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Research article

Quadruplex real-time PCR targeting airborne spores of grapevine pathogens

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ABSTRACT

Grapevine cultivation in Europe is severely affected by major fungal pathogens, such as *Plasmopara viticola* (downy mildew), *Erysiphe necator* (powdery mildew), and *Botrytis cinerea* (gray mold), which necessitate frequent fungicide applications. To support the monitoring of airborne inoculum, we developed a novel quadruplex quantitative PCR assay that allows simultaneous detection and quantification of *P. viticola*, *E. necator*, *B. cinerea* spores, and an internal positive control in a single reaction. Airborne spores were collected from a Swiss vineyard during the 2023 growing season using a rotating-arm sampler, and the results were compared with disease assessments and meteorological data. The quadruplex qPCR exhibited high sensitivity and specificity, detecting spores even at low densities. The assay revealed pathogen-specific dispersal patterns strongly associated with climatic conditions: *E. necator* and *B. cinerea* spore concentrations decreased after rainfall but exhibited subsequent peaks under favorable humidity and temperature, whereas *P. viticola* spores persisted throughout the season, with maximum numbers occurring after rainfall and the reactivation of sporulation from oil spots. This work introduces a robust molecular tool for the simultaneous monitoring of the three most important grapevine pathogens and highlights the potential of qPCR-based spore quantification to support more precise, data-driven control strategies.

1. Introduction

Grapevine is a major crop in Europe, with about 3.2 million ha dedicated to its cultivation, including nearly 15,000 ha in Switzerland. The three most damaging grapevine pathogens are the oomycete *Plasmopara viticola* (downy mildew), the ascomycetes *Erysiphe necator* (powdery mildew), and *Botrytis cinerea* (gray mold) (Wilcox et al., 2015). All three pathogens cause polycyclic diseases with asexual reproduction during the growing season and releasing a large number of spores into the air whenever climatic conditions are favorable (Gessler et al., 2011). To prevent infections and crop losses, grape growers rely mostly on repeated preventive fungicide applications, with an average of 8–10 fungicide applications per season (May–August) in Swiss conventional grapevine production systems. However, growing concerns about the environmental and health impacts of plant protection products have intensified societal and political pressure to reduce their use (Komárek et al., 2010).

Disease forecasting models and decision-support tools have been developed to help winegrowers optimize treatments by estimating infection risk based on climatic parameters (Velasquez-Camacho et al., 2023). Complex models, such as mechanistic models, allow for the modeling of all stages of the pathogen's biological cycle (Park et al., 1997; Siegfried et al., 2004; Rossi et al., 2008). However, all models developed to date are based almost exclusively on climatic parameters (Velasquez-Camacho et al., 2023). During early spring, which is a critical period in the onset of *P. viticola* epidemic, disease risk forecasting models often fail to predict the occurrence of primary infections. Incorporating biological parameters, such as spore detection, could therefore improve model performance by providing direct evidence of pathogen presence before symptom development (Brischetto et al., 2020; Mahaffee et al., 2023). This new parameter is useful not only for improving modeling but also for disease monitoring and controlling spray timing. Spore traps are increasingly being deployed in fields (West and Kimber, 2015), particularly in vineyards (Brischetto, 2020; Carisse

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et al., 2021; Douillet et al., 2022). Moreover, several commercial services now offer grapevine pathogen spore quantification (AgriSamplers (2020), Little Kingshill, UK; Scanit Technologies (2021), Fremont, US; DAC ADN (2022) Venejan, FR; Biology as a Solution (2022) Martillac, FR; BioScout (2024) Marickville, AU).

Although critical questions remain, such as defining spore density thresholds associated with infection risk, spore detection approaches have the potential to refine fungicide application strategies by adapting them to local inoculum presence and quantity (Douillet et al., 2022). Quantitative polymerase chain reaction (qPCR) is a powerful molecular tool for precisely estimating the abundance of target DNA molecules in environmental samples (McCaustland, 2005). Widely applied in plant pathology, qPCR has been used to monitor diverse pathogens on lettuce (Dhar et al., 2020), wheat (Araujo et al., 2021; Wang et al., 2025), apple (Meitz-Hopkins et al., 2014), cucumber (Bello et al., 2021), onion (Van der Heyden et al., 2020), spinach, and beet (Klosterman et al., 2014). In grapevine, qPCR-based monitoring systems have been developed for *Botrytis cinerea* spores (Carisse et al., 2014) and two downy mildew clades in North America: *Plasmopara viticola aestivalis* and *Plasmopara viticola riparia* (Carisse et al., 2021).

This paper presents the development of a novel quadruplex qPCR assay for the simultaneous detection and quantification of three major grapevine pathogens: *P. viticola* clade *aestivalis*, the only clade present in Europe (Fontaine et al., 2021), *E. necator*, and *B. cinerea*. The assay also includes an internal positive control to verify DNA extraction efficiency and the absence of reaction inhibitors, which are commonly found in environmental samples (Schrader et al., 2012). This method provides a rapid, sensitive, and specific tool for monitoring airborne inoculum of major grapevine pathogens, with potential applications in both epidemiological studies and disease management.

