumetric Burkard spore traps under indoor and outdoor conditions. The relative efficiency of the Rotorod (= spores per unit of air sampled by Rotorod divided by spores per unit of air sampled by the Burkard sampler) was higher outdoors compared to the study in the laboratory. Aylor (1993) also found that the Burkard spore trap captured more spores per volume of air in both conditions. These data appear to contrast with our results; it can be explained, however, by our choice not to express the numbers of captured spores per volume of air, which theoretically is larger for the rotating-arm sampler, and which should therefore increase at higher rotational speeds. Based on our observations from a preliminary study in the well-controlled environment we found no significant difference in spore capture within the rotational speed range of the rotating-arm samplers (2300 to 3150 RPM) used in our experiments. This did not allow us to take rotational speed and volume of sampled air into account. Direct comparison of the sampler types will remain difficult until the true volume of air sampled by the rotating-arm samplers is known. However, in case one would like to compare the data based on the theoretical volumes of air sampled, then the flow rates of 10 L/min for the Burkard sampler and 69.4 L/min (2300 RPM) to 95 L/min (3150 RPM) for the rotating-arm samplers should be taken into account. Based on these theoretical flow rates and the actual numbers of spores measured, the capture efficiency of the rotating-arm samplers would be 9% (3150 RPM) to 12% (2300 RPM) of the capture efficiency of the Burkard sampler. Crisp et al. (2013) and Latorre et al. (2008) found little or no difference in actual counts of several fungal spores and pollen from multiple species when comparing the two types of traps outdoors. Sutton and Jones (1976) also observed negligible differences when monitoring *V. inaequalis* ascospores with a Rotorod or Burkard sampler. Also during our outdoor experiments, no significant difference was observed between the Burkard sampler and the rotating-arm sampler when quantified by the same technique. In an outdoor environment differences in efficacy of spore traps are often attributed to wind speed and atmospheric turbulence (Aylor 1993; Frenz 2000). In our outdoor experiments, wind speed did not influence the results.

Over the course of the three-year experiments, the molecular and microscopic quantification of the Burkard samples correlated well (ρ ranging from 0.62 to 0.74) with the molecular quantification being more sensitive in 74 out of 85 occasions. Also, the rotating-arm samples quantified microscopically correlated well with those quantified molecularly (and those triggered by the rain sensor ($\rho = 0.83$)), but the molecularly quantified samples did not yield significantly higher numbers. We found a correlation between the molecularly quantified samples from the rotating-arm sampler rotating continuously and those of the rotating-arm sampler triggered by the rain sensor.

The Spearman rank correlation test, although quite robust and less affected by outliers, could be affected by an uneven distribution of the ascospore counts, such as a combination of very high and very low values. However, when we only took the large ascospore counts (>10,000 spores) into account for the calculation of the correlations, this resulted in only minor shifts in the correlation coefficients ($\Delta\ \rho = 0.048 \pm 0.12$). This provides confidence in the observed correlations and shows that the techniques correspond well for the large ascospore release events, which are most relevant for disease warnings.

Occasionally the rotating-arm sampler triggered by the rain sensor captured a higher number of ascospores compared to the continuously rotating arm-sampler. Because sample time differed, it is plausible that the samples from the continuously rotating devices were saturated with other airborne particles, while samples from devices that were activated by rain were not. However, under molecular analysis, the continuously-rotating sampler correlated better with the Burkard than the rotating-arm sampler triggered by the rain sensor. Yields from continuously capturing devices would indeed correspond better if some captures were also taken outside of rain events. Although conventionally it is assumed ascospores are released only during daylight, until two hours after a rain event, there are also reports of limited ascospore release during presence of dew, at night and over longer periods of time (Gadoury et al. 1998; Rossi et al. 2001; Stensvand et al. 1998).

In a controlled environment, all spore traps and quantification techniques showed a very strong correlation and a low variance. This suggests the possibility for converting spore counts among them, allowing an interpretation of the data as they can be translated to reference values established with traditional systems. During the semi-field experiment, the rotating-arm samplers quantified molecularly tended to yield more spores than the Burkard spore trap. This trend was consistent with the tests in the controlled environment but due to the larger variance the differences between sampler types was not significant ($p = 0.08$ to >0.99) in the semi-field experiments. The variability observed during the semi-field trial might be attributed to the position of the spore samplers as the Burkard sampler was always positioned in the middle of the inoculum source and the rotating-arm samplers more to the side of the pit; they may therefore have been influenced by wind direction. Similar to our data, Crisp et al. (2013) concluded that in general the yields from the rotating-arm samplers follow the same trend as those from the Burkard spore trap, especially when the same quantification technique is used.

We conclude that the rotating-arm samplers combined with our new molecular quantification technique is a sensitive and flexible method that holds promise for large-scale monitoring of ascospore levels in commercial orchards, direct application in disease warning systems, and targeted pest management. This method can also be used to study the dynamics of ascospore release in orchards, to further validate and improve climate-based ascospore release models.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals Not applicable.

Informed consent Not applicable.

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