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# Development of a grower-conducted inoculum detection assay for management of grape powdery mildew

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Management of grape powdery mildew (*Erysiphe necator*) and other polycyclic diseases often relies on calendar-based pesticide application schedules that assume the presence of inoculum. An inexpensive, loop-mediated isothermal amplification (LAMP) assay was designed to quickly detect airborne inoculum of *E. necator* to determine when to initiate a fungicide application programme. Field efficacy was tested in 2010 and 2011 in several commercial and research vineyards in the Willamette Valley of Oregon from pre-bud break to véraison. In each vineyard, three impaction spore traps were placed adjacent to the trunk. One trap was maintained and used by the grower to conduct the LAMP assay (G-LAMP) on-site and the other two traps were used for laboratory-conducted LAMP (L-LAMP) and quantitative PCR assay (qPCR). Using the qPCR as a gold standard, L-LAMP was comparable with qPCR in both years, and G-LAMP was comparable to qPCR in 2011. Latent class analysis indicated that qPCR had a true positive proportion of 98% in 2010 and 89% in 2011 and true negative proportion of 96% in 2010 and 64% in 2011. An average of 3·3 fewer fungicide applications were used when they were initiated based on spore detection relative to the grower standard practice. There were no significant differences in berry or leaf incidence between plots with fungicides initiated at detection or grower standard practice plots, suggesting that growers using LAMP to initiate fungicide applications can use fewer fungicide applications to manage powdery mildew compared to standard practices.

*Keywords: Erysiphe necator*, grapevine, powdery mildew, *Vitis vinifera*

## Introduction

Grape powdery mildew, caused by the biotrophic fungus *Erysiphe necator*, is a polycyclic disease of grape that causes losses to crop quality and yield worldwide (Gadoury *et al.*, 2012). Both the foliage and fruit are affected, and as little as 3% incidence of fruit infection has been shown to cause off flavours in wine (Ough & Berg, 1979; Stummer *et al.*, 2003). In the Pacific Northwest United States, grape powdery mildew epidemics are managed with fungicide applications that are initiated with the availability of susceptible host tissue and aimed at reducing the rate of epidemic development (Pearson, 1994; Gadoury *et al.*, 2012). This approach is based on the assumption that the host's development of susceptible tissue occurs in synchrony with the pathogen's production and dispersal of spores. However, this assumption might not be accurate in regions where both the host, *Vitis vinifera*, and pathogen do not have a long history of co-evolution (Zohary, 1995; Brewer & Milgroom, 2010). As the origin of *E. necator* is in the eastern United States (Brewer & Milgroom, 2010) and the origin of *V. vinifera* is in southern Europe and the Mediterranean basin (Zohary, 1995), each organism has evolved under very different climates, which may require different environmental conditions to break winter dormancy. Pscheidt *et al.* (2000) and Hall (2000) demonstrated that there was a delay in inoculum availability and epidemic onset in western Oregon that resulted in multiple unwarranted fungicide applications prior to inoculum availability. This asynchrony appears to result in the host escaping some or all of the overwintering inoculum through the occurrence of ascospore release prior to bud break (BBCH stages 00–07; Rossi *et al.*, 2010; Caffi *et al.*, 2012), delayed pathogen development compared to that of the host (Hall, 2000), or environmental stresses that impact pathogen development and rate of disease development (Moyer *et al.*, 2010).

Spore trapping and microscopy have been used in numerous pathosystems for the management of disease, including apple scab, downy mildew of hops, and sclerotinia stem rot of oilseed rape crops (Kremheller & Diercks, 1983; Aylor, 1995; Temple & Johnson, 2011). For exam-

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ple, the use of visual detection and quantification of the sporangia of *Pseudoperonospora humuli* on hops in conjunction with weather monitoring (Royle, 1973; Kremheller & Diercks, 1983) is still used to guide fungicide programmes in the Hallertau region of Germany. However, it is difficult to implement this approach in the management of grape powdery mildew due to the difficulty of visually identifying infective propagules. In the Willamette Valley of Oregon, 23 different powdery mildew species were found on hosts in or immediately adjacent to a vineyard (W. F. Mahaffee, personal observation), further complicating visual identification of *E. necator* spores. Alternatively, various nucleic acid-based technologies have been developed that are suitable for detecting and quantifying airborne pathogens and reduce the time required for assessing samples while increasing confidence in inoculum identification (Calderon *et al.*, 2002; Luo *et al.*, 2007; Carisse *et al.*, 2009a; Gent *et al.*, 2009; Rogers *et al.*, 2009). Falacy *et al.* (2007) demonstrated that inoculum of *E. necator* can be monitored using molecular tools, specifically PCR, and suggested that this information can be used to reduce the number of fungicide applications. PCR detection of airborne *E. necator* was shown to be effective for timing the initiation of a fungicide application programme to manage grape powdery mildew in the Yakima Valley of Washington State, USA (Falacy *et al.*, 2007). Similarly in Canada, it was shown that modelling could be improved, and action thresholds could be developed, based on PCR detection of airborne *E. necator* (Carisse *et al.*, 2009a). Others have also shown that PCR assays for detection or quantification of inoculum can be used to improve the sustainability of the disease management through more targeted fungicide applications (West *et al.*, 2008; Gent *et al.*, 2009). Unfortunately, these assays must be performed in well-equipped laboratories with skilled staff (Notomi *et al.*, 2000; West *et al.*, 2008) because the sensitivity can be adversely impacted by PCR inhibitors, particularly when inoculum levels are low or near detection limits and background particle density is high (Vrana, 1996).