2. Materials and methods

2.1. Environmental samples and experimental site

Airborne spores were actively collected by air sampling using a SporeStick rotating-arm sampler (OptiSense Ltd, Horsham, UK) bearing two wood matches of 43 × 2 mm (10 Doigts, Leers, FR, Cat. no. 14937) coated with petroleum jelly (Cooper, Melun, FR) (Fig. 1). The matches rotated at 2400 rpm, continuously sampling air at a volumetric rate equivalent to 6.3 m^3 per h (McCartney et al., 1997). Samples were collected three times a week at noon on Monday (72 h sampling), Wednesday (48 h sampling), and Friday (48 h sampling) from 1 May to 30 August 2023. Samples were stored in 5 ml tubes and kept at 8°C until DNA extraction.

The SporeStick was placed 60 cm above the ground at the beginning of the season in the middle of a row of five *Vitis vinifera* cv. Chasselas vines in an experimental field in Changins, Switzerland (46°23′47.7"N, 6°13′54.0"E) and was progressively raised during the season to remain within the canopy as vegetation developed. This experimental field has been used since 2003 to monitor the primary infections of downy mildew and the initial stages of the epidemic. For this purpose, the vine shoots are positioned so that they lie on the ground to facilitate primary infection detection. In addition, grapevine leaves containing a high quantity of oospores were placed on the soil each autumn. As an untreated plot, no fungicide was applied since the planting of the grapevines.

2.2. Disease assessment

During the early growing season, downy mildew incidence was assessed on leaves and bunches at four time points, with assessments stopping on 15 June 2023 as monitoring at the outdoor laboratory focused specifically on primary infections. Powdery mildew was also monitored during this early period; however, no visible symptoms were observed up to the end of the assessments. Disease incidence was defined as the proportion of organs (leaves or bunches) showing symptoms. For each assessment, all leaves and bunches from five selected Chasselas vines were examined. Incidence was calculated as the percentage of symptomatic leaves or bunches. In the absence of a specific gray mold assessment, *B. cinerea* qPCR results were interpreted solely as measures of airborne inoculum dynamics.

2.3. DNA extraction

DNA was extracted from matches using CTAB and the phenol-chloroform extraction protocol, as described by Douillet et al. (2022). Briefly, the ends of the matches (without petroleum jelly) were cut off,



Fig. 1. Rotating arm-sampler SporeStick® with the two matches in place (Changins, Switzerland).

and the matches were placed in 2 ml microcentrifuge tubes. We then added 1 ml of CTAB and 6×10^4 copies of an exogenous internal positive control (EIPC) consisting of double-stranded DNA genomic blocks (Integrated DNA Technologies Inc., US), as was used by Carisse et al. (2021). After 2 h of incubation at 65°C, about 800–900 μl of solution was transferred into a new tube, and phenol:chloroform:isoamyl alcohol 25:24:1 (Sigma Aldrich, US, Cat. no. 77617) kept at 4°C was added at a 1:1 volume. Samples were gently mixed by inverting the tubes before centrifuging them at 13,000 rpm for 10 min. The aqueous phase (on top) was transferred into a new tube, and 56 μl of isopropanol per 100 μl of sample solution was added. After a gentle mix, the tubes were incubated overnight at −20°C. The following day, the samples were centrifuged at 13,000 rpm for 5 min, the supernatants were discarded, and the pellets were washed twice with 700 μl of 70% ethanol at −20°C. The pellets were dried for 20 min at room temperature using SpeedVac (Thermo Fisher Scientific, US), resuspended in 50 μl of nuclease-free water (Qiagen, Hilden, DE), and kept at −20°C until used in the qPCR reaction.

2.4. Erysiphe necator qPCR primers and probe design

DNA extracted from *E. necator* samples collected in this study was amplified using the forward primer EnITSstd1F (ACGTTGTCATGTAGTCTA) and the reverse primer EnITSstd1R (CGCTTATTGATATGCTTA) to generate a 435-bp amplicon of the internal transcribed spacer region. The amplicons were sent for Sanger sequencing (Génome Québec Innovation Center, Québec, Canada). The resulting sequences were aligned with additional sequences of *Erysiphe* spp. and other fungi available on NCBI GenBank (www.ncbi.nlm.nih.gov/genbank/). Using Beacon Designer 8.13 software (Premier Biosoft, Palo Alto, CA), the forward primer EnITS2F (ACGCGTAGTAACTTGTTC), the reverse primer EnITS1R (GAGGTCAACCTGTCAATC), and the probe EnITS2P (TCGTGATCAGCCAGAACCACC) were designed to amplify a 95-bp amplicon for the absolute quantification of *E. necator*. The specificity of the qPCR assay was tested on several *Erysiphales*, including *Podosphaera aphanis, Podosphaera xanthii*, and *Erysiphe cichoracearum*, as well as grape pathogens, such as *Botrytis cinerea*, *Plasmopara viticola*, and *Elsinoe ampelina*.

2.5. Real-time PCR setup

Primer and probe sequences targeting the pathogens studied—*Plasmopara viticola* (Carisse et al., 2021), *Erysiphe necator* (described above in 2.4), and *Botrytis cinerea* (Carisse et al., 2014)—and the EIPC (Carisse et al., 2021) are described in Table 1. The qPCR was performed in a 96-well plate (BioRad, Hercules, US, Cat. no. MLL9651) using a Biorad CFX96 thermal cycler. The reactions were conducted in triplicate with the following mix per well: 1x GoTaq Probe qPCR Master Mix (Promega, Madison, US, Cat. no. A6102), 0.15 μg/ml of bovine serum albumin, 0.4 μM of each Pv primer, 0.2 μM of Pv probe, 0.3 μM of each En primer, 0.2 μM of En probe, 0.3 μM of each Bc primer, 0.2 μM of Bc probe, 0.2 μM of each EIPC primer, 0.15 μM of EIPC probe, and 3 μl of DNA sample in a total volume of 20 μl. The thermal cycler conditions included a first step of 5 min at 95°C, then 30 s at 95°C, and 30 s at 60°C for 45 cycles. Multiple channels were selected for FAM, ROX, HEX, and Cy5 fluorophore reads.

2.6. Standard curves

Sporangia and conidia used for the standard curves were obtained from laboratory cultures of *P. viticola*, *E. necator*, and *B. cinerea* maintained at Agroscope, Changins, Switzerland. To estimate the number of spores from the number of specific internal transcribed spacer (ITS) copies, Pv sporangia and En or Bc conidia were aspirated into a 200-μl filtered pipette tip and then blown into 200 μl of isopropanol. Sporangia and conidia were counted using a hemocytometer-type grid (KOVA, Austin, US, Cat. no. 87144). The equivalent of 3125 spores was put onto a match coated with petroleum jelly. Given the potential influence of petroleum jelly on spore release and DNA recovery, standard curves were established on petroleum-jelly-coated matches and processed identically to field samples, ensuring that matrix-related effects were accounted for in the calibration. DNA was extracted as described in Section 2.3. Serial dilutions of 1:5 from 3125 spores to 1 spore were made and used in qPCR in quintuplicate.

This calibration accounted for matrix-related effects during DNA extraction and qPCR quantification, but it did not include an independent correction for the airborne collection efficiency of the rotating-arm sampler. Therefore, spore quantities should be interpreted as spore equivalents recovered from the sampling surface, or normalized to the sampled air volume, rather than as absolute atmospheric spore concentrations.

2.7. Data analysis

The threshold fluorescence line for the quantification cycle (Cq) was set at 150 relative fluorescent units (RFU) for FAM, 100 RFU for HEX and Cy5, and 75 RFU for ROX. The Cq values obtained after qPCR cycles were converted into spore quantity using the following equation (Kubista et al., 2006):

$$x = 10^{\frac{Cq-b}{a}}$$

Where x is the estimated number of spores in the sample, Cq is the quantification cycle, a is the slope, and b is the intercept of the regression line of the standard curve. The significance of the standard curve slope was assessed using a t-test to confirm that Cq values were significantly correlated with log-transformed standard concentrations.

Amplification efficiency was estimated from the slope of each standard curve to assess qPCR performance and verify that target amplification was close to the theoretical doubling of DNA during each cycle. Efficiency was calculated according to Kubista et al. (2006) using the equation:

$$E = 10^{\frac{-1}{a}} - 1$$

where E is the amplification efficiency and a is the slope of the standard curve obtained by plotting Cq values against the log10-transformed initial target quantity. Efficiency values were expressed as percentages by multiplying E by 100.

The total number of spores collected on each sample was divided by

Table 1
List of primers and probes used in this study.

Target Name	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)	Probe (5′ – 3′)	Reference
Plasmopara viticola ITS2 **PvB-ITS2**	GGTTGTTAGACTTTGTGATTAGTAA	GGCAGAAAGCATACTATATAAGC	5′ 6-FAM/ZEN – CTCGACAAACAAACCGGGA – 3′ IBFQ	Carisse et al. (2021)
Erysiphe necator ITS2 **EnITS2**	ACGCGTAGTAACTTGTTC	GAGGTCAACCTGTCAATC	5′ Cy5/TAO – TCGTGATCAGCCAGAACCACC – 3′ IBRQ	This paper
Botrytis cinerea IGS **Bc3**	GCTGTAATTTCAATGTGCAGAATC	GGAGCAACAATTAATCGCATTTC	5′ ROX NHS –TCACCTTGCAATGAGTGG – 3′ IBRQ	Carisse et al. (2014)
Exogenous internal positive control **EIPC99**	ACGAGAATTAAGGTCACTTTC	CCCTTCATACCCGATCTG	5′ HEX/ZEN –TTCTTGCCTGTTCGCCGCTG – 3′ IBFQ	Carisse et al. (2021)

2 or 3 days depending on the sampling period and reported as the average spore number per day for that period. Meteorological data were obtained from www.agrometeo.ch for Changins (Campbell weather station, 46°23′53.4"N 6°13′50.4"E), and the following daily parameters were chosen: average temperature at 2 m above ground, average relative humidity, total precipitation, and total leaf wetness. Wind data were collected from the MeteoSwiss weather station at Nyon/Changins (46°24′03.8"N 6°13′39.8"E), with the following daily parameters: average wind speed and maximum wind speed. The two weather stations were separated by 390 m.

To explore the relationships between airborne spore concentrations and meteorological variables, Gaussian generalized linear models (GLMs) were fitted separately for each pathogen using log-transformed spore-equivalent concentrations [log(spore-equivalents/$m^3 + 1$)] as the response variable. Mean temperature, cumulative rainfall, and their interaction were included as explanatory variables. Meteorological variables were aggregated over each 2- to 3-days spore-sampling period to match the temporal resolution of the qPCR measurements. Pairwise relationships between log-transformed spore concentrations and meteorological variables were visualized using linear regressions.