Loop-mediated isothermal amplification (LAMP) is a DNA amplification method that has been used to detect pathogens in a wide variety of disciplines including human, veterinary and plant sciences (Notomi *et al.*, 2000; Ohtsuka *et al.*, 2005; Boehme *et al.*, 2007; Kubota *et al.*, 2008; Tsai *et al.*, 2009; Temple & Johnson, 2011). Because thermal cycling is not necessary, LAMP assays may be conducted using relatively inexpensive heat sources, such as a water bath or block heater (Notomi *et al.*, 2000). A by-product of the LAMP assay is a large amount of magnesium pyrophosphate precipitate, which allows for the visual assessment of target DNA amplification (Mori *et al.*, 2001). The amount of DNA amplification and resulting precipitate generated is relatively independent of initial target DNA concentration, thus allowing for unambiguous determination of positive and negative test results (Tomlinson *et al.*, 2007). LAMP has also been shown to be less sensitive to PCR inhibitors, thereby requiring less DNA purification for high sensitivity (Poon *et al.*, 2006; Tomlinson *et al.*, 2007). Given these traits, LAMP assays may be suitable for growers or crop consultants to perform for in-house detection of pathogens, where results may be used to initiate or time fungicide applications.

The purpose of this research was to develop a rapid and inexpensive molecular assay for detection of airborne *E. necator* inoculum that was sensitive and specific enough for commercial implementation, and thus be used on-site to signal initiation of fungicide applications for management of grape powdery mildew. The specific objectives were to: (i) develop a LAMP assay suitable for in-field use, consisting of both the DNA extraction and LAMP amplification protocol to allow rapid and inexpensive detection of *E. necator*; (ii) determine the sensitivity and specificity of the LAMP assay for detection of airborne *E. necator* inoculum in vineyards; and (iii) test the implementation of a LAMP assay conducted by vineyard managers in commercial vineyards for the initiation of fungicide programmes without compromising the level of disease control.

## Materials and methods

### Sampling rod preparation

Stainless steel sampling rods 1·1 mm in diameter were cut to 36 mm lengths from 308LSI welding rods (Weldcote Metals). Rods were first soaked in hexane for 24 h, and then rinsed with dishwashing detergent and water. Next, the rods were shaken in 10% Clorox bleach solution (0·83% NaOCl) for 15 min, and then rinsed with deionized water in 3–4 successive rinses. Rods were autoclaved for 30 min and aseptically air dried in a laminar flow hood. Rods were then transferred to a surface-sterilized biocontainment hood and coated with a very thin layer of silicone vacuum grease (Dow Corning) by gloved hand. Pairs of greased rods were then embedded in a small quantity of plumbers' putty fixed in the lid of a sterile 14 mL Falcon snap-cap tube (Corning Inc.).

To develop a standard curve for spore quantification, test primer specificity and sensitivity, and produce positive controls for each assay, rod pairs were coated with known quantities of conidia. *Erysiphe necator* conidial spore suspensions were produced by suspending spores from *V. vinifera* 'Chardonnay' vines in 0·05% Tween 20 (Sigma-Aldrich) in nuclease-free water then pipetted onto sterile prepared sampling rods. The concentration of the conidia suspension was estimated using a haemocytometer, and the suspension was pipetted onto pairs of coated stainless steel rods such that *c.* 100, 500, 1000 or 10 000 conidia were present on the rods (depending on experiment). One- and 10-conidia concentrations were created by using an eyelash brush to manually transfer conidia to coated stainless steel rods. The rods were allowed to air dry, and were either processed, as above, or stored at −20°C until processing.

### Quantitative PCR (qPCR) assay

DNA was extracted from rod pairs using the PowerSoil DNA extraction kit (MO BIO Laboratories) following the manufacturer's protocol. A set of silicone vacuum grease-coated stainless steel rods containing *c.* 500 *E. necator* conidia was included in

each set of DNA extractions as a positive control for extraction efficiency. DNA samples were analysed using qPCR the same day as processing, and then stored at −20°C for subsequent analyses.

Species-specific primers from Falacy *et al.* (2007), which produce a 367 bp PCR product from the internal transcribed spacer (ITS) region, were paired with a TaqMan probe with minor groove binder (Table 1). qPCR reactions were performed on an ABI StepOne Plus qPCR machine (Applied Biosystems). Each 15 μL qPCR reaction included 7·5 μL Path-ID qPCR Master Mix (Invitrogen), 400 nM final concentration of each *E. necator* forward and reverse primers and the probe, and 1·5 μL extracted DNA. PCR conditions were 95°C for 10 min, followed by 55 cycles of 95°C for 15 s and 65°C for 40 s.

All qPCR reactions were performed in triplicate and every reaction plate contained the 500 conidia extraction control, 100 and 10 000 conidia positive reaction controls, and template-free controls. Data acquisition and cycle threshold ($C_t$) analysis was conducted using ABI StepOne software. For every reaction plate, an automatic baseline was set by the StepOne software and the threshold was manually set to a value of 0·02 to allow for plate-to-plate relative comparison. The baseline was manually manipulated only when the automatic baseline yielded abnormal amplification curves. Conidia quantification was determined for each unknown field sample by identifying the average $C_t$ value for each triplicate reaction at which the log-linear phase intercepted the 0·02 threshold value and comparing this value to the standard curve described below. Average *E. necator* $C_t$ values of the known positive controls (100, 500 and 10 000 conidia) from each 96-well plate were used to confirm the efficiency of each qPCR reaction plate and to assess the suitability of the standard curve for converting $C_t$ values to conidia concentration.

Quantification of experimental samples was determined by comparing the $C_t$ value of each unknown field sample to a standard curve. An *E. necator* standard curve was prepared by placing a tenfold conidial dilution series (1–$10^5$ conidia) on the stainless steel sampling rods as described above. DNA extractions were conducted using the PowerSoil DNA extraction kit. Five separate *E. necator* conidia dilution series were prepared in this manner and analysed using qPCR as described. The standard curve was then generated by averaging the $C_t$ values for each conidia quantity from the five independent DNA extractions.

## LAMP primer development

A consensus sequence of the ITS1, ITS2 and 5S ribosomal RNA encoding regions of the *E. necator* rDNA was derived from 25 *E. necator* isolates from Oregon, Washington, California, New York (Brewer *et al.*, 2011) and Europe (Brewer & Milgroom, 2010). Sequences were used for primer design in PrimerExplorer v. 3.0 (Eiken Chemical Co.). Over 300 primer combinations were initially identified; however, only those in the ITS2 region, which is highly heterogeneous among Erysiphales (data not shown), were considered further. The final set of six primers specific to the ITS2 region of *E. necator* are presented in Table 1.

## LAMP assay

Pairs of prepared stainless steel sampling rods (described above) were collected from spore traps (described below) twice a week and transported in 14 mL Falcon tubes. For DNA extraction, rods were aseptically transferred to sterile 2 mL screw-cap tubes containing 200 μL Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7·5) for DNA extraction. The tube contents were thoroughly mixed using a vortexer at maximum speed for 5 s, centrifuged at 16 000 ***g*** for 1 min, boiled for 5 min, and then centrifuged at 16 000 ***g*** for 5 min. The supernatant was used as template for the LAMP reaction described below, and the rods were aseptically removed from the extraction tubes and discarded. The remaining supernatant was then stored at −20°C for subsequent analyses.

LAMP reaction procedures followed those of Notomi *et al.* (2000) with some modification through the addition of bovine serum albumin (BSA) and adjustment of buffer concentrations to account for high amounts of inhibitors present in field DNA extractions. The master mix was altered by manipulating the concentration of $MgSO_4$ (6–14 mM) and betaine (0·6–1·2 M). The final reagent mix contained ThermoPol buffer (1×; New England BioLabs), dNTP mix (1·4 mM), betaine (0·6 M), BSA (0·6 mg $mL^{-1}$), $MgSO_4$ (7 mM), internal primers FIP EN and BIP EN (2·4 μM), external primers F3 EN and B3 EN (0·24 μM), and loop primers FL EN and RL EN (1 μM), *Bst* DNA polymerase (0·32 U $\mu L^{-1}$; New England BioLabs), 5 μL of extracted DNA, and nuclease-free DEPC-treated water (Growcells) for a final volume of 50 μL per reaction. Reactions were incubated in a 65°C heat block for 45 min and then transferred to a heat block at 80°C for 5 min to inactivate the polymerase and then allowed to cool.

Visual inspections of turbidity were used to determine if extractions contained *E. necator* DNA. Turbidity is caused by the precipitation of magnesium pyrophosphate from a positive LAMP reaction. If turbidity was observed, then the reaction was

Table 1 Primers and probes used for detecting for the detection of *Erysiphe necator* internal transcribed spacer region

| Reaction | Primer/probe | Sequence (5′–3′) |
|---|---|---|
| qPCR[a] | Uncin144 | CCGCCAGAGACCTCATCCAA |
| | Uncin511 | TGGCTGATCACGAGCGTCAC |
| | Unc TaqMan Probe | 6FAM-ACGTTGTCATGTAGTCTAA-MGBNFQ |
| LAMP[b] | FIP EN (internal) | ACCGCCACTGTCTTTAAGGGCCTTGTGGTGGCTTCGGTG |
| | BIP EN (internal) | GCGTGGGCTCTACGCGTAGTAGGTTCTGGCTGATCACGAG |
| | F3 EN (external) | TCATAACACCCCCCTCAAGCTGCC |
| | B3 EN (external) | AACCTGTCAATCCGGATGAC |
| | FL EN (loop) | AAACTGCGACGAGCCCC |
| | RL EN (loop) | ACTTGTTCCTCGCGACAGAG |

[a]Primer concentrations in the reaction mix were 400 nM for Uncin144 (forward), Uncin511 (reverse), and for the Unc TaqMan probe. Melting temperatures for the primers were 59·2 and 59·9°C, respectively.

[b]Primer concentrations in the reaction mix were 2·4 μM for FIP, BIP, FL and RL, and were 0·24 μM for F3 and B3. Melting temperatures for the primers were between 64 and 99°C.

deemed positive; if no turbidity was observed, the reaction was deemed negative. LAMP reaction results were compared to concurrent qPCR reaction results to confirm sensitivity and specificity to *E. necator* DNA. In addition, a subset of samples was confirmed using *Nru*I (New England Bio Labs) restriction digestion and gel electrophoresis on a 3% agarose gel at 70 V for 60 min. To test the ability of the growers to perform the LAMP assay, they were provided with equipment to conduct the LAMP extraction and reaction, which included the heating block, centrifuge, vortex, pipette, extraction tubes with buffer, and reaction tubes with frozen master mix. The sensitivity of the grower-conducted LAMP assay (G-LAMP) was tested by providing each grower with blind samples of 0, 1, 10, 100, 500 and/or 1000 spores periodically throughout the monitoring period, with each grower conducting at least three independent extractions of each concentration. Sample rods were prepared as described above and placed at −20°C until used by growers.