3. Results

3.1. Specificity and performance of the multiplex qPCR

To establish a quadruplex qPCR targeting *P. viticola* (Pv), *E. necator* (En), *B. cinerea* (Bc), and the positive control EIPC, different combinations of primers and probe concentrations were tested. The optimized mix that yielded the best amplification efficiencies consisted of 0.4 μM of each Pv primer, 0.2 μM of Pv probe, 0.3 μM of each En and BC primer, 0.2 μM of En and Bc probe, 0.2 μM of each EIPC primer, and 0.15 μM of the EIPC probe. Under these conditions, the assay produced clear and channel-specific amplification signals for all four fluorophores (FAM, Cy5, ROX, and HEX), without detectable fluorescence spectral cross-talk between channels. No signal was detected in the no-template controls, confirming that the primers and probes did not cross amplify each other. Comparable signals were obtained when one or two pathogen targets were omitted from the reaction, demonstrating that the assay performed robustly, even in the absence of one or more specific targets. The EIPC control allowed us to verify the quality of the DNA extraction and the amplification conditions. The EIPC was consistently amplified with similar Cq values across samples when DNA extraction was efficient, whereas delayed or absent amplification indicated potential qPCR inhibition or DNA loss.

Amplification efficiencies were initially evaluated using serial dilutions of amplicons as templates. In the quadruplex, amplifications in the same reaction showed 91.7% efficiency for Pv ($y = -3.54x + 37.05$, $R^2 = 0.997$, $p < 0.0001$), 92.7% efficiency for En ($y = -3.51x + 37.77$, $R^2 = 0.999$, $p < 0.0001$), and 95.6% efficiency for Bc ($y = -3.43x + 38.50$, $R^2 = 0.995$, $p < 0.0001$) (Fig. 2). Standard curves were calibrated with serially diluted spore suspensions (1:5), starting with 3125 spores up to 1 spore. Detection was possible down to a single spore for each of the three pathogens, showing the high sensitivity of the assay. Based on spore suspensions, efficiencies were 90.4% for Pv ($y = -3.57x + 36.89$, $R^2 = 0.995$, $p < 0.0001$), 94.9% for En ($y = -3.45x + 39.37$, $R^2 = 0.975$, $p < 0.0001$), and 101.8% for Bc ($y = -3.28x + 39.82$, $R^2 = 0.996$, $p < 0.0001$). All amplification efficiencies fell within the commonly accepted optimal range for qPCR assays (Kubista et al., 2006). The slightly above-theoretical apparent efficiency obtained for *B. cinerea* (101.8%) likely reflects minor variation in spore counting, serial dilution, DNA recovery, or standard-curve fitting rather than a biologically meaningful over-amplification or systematic technical bias, as the standard curve remained highly linear ($R^2 = 0.996$).

To relate qPCR results to spore counts, standard amplicon curves were converted into pathogen-specific spore calibration curves. This was achieved by comparing the number of ITS copies quantified per sample with the direct spore counts obtained by microscopy, yielding conversion factors for each pathogen. Under our conditions, one *P. viticola* sporangium corresponded to 1.3 ITS amplicons, one *E. necator* conidium corresponded to 0.25 ITS amplicon, and one *B. cinerea* conidium corresponded to 0.168 ITS amplicon.