## Primer specificity and sensitivity

Primer specificity and sensitivity of the LAMP reaction was examined in the laboratory. Air biota samples from vineyards with no known occurrence of grape powdery mildew, and hop yards with no vineyards within 5 km, were collected to test primer specificity against large quantities of background DNA. Primer sets that had no reaction with these samples were further tested against DNA from powdery mildew species found in and around vineyards in the region (Table 2). Conidia from various Erysiphales species were manually collected from plant leaves using vacuum grease-coated, stainless steel rods. *Erysiphe necator* DNA (confirmed by sequencing) was often present in samples that were obtained from vineyards with grape powdery mildew, causing amplification in the specificity testing of other powdery mildews. Therefore, the ITS1 and ITS4 primers (Tomlinson *et al.*, 2007) were used to amplify the ITS region from these samples, and the products were then cloned into the pTOPO 2.1 vector (Invitrogen) according to the manufacturer protocols. Plasmid DNA was purified using the Wizard Plus Miniprep DNA Purification System (Promega). The cloned ITS regions were then sequenced at the Center for Genome Research and Biocomputing at Oregon State University using the M13 primer, and were compared to known sequences found in GenBank. Plasmids containing the ITS region of other members of the Erysiphales were then used to test the specificity of the LAMP primers. The specificity of the qPCR primers was tested previously in this same manner (data not shown).

To test the sensitivity of the LAMP primers, *E. necator* conidial spore suspensions were created as above. Ten independent extractions of each concentration were examined. In addition, DNA samples ($n = 42$) of vineyard air biota, collected twice a week through a growing season and that had tested negative after three sequential qPCR amplifications, were added to provide a source of background DNA in place of the nuclease-free water.

## Field inoculum detection

Custom impaction spore traps (Fig. 1), similar to Rotorods (Sampling Technologies Inc.), were placed in 10 and 8 commercial vineyards within the Willamette Valley of Oregon in 2010 and 2011, respectively. Within each commercial vineyard, the impaction traps were located in areas where disease is perennially most severe or at locations where disease levels were the highest the previous autumn. Impaction spore traps were also placed at the Oregon State University Botany and Plant Pathology Research Vineyard (Corvallis, OR) in both years. In each vineyard, spore traps were placed such that the sampling arm was within 10 cm of a trunk or cordon until 30 cm of shoot growth occurred. Traps were then placed so that the sampling arm remained above the canopy for the rest of the growing season using 19 mm (ID) PVC pipe extensions. Each trap was capable of sampling $48{\cdot}3 \pm 1{\cdot}2$ L air $min^{-1}$ by spinning two stainless steel rods ($1{\cdot}1 \times 36$ mm effective surface area) coated with vacuum grease at $1{\cdot}05 \pm 0{\cdot}03$ m $s^{-1}$. Sampling rods were placed in traps every 3–4 days, and traps were run continuously from 26 April to 26 August 2010 and from 21 April to 22 August 2011.

Three traps were placed adjacent to one another at each location: one for G-LAMP, one for the laboratory-conducted LAMP (L-LAMP), and one for the qPCR. For the G-LAMP, sample rods were placed, collected and processed by the growers using the LAMP extraction protocol, and the LAMP reaction was conducted on location. The two other traps were maintained and processed by laboratory personnel. Each vineyard was treated as the experimental unit due to each grower individually conducting the LAMP assay and making subsequent management decisions based on the assay results. At the OSU Research Vineyard,

Table 2 Summary of specificity testing of *Erysiphe necator* ITS2 primers by loop-mediated isothermal amplification (LAMP) using other species of powdery mildew fungi found on various host species present in the Pacific Northwest. None of the isolates were detected using the LAMP primers specific to the ITS2 region of *E. necator*

| Pathogen species | Host |
|---|---|
| *Blumeria graminis* | *Poa* sp. |
| *Erysiphe aquilegiae* var. *ranunculi* | *Aquilegia canadensis* |
| *E. chicoracearum* | *Callistephus* |
| | *Cirsium arvense* |
| | *Coreopsis* sp. |
| | *Lactuca serriola* |
| | *Mentha arvensis* |
| | *Rudbeckia laciniata* |
| | *Taraxacum officinale* |
| *E. convolvuli* | *Convolvulus arvensis* |
| *E. cruciferarum* | *Brassica rapa* |
| *E. magnicellulata* var. *magnicellulata* | *Phlox* sp. |
| *E. pisi* | *Medicago sativa* |
| *E. polygoni* | *Beta vulgaris* |
| *E. rhododendri* | *Rhododendron* |
| *E. syringae* (syn. *Microsphaera syringae*) | *Caragana arborescens* |
| *E. trifolii* | *Trifolium pratense* |
| *Leveillula taurica* | *Allium cepa* |
| *Microsphaera nemopanthis* | *Ilex verticillata* |
| *Podosphaera aphanis* (formerly *Sphaerotheca macularis)* | *Rubus ursinus* |
| | *Rubus idaeus* |
| *Podosphaera aphanis* (formerly *S. macularis* f. sp. *fragariae*) | *Fragaria* sp. |
| *Podosphaera clandestina* | *Prunus* sp. |
| *P. delphinii* (formerly *Sphaerotheca delphinii*) | *Ranunculus abortivus* |
| *P. fusca* (formerly *Sphaerotheca fusca*) | *Cucurbita pepo* |
| *P. macularis* (formerly *S. macularis*) | *Humulus lupulus* |
| *P. leucotrica* | *Malus domestica* |
| *P. pannosa* (formerly *Sphaerotheca pannosa*) | *Prunus persica* |
| *Sawadea* sp. | *Acer* sp. |
| *Uncinuliella flexuosa* | *Aesculus* sp. |

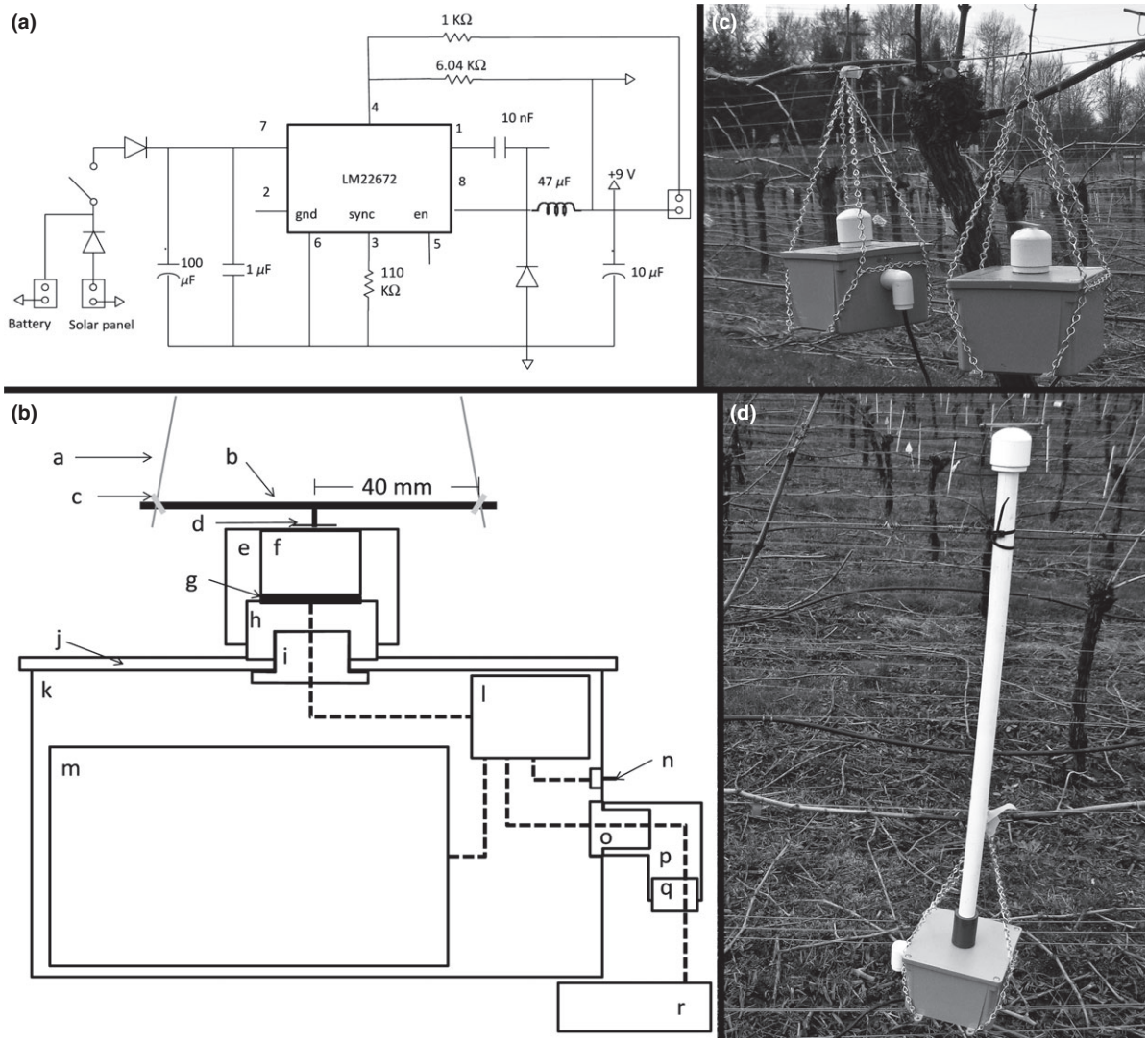


Figure 1 Impaction spore trap design as used in 2010 and 2011 monitoring years. (a) Circuit diagram of the voltage regulator used for impaction spore trap. (b) Spore trap components: a, 1·1 × 40 mm stainless steel rods; b, 4·7 × 90 mm aluminum sampling arm with 40 mm sampling radius; c, silicone o-ring (4·5 mm ID); d, Teflon disk (6·5 mm) with a 1·5 mm hole in the centre and vacuum grease placed underneath; e, 32 mm PVC endcap; f, Mabuchi RF-500T-10750 DC motor; g, silicone O-ring (24 mm ID); h, 32 to 19 mm PVC reducer; i, 19 mm PVC bushing; j,k, 153 × 153 × 102 mm PVC junction box with gasketed lid; l, circuit board in (a); m, 5 Ah sealed lead acid battery; n, toggle switch; o, 13 mm PVC bushing; p, 13 mm PVC elbow; q, 13 mm PVC plug; r, 18 W 12 V solar panel (SunWise, Inc.). Dashed lines are 22 gauge paired electrical wire except for the 14 gauge wires connecting the solar panel. (c) Early season traps placed on either side of the trunk to capture ascospore release. (d) 19 mm ID PVC pipe extension used to raise the sampling arm of the impaction spore trap above the grapevine canopy.

two traps were used; one trap was processed using the LAMP assay (LAMP DNA extraction and LAMP reaction), and the other trap was processed using the PowerSoil DNA extraction kit and qPCR reaction as described above. The amplicons from all initial positives for the L-LAMP and G-LAMP were confirmed using gel electrophoresis, as described above.