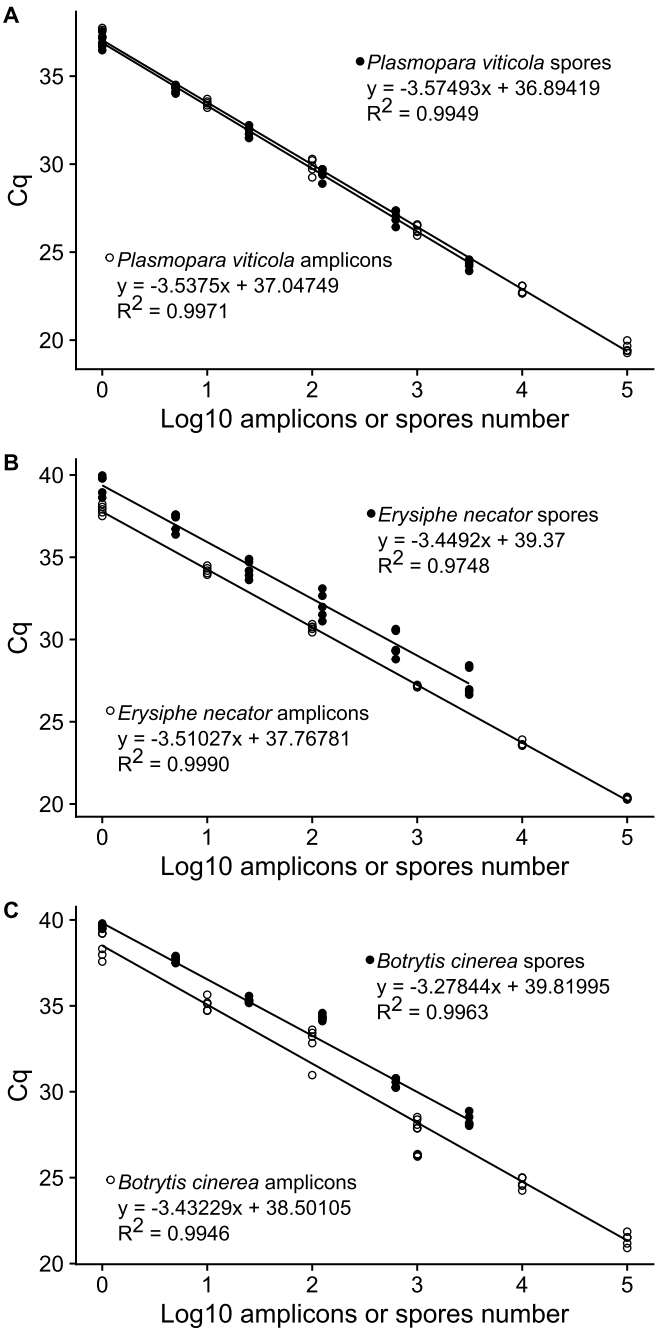


Fig. 2. Relationship between quantification cycle (Cq) value and the $\log_{10}$ spore concentration in black dots, and between Cq and the $\log_{10}$ of ITS amplicon copies in white dots for **A**. *Plasmopara viticola*, **B**. *Erysiphe necator*, and **C**. *Botrytis cinerea*.

Specificity assays for *E. necator* probe and primers performed with non-target *Erysiphales* (*Podosphaera aphanis, Podosphaera xanthii*, and *Erysiphe cichoracearum*) and other grapevine pathogens (*B. cinerea*, *P. viticola*, and *Elsinoë ampelina*) produced no amplification, confirming the assay specificity.

3.2. Field monitoring

3.2.1. Erysiphe necator and Botrytis cinerea

At Changins, the spore release patterns of *E. necator* and *B. cinerea*—both dispersed by wind—showed a similar seasonal dynamic (Fig. 3). During the study period of April–September 2023, the spores of *E. necator* were first detected in early May (15 spores detected during the 10-day period from May 6–15), coinciding with rainfall (totaling 28.9 mm). These conditions followed two previous rain periods (22 mm from 19 to 25 April 2023, and 18.5 mm from April 28–May 1). During the study period, May 6–8 had a low total rainfall of 1.2 mm, with the leaves remaining wet for slightly more than 25 h, and the average temperature was 16°C. These favorable conditions were confirmed with the detection of the first set of spores. After May 15, the weather was warm and dry, and limited *E. necator* spores were captured until June, indicating a latent period between infection and the onset of sporulation. Thereafter, spore numbers gradually increased, peaking at > 3000 spores/day from June 13–16, following rainfall on June 9–10. The most important peak of the season was observed during the period from June 17–23, with about 10,000 spores per day under favorable conditions (average RH 62.8%, daily mean > 20°C) and strong winds (gusts up to 19.7 m/s on June 22). Following 12.4 mm rainfall on June 22, spore numbers declined sharply, recovering briefly before another heavy rainfall (44.6 mm on June 30), followed again by reduced counts. Sporulation peaks were observed through July, each followed by sharp post-rainfall declines.

However, extreme summer heat with maxima of 33.6°C in July and 37.9°C in August limited powdery mildew epidemics, and En spore levels remained low after late July.

Botrytis cinerea spores were already present in the first sample collected on May 1. During the season, *B. cinerea* spore quantity decreased after rainfall events. Nevertheless, a strong decrease in spore quantity not linked to rainfall events was observed mid-May, when the *B. cinerea* spore count dropped from 985 spores/day (16-17 May) to 33 spores/day (May 18–19). Strong winds (>7 m/s average, with gusts up to 23.9 m/s) likely explain this fluctuation. No gray mold symptoms were observed on grapes in 2023, suggesting that the spores originated mainly from external sources rather than the local grapevine.

3.2.2. Plasmopara viticola

Unlike *E. necator* and *B. cinerea*, the oomycete *P. viticola* requires free water for dispersal and infection. In Changins, *P. viticola* spores were detected from May 2 until late August. However, despite an early-season spore presence, disease development remained limited and no sustained epidemic progression was observed (Fig. 4). From May 2–10, a total of 9.4 spores were captured on this period, coinciding with an average daily leaf wetness of ~6 h. The first oil spots appeared on May 11, followed by a spore peak of 46 spores/day on May 11–12, probably originating from both sporulating lesions and the germination of oospores in the soil. A second peak of 65 spores/day (May 20–22) corresponded to the primary infections of May 9 with 12.5 mm rainfall. The disease frequency on leaves on May 22 was 5.4%, and increased up to 18.4% 8 days later on May 30. However, rainfall on June 9–10 (8.4 mm) triggered the reactivation of oil spots, leading to a major peak of 4311 spores/day (June 10–16). The highest seasonal peak occurred June