### *Analysis of LAMP performance assuming qPCR as a gold standard*

The LAMP assay results were compared to the qPCR assay results via 2 × 2 contingency table for both the L-LAMP and the G-LAMP assays. For this analysis, the qPCR assay was assumed to be correct and treated as the 'gold standard' test. From the contingency table, true positive proportion, true negative proportion, accuracy, and the positive and negative predictive values of the LAMP assays were calculated (Fawcett, 2006). The true positive proportion was calculated as [1 − (false positive/total negative)], and the true negative proportion as [1 − (false negative/total positive)]. The accuracy is defined as [(true positive + true negative)/total observations], and the positive predictive value was the probability of being truly positive given a specific set of test results. The misclassification rate is defined as [(false positive + false negative)/total observations]. A Fisher's exact test was conducted on 2 × 2 contingency tables, whereby the qPCR assay was assumed to be correct for the detection of *E. necator* DNA on the sampling rods; the null

hypothesis for the Fisher's exact test was that results of the LAMP and qPCR assays were not correlated.

### *Analysis of LAMP performance assuming no 'gold standard'*

The assumption that the qPCR results always correctly indicated *E. necator* presence is probably not true due to errors inherent to the qPCR assay and due to independent spore samples being used by each of the experimental assays that may not have always contained similar quantities of *E. necator* DNA for each assay at every location. Therefore, a latent class analysis (LCA) was used to estimate the test characteristics for both the qPCR and LAMP assays using the SAS PROC LCA (Lanza *et al.*, 2007, 2011). PROC LCA is an add-on procedure available through the Pennsylvania State University Methodology Center for SAS v. 9.3 (SAS Institute). PROC LCA fits latent class models by treating the presence of spores as a two-class latent variable (Turechek *et al.*, 2013). As used here, LCA is a statistical procedure used to evaluate the performance of diagnostic tests in the absence of a gold standard. The methodology exploits the use of the cross-classified test results and uses a maximum likelihood approach to designate individual test results in to one of two mutually exclusive categories (spores present or absent) and uses this information to estimate the true positive and negative proportions for the individual tests. This method does not make the assumption that the qPCR is the gold standard. A full description of the procedure in a plant pathology setting can be found in Turechek *et al.* (2013). Two separate LCA analyses were conducted: a 2-test LCA (comparing qPCR and L-LAMP) and a 3-test LCA (comparing the qPCR, L-LAMP and G-LAMP assays, resulting in eight comparison combinations). The assay true positive and true negative proportions could not be estimated for the individual years in the LCA because there were not enough degrees of freedom to estimate five parameters (Hui & Walter, 1980), so the yearly data was treated as two independent populations and it was assumed that the tests' true positive and true negative proportions were equivalent for the two populations. Posterior probability values, generated from SAS PROC LCA, were used to describe the probability of the presence of *E. necator*, where the *x*-axis represents the probability of a positive detection and the *y*-axis represents the probability of a true positive detection for a given assay.

## Commercial vineyard test sites

At each vineyard ($n$ = 10 and 8 in 2010 and 2011, respectively), growers established paired treatment plots consisting of their standard management programme (control plot) and a detection treatment (detection plot). Control plot fungicides were initiated at 6 inches of growth or when a risk model indicated a high risk for spore release, and detection treatment plot fungicide applications were withheld until inoculum was detected or bloom had occurred (BBCH growth stage 61). Subsequent applications of fungicides followed manufacturer recommendations for reapplication depending on chemistry. Plot size varied from six 30 m rows to 1 ha. After a fungicide programme was initiated, additional applications in both the control and detection plots were made using the grower's standard fungicide programme. Detection plots were strategically placed in powdery mildew 'hot spots' because they are more likely to have greater numbers of overwintering cleistothecia and greater potential for early inoculum detection. True negative control plots at each vineyard were not possible to include in the experimental design due to the crop value (>\$74 000 $ha^{-1}$) and the potential for interplot interference (Campbell & Madden, 1990), and negative control plots were not required to determine if the delayed fungicide treatments were as effective as the standard grower practice. All management decisions were made by the grower associated with each sampling site, based on the standard disease management procedures for the region and using inoculum detection data to initiate fungicide application schedules. Rainfall, temperature, relative humidity and leaf wetness were recorded in 15 min intervals at all field locations to assess the suitability of environmental conditions for disease development using the Gubler/Thomas index (Gubler *et al.*, 1999).