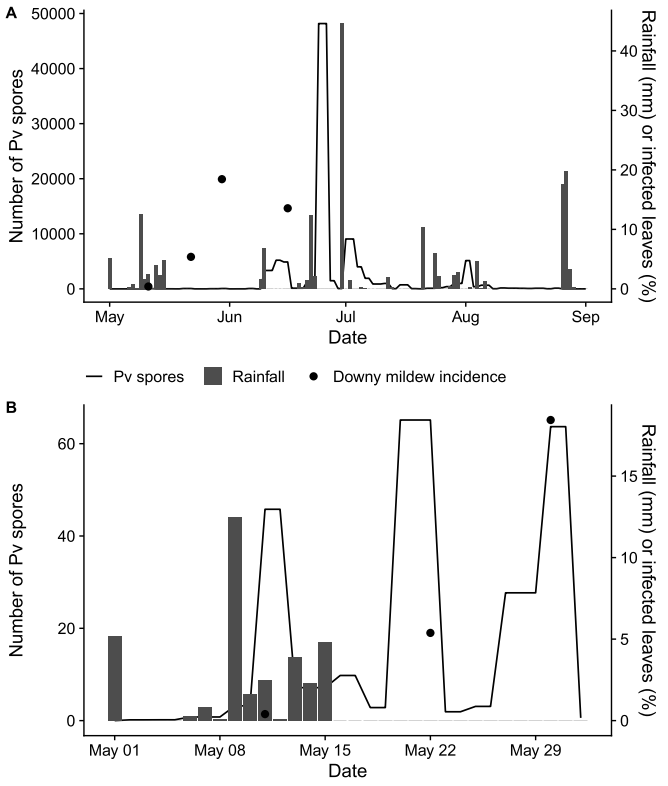


Fig. 4. Seasonal spore quantity monitoring of *Plasmopara viticola* (black line). The total percentage of leaves infected by downy mildew is represented in black dots, and total daily rainfall is represented in dark gray bars. Disease assessments were conducted until 15 June, as monitoring focused specifically on primary infections. **A**. Whole season 01/05/2023–01/09/2023, square corresponds to Fig. 4B. **B**. Zoom in the early season 01/05/2023–01/06/2023.

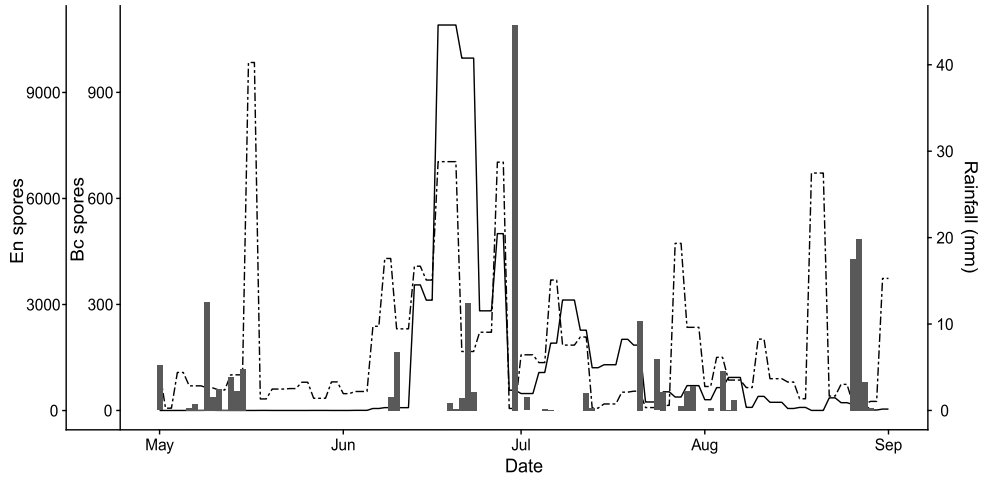


Fig. 3. Seasonal monitoring of spore quantities in 2023: *Erysiphe necator* (En, solid line) and *Botrytis cinerea* (Bc, dashed line) in Changins, Switzerland. The total daily rainfall is shown as dark gray bars.

24–26, with 48,176 spores/day following 17 mm rainfall. On June 30, a heavy storm 44.6 mm in 1 h and hail was followed by another peak of 9058 spores/day (July 1–3). Hot, dry conditions in July, with 28 mm rainfall over the month, limited epidemic development as lesions necrosed. Sporulation resumed sporadically after late-July rainfall, with some peaks (e.g., 5136 spores/day, August 1–2). Thereafter, the lesion necrosed, and spore concentration was minimal until September.

4. Discussion

The new quadruplex qPCR assay presented in this paper was first validated under laboratory conditions by testing primer and probe specificity and sensitivity for the detection of the most common grapevine pathogens. Subsequently, it was assessed on field samples. This quadruplex allows for the rapid and accurate detection of *E. necator*, *B. cinerea*, and *P. viticola* spores in a single reaction. Its performance was confirmed through calibration curves similar to those used in other agronomic qPCR assays (Van der Heyden et al., 2020; Araujo et al., 2021; Carisse et al., 2021). Unlike previously available simplex or duplex qPCR assays, this quadruplex reduces the number of reactions, reagent consumption, sample volume required, and handling steps, while limiting technical variability between assays. In Canada, for instance, a duplex qPCR assay was developed to monitor the spread of two clades of the downy mildew pathogen, *P. viticola aestivalis* and *P. viticola riparia* (Carisse et al., 2021). Similarly, LAMP-based assays offer rapid detection and potential field deployment for airborne grapevine downy mildew monitoring (Douillet et al., 2022), but they are generally designed as single-target assays and therefore do not provide simultaneous multi-pathogen quantification. The main operational advantage of the present assay is therefore its capacity to generate quantitative data for three major grapevine pathogens and an internal positive control from the same environmental sample, which is particularly valuable for spore-trap monitoring and epidemiological studies.

The challenge in developing the quadruplex described here was to integrate three sets of primers and probes—those for the European clade *P. viticola aestivalis*, those for *B. cinerea*, which was previously established for gray mold detection (Carisse et al., 2014), and the newly designed set for *E. necator* detection—all within a single reaction. Optimization using different spore quantities allowed the identification of concentrations of primers and probes, providing robust detection of all three targets together with the positive control. Under these conditions, the assay showed high sensitivity, detected as little as single spores in standard curves, and remained reliable when applied to highly variable field samples.

The proposed assay represents a powerful tool for vineyard pathogen inoculum monitoring, as it makes it possible to quantify airborne spores of interest directly from spore trap samples. More broadly, field monitoring is increasingly recognized as a key strategy in agronomy to prevent or limit infections (Dhar et al., 2020; Basha et al., 2021; Douillet et al., 2022). In viticulture, timely disease information is therefore critical for deciding whether to apply preventively protective sprays before infection occurs. Several rapid methods have been developed to address this need, such as direct PCR on crushed tissues without DNA extraction (Gindro et al., 2014) and loop-mediated isothermal amplification (LAMP) assays for airborne downy mildew spores (Douillet et al., 2022). While direct PCR and LAMP assays provide faster results, they generally target single pathogens and do not provide quantitative information. By contrast, the quadruplex qPCR provides both sensitivity and multiplexing, offering both quantitative data and simultaneous monitoring of three pathogens, a unique advantage for vineyard surveillance and decision support.

In this context, in 2023, the assay was demonstrated to be valuable, and the results were in good agreement with the observed disease symptoms. Notably, it could detect at the beginning of the season extremely low spore numbers as few as 1 spore-equivalent, as estimated from standard curves established under controlled calibration conditions. The assay also captures very high numbers of spores during peak infection periods, with up to 144,528 spores-equivalents detected over a three-day sampling period. Field deployment illustrates how this assay can be used to characterize pathogen-specific airborne inoculum dynamics in relation to weather conditions. For *E. necator*, spore numbers declined after rainfall or prolonged leaf wetness, followed by peaks a few days later. This is consistent with the inhibition by free water and stimulation of infections by high relative humidity, followed by sporulation and spore release after incubation (Wilcox et al., 2015). Moreover, the timing of the first spore detections is consistent with the requirement for ascospore release from cleistothecia, which is induced by rainfall and temperatures above 10°C (Gadoury et al., 2012; Gadoury and Pearson, 1990). Rainfall, while initially required for primary release of ascospores, reduced secondary infections, explaining observed declines after rain events. By contrast, temperature was the dominant driver of secondary development, with optimal infection at 26°C (Delp, 1954). Between June and September, daily means exceeded 20°C, and spore peaks coincided with increases in relative humidity, optimal near 85% (Wilcox et al., 2015). Despite a favorable spring for powdery mildew epidemic initiation, July–August heatwaves in July and August limited disease progression, reflected in the low spore counts from late June until harvest.

By comparison, *Botrytis cinerea* spore levels were more stable across the season. Spores were continuously detected before, during, and after grapevine growth, reflecting the necrotrophic and saprophytic nature of this pathogen able to develop on more than 200 host species (Elmer and Michailides, 2007; Williamson et al., 2007). In Swiss vineyards, gray mold is usually well managed through viticultural practices (e.g., vigor limitation and partial leaf-stripping) and causes limited damages under modern conditions, although localized outbreaks still occur. Unlike *E. necator* and *P. Viticola*, which are obligate biotrophs, *B. cinerea* does not depend on the grapevine cycle. Grapevine susceptibility peaks during flowering and ripening (Viret et al., 2004). *Botrytis cinerea* infection requires 15–20°C with free water or >93% RH (Nair and Allen, 1993). In 2023, flowering in Changins occurred under dry conditions (no rain and an average RH of 56.7%), preventing infection. The observed pattern of decreased spore counts following rainfall events is likely to be due to rain washout (Ladin et al., 2021). Spore peaks typically follow rain or high humidity events, released by air turbulence, water splash, or mechanical disturbance (Wilcox et al., 2015). Continuous detection of *B. cinerea* spores even outside the vine growing season likely reflects its saprotrophic presence on decaying plant material within and around vineyards. However, despite detectable spores, no gray mold symptoms developed in autumn, consistent with the season's dry conditions.

Plasmopara viticola spores were first detected in early May 2023. The site was used for long-term downy mildew monitoring and contained abundant oospores in soil due to repeated leaf litter deposits over 20 years, likely explaining the early detection. According to the forecasting model VitiMeteo-Plasmopara (VM) (Siegfried et al., 2004), rainfall on 1 May would have triggered primary infection, consistent with the first oil spot symptom observed on 11 May. Additional soil infections would have occurred on May 9 and 15, followed by sporulation. The spore peaks observed in this study corresponded well with the VM predictions and field symptom assessments. Subsequent warm, dry conditions limited new infections, while rapid vine growth diluted lesion impact. Later in the season, spore peaks followed rain events, consistent with sporulation requirements of 95–100% RH or leaf wetness (Wilcox et al., 2015). The highest peak of a total of 144,528 spores over three days (24–26 June) corresponded with VM predictions of 2 primary and 10 secondary infections. A final peak in early August of 5136 spores/day was associated with multiple rain events at the end of July and the strong winds that facilitated spore production and dispersion. Overall, the *P. viticola* spore dynamics mirrored low field disease pressure and were consistent with the model predictions.