## Vineyard disease monitoring

To monitor disease progress, each plot was visually scouted weekly for powdery mildew incidence starting on 16 June 2010 and 24 June 2011 (BBCH 15–19, when leaf pubescence has decreased), for the respective years, until véraison by inspecting 10 arbitrarily selected leaves from each of 50 vines in each plot with a hand lens. Disease severity was not assessed due to the low disease incidence observed before véraison on foliar tissue. Because there were few, if any, signs or symptoms of powdery mildew visually observed on the fruit (<0·1%), below the economic threshold (Ough & Berg, 1979; Stummer *et al.*, 2003), berry disease incidence was determined by destructively sampling one cluster per vine from 50 vines per plot at the onset of véraison (BBCH 81). Clusters were frozen after collection at −20°C until microscopically assessed for powdery mildew presence. After freezing, berries were stripped from the rachis and 25 berries were arbitrarily assessed for the presence of powdery mildew under ×40 magnification (Ficke *et al.*, 2003). A berry was rated to have disease if a single penetration site was observed.

Leaf disease incidence from the detection and control plots were compared by determining the area under disease progress curve (AUDPC) for each grower plot, which were then compared by a one-tailed Student's *t*-test. Berry disease incidence was also compared using a one-tailed Student's *t*-test.

# Results

## Primer specificity and sensitivity

The LAMP primers did not cross-react with any of the Erysiphales species tested (Table 2), nor with the air biota samples from hop yards or vineyards without a history of powdery mildew, suggesting a level of pathogen specificity sufficient for testing in Pacific Northwestern vineyards. The LAMP primers provided positive reactions when *E. necator* DNA was introduced at 1, 10, $10^2$, $10^3$, $10^4$ and $10^5$ conidial quantities, showing high primer sensitivity to low spore quantities (1–10 spores; Fig. 2). G-LAMP showed less sensitivity than L-LAMP in both years (Fig. 2).

## Field inoculum detection

### *Analysis of LAMP performance assuming qPCR as a 'gold standard'*

The Fisher's exact test showed that the L-LAMP and qPCR results were in agreement for both 2010 and 2011 ($P < 0{\cdot}0001$; Table 3). The L-LAMP had true positive

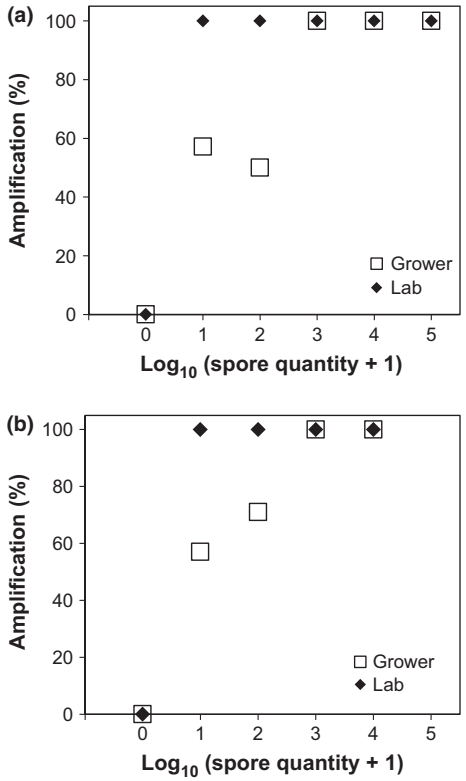


Figure 2 LAMP primer sensitivity to $\log_{10}$ conidial quantities +1 as tested by the laboratory (solid diamond) ($n$ = 53) and growers (square) ($n$ = 42) in both (a) 2010 and (b) 2011 blind samples.

proportions of 96 and 92% in 2010 and 2011, respectively. The L-LAMP had a misclassification rate of 8 and 19% in 2010 and 2011, respectively. The L-LAMP accuracy for both years was 92 and 80% in 2010 and 2011, respectively.

Commercial vineyard managers from the Willamette Valley were able to assess grape powdery mildew presence using the LAMP assay. A Fisher's exact test showed that there was an agreement in 2011 ($P$ = 0·049) between the G-LAMP and qPCR assays (Table 3). The G-LAMP had true negative proportion of 76 and 94% in 2010 and 2011, respectively. G-LAMP did not perform as well as the L-LAMP, with lower assay true positive proportions (48% in 2010 and 33% in 2011) compared to the L-LAMP assay true negative proportions (82% in 2010 and 67% in 2011). However, the G-LAMP had a misclassification rate of 38 and 39% in both 2010 and 2011. In addition, the G-LAMP assay was 62% accurate in both 2010 and 2011.

### *Analysis LAMP performance assuming no 'gold standard'*

Considering only the laboratory results in Table 4 (i.e. qPCR and L-LAMP), complete agreement occurred for 89% [i.e. (8 + 11 + 5 + 19)/48] and 69% of the samples in 2010 and 2011, respectively. The G-LAMP assay agreed with qPCR results *c.* 63 and 62% of the time and with L-LAMP results *c.* 60 and 87% of the time for 2010 and 2011, respectively (Table 4). Complete agreement among all three test results – either all positive or all negative – was found in *c.* 56 and 59% of the cases in 2010 and 2011, respectively (Table 4). Both the 2-test and 3-test LCA (Table 5) indicated that the G-LAMP and L-LAMP assay results had lower true positive and true negative proportions than the qPCR assay results in 2010 and 2011. While the qPCR had a higher true positive proportion than the LAMP assays, the LAMP assays still had a true negative proportion of 80% or greater in both 2-test and 3-test LCA (Table 5). A positive result