To complement the descriptive interpretation of pathogen-specific

dynamics, pairwise relationships between airborne spore concentrations and meteorological variables were visualized using linear regressions in Supplementary Fig. S1. However, these relationships should be interpreted cautiously because the biological processes underlying spore detection are not expected to be strictly linear. Mean temperature and cumulative rainfall were aggregated over 48- to 72-h sampling periods, whereas airborne spore concentrations reflect integrated inoculum release over the same windows. To better account for the joint effects of meteorological variables, exploratory Gaussian GLMs were therefore fitted using log-transformed spore-equivalent concentrations as the response variable and mean temperature, cumulative rainfall, and their interaction as explanatory variables. For *P. viticola*, temperature showed only a weak, non-significant trend ($p = 0.095$), while cumulative rainfall and the temperature × rainfall interaction were not significant ($p = 0.681$ and $p = 0.728$, respectively). For *E. necator*, temperature had a significant positive effect on airborne spore concentrations ($p = 0.0035$), whereas cumulative rainfall and the interaction term were not significant ($p = 0.311$ and $p = 0.258$, respectively). For *B. cinerea*, neither temperature, cumulative rainfall, nor their interaction significantly explained airborne spore concentrations ($p = 0.583$, $p = 0.795$, and $p = 0.939$, respectively). Overall, these results indicate that simple statistical relationships with aggregated meteorological variables explain only part of the observed airborne inoculum dynamics, which likely depend on pathogen-specific biology, seasonal stage, threshold effects, and time-lagged responses to environmental conditions.Despite its sensitivity, the assay should not be considered a direct measure of infection risk or used as the sole basis for spray decisions. A key limitation of DNA-based qPCR is its inability to distinguish viable from non-viable spores. Consequently, DNA from dead or damaged spores, as well as fungal debris, may contribute to the signal and overestimate the amount of biologically active inoculum (Wolffs et al., 2005; Chen et al., 2018). This is particularly important when defining inoculum thresholds, because a given number of DNA-based spores may not correspond to the same infection potential depending on spore viability, host susceptibility, and environmental conditions. Detection alone is therefore insufficient for actionable decision support unless biologically meaningful threshold values linked to infection risk are established (Brischetto et al., 2020). Future work aimed at supporting the integration of molecular monitoring into vineyard management should define such thresholds and validate them across multiple sites, seasons, cultivars, and disease-pressure conditions. In addition, complementary approaches such as viability-qPCR using DNA-intercalating dyes or RNA-based assays could help distinguish viable or metabolically active spores from residual DNA, thereby improving the biological interpretation of qPCR-based inoculum monitoring (Nocker et al., 2007; Fittipaldi et al., 2012; Zhang et al., 2023).Taken together, spring is a critical period for all three pathogens, as primary infections establish the onset of epidemics for the season. Detecting overwintering spore release is therefore essential for defining the timing of initial sprays. Although weather-driven models are widely used, they often fail to predict the first infections of downy mildew (Rossi et al., 2008) and powdery mildew (Valori et al., 2023). A highly sensitive approach, such as qPCR, can refine the timing of primary inoculum release and germination, potentially improving early warning accuracy, provided that within-plot spatial heterogeneity is adequately considered. Our field results suggest that integrating qPCR-based spore monitoring with forecasting models could provide a more precise understanding of epidemic onset and may help to better identify situations where model-predicted risk is not accompanied by detectable inoculum, and vice versa.

5. Conclusion

The multiplex qPCR assay described here combines high sensitivity and specificity with the capacity to monitor three major grapevine pathogens simultaneously. Beyond research applications, it has the potential to improve forecasting models and decision support systems by providing almost real-time data on pathogen dynamics. Nevertheless, some limitations remain, including the need for specialized laboratory infrastructure and the time required for sample accumulation, collection and processing, which currently limit its use for true real-time decision-making. Moreover, qPCR detects DNA regardless of spore viability, which may lead to an overestimation of actual infection risk. Future work should define spore threshold values that translate into potential infection risk, which could support the integration of molecular monitoring into vineyard management. Such integration would allow fungicide applications to be more closely aligned with epidemic risk, reducing unnecessary treatments and promoting more sustainable viticulture.

CRediT authorship contribution statement

Sara Leoni: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Odile Carisse:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Livio Ruzzante:** Writing – review & editing, Formal analysis, Data curation. **Anne-Lise Fabre:** Writing – review & editing, Investigation, Data curation. **Sylvain Schnée:** Writing – review & editing, Investigation. **Jérôme Kasparian:** Writing – review & editing, Supervision. **Jean-Pierre Wolf:** Project administration, Funding acquisition. **Pierre-Henri Dubuis:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies

During the preparation of this work, the authors used AI-assisted technologies to improve language clarity and readability. The authors reviewed and edited the content and take full responsibility for the final manuscript.

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Declaration of competing interest

My co-authors and I do not have any conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2026.107925.

Data availability

Data will be made available on request.

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