Table 3 Contingency table representing grower-conducted LAMP (G-LAMP) assay and laboratory-conducted LAMP (L-LAMP) assay to quantitative PCR (qPCR) results for the presence of *Erysiphe necator* sampled from custom-made impaction spore traps from commercial vineyards and research plots at the Oregon State University Botany and Plant Pathology Research Vineyard

| Year | Test[a] | | qPCR[b] Positive | qPCR[b] Negative | Fisher's exact test (probability)[c] |
|---|---|---|---|---|---|
| 2010 | G-LAMP | Positive | 11 (23%) | 6 (13%) | 0·13 |
| | | Negative | 12 (25%) | 19 (39%) | |
| | L-LAMP | Positive | 54 (20%) | 9 (3%) | <0·0001* |
| | | Negative | 12 (5%) | 191 (72%) | |
| 2011 | G-LAMP | Positive | 7 (18%) | 1 (3%) | 0·049* |
| | | Negative | 14 (36%) | 17 (44%) | |
| | L-LAMP | Positive | 37 (32%) | 5 (4%) | <0·0001* |
| | | Negative | 18 (15%) | 57 (49%) | |

[a]'Positive' and 'negative' indicate the number of samples for which *E. necator* DNA was detected and not detected, respectively, as tested by G-LAMP ($n$ = 48 in 2010, $n$ = 39 in 2011) and L-LAMP ($n$ = 266 in 2010, $n$ = 117 in 2011) assays as described in the text.
[b]qPCR results based on TaqMan probe with minor groove binder for detecting *E. necator* DNA. 'Positive' and 'negative' indicate the number of samples for which *E. necator* DNA was detected and not detected, respectively.
[c]Fisher's exact test was used to assess the null hypothesis that the LAMP assay was not significantly different from the qPCR assay.
*Significant chi-squared test at $\alpha$ = 0·05.

Table 4 The eight possible test response patterns of the quantitative PCR (qPCR), laboratory-conducted LAMP (L-LAMP), and grower-conducted LAMP (G-LAMP) used in the 3-test latent class analysis (LCA), and the number of samples each year with each pattern

| Test response pattern | | | Year | | |
|---|---|---|---|---|---|
| qPCR | L-LAMP | G-LAMP | 2010 | 2011 | Total |
| + | + | + | 8 | 6 | 14 |
| + | + | − | 11 | 4 | 15 |
| + | − | + | 3 | 1 | 4 |
| + | − | − | 2 | 10 | 12 |
| − | + | + | 0 | 1 | 1 |
| − | + | − | 0 | 0 | 0 |
| − | − | + | 5 | 0 | 5 |
| − | − | − | 19 | 17 | 36 |
| *n* | | | 48 | 39 | 87 |

Table 5 Estimates of the true positive and true negative proportions of quantitative PCR (qPCR), laboratory-conducted LAMP (L-LAMP), and grower-conducted LAMP (G-LAMP) assay results from 2010 and 2011 commercial vineyards and research plots at the Oregon State University Botany and Plant Pathology Field Lab based on 2-test and 3-test latent class analyses (LCA)[a]

| | 2-test LCA | | 3-test LCA | | | |
|---|---|---|---|---|---|---|
| | 2010/11 | | 2010 | | 2011 | |
| Test | True positive proportion[b] | True negative proportion[c] | True positive proportion | True negative proportion | True positive proportion | True negative proportion |
| qPCR | 0·9101 | 0·9957 | 0·9874 | 0·9573 | 0·8993 | 0·6372 |
| L-LAMP | 0·7551 | 0·9780 | 0·8074 | 0·9929 | 0·8474 | 0·9896 |
| G-LAMP | – | – | 0·4576 | 0·7857 | 0·6183 | 0·9924 |

[a]LCA was conducted using PROC LCA in SAS v. 9.3 (SAS Institute).
[b]True positive proportion was defined as the conditional probability of a positive assay result given the sample is truly positive.
[c]True negative proportion was defined as the conditional probability of a negative assay result given the sample is truly negative.

from both assays had a probability of >0·98 that *E. necator* was present even for a prior *P* as low 0·01. A positive result by qPCR but a negative result by LAMP decreased the probability the detection was truly positive but not as substantially as a negative test result by qPCR and a positive result by L-LAMP decreased the probability of detection. It is still possible that *E. necator* was present when neither assay was positive (Fig. 3).

### Commercial vineyard test sites

Of the 10 and 8 commercial test locations in 2010 and 2011, respectively, only five vineyard managers in each year kept the inoculation and control plots under independent management. Across both testing seasons, fungicide application savings were variable, *c.* 3·3 fungicide applications per vineyard, depending on fungicide chemistry (either organic or synthetic chemistry) and location. On average 2·6 fewer conventional fungicide applications and four fewer organic fungicide applications were used in the duration of the project. Vineyard managers that did not follow protocol used the inoculum detection to time their initial fungicide applications for their entire vineyard. Of the five sites that were managed according to experimental protocols, there were not significant differences in the disease progress curves of leaf incidence for the detection treatment and the commercial standard control (Fig. 4). Nearby abandoned vineyards had 100% disease incidence by 15 July in both years (data not shown) and the Gubler/Thomas index (Gubler *et al.*, 1999) remained mostly above 80 from 15 June to 1 September (data not shown). In addition, all vineyards had >50% leaf incidence on young leaves at harvest (30–60 days after last fungicide application depending on vineyard). These observations indicated that the pathogen was present and that conditions were suitable for disease development when not managed.

### Vineyard disease monitoring

The leaf incidence AUDPC for 2010 (five sites) detection treatment and commercial standard control were extremely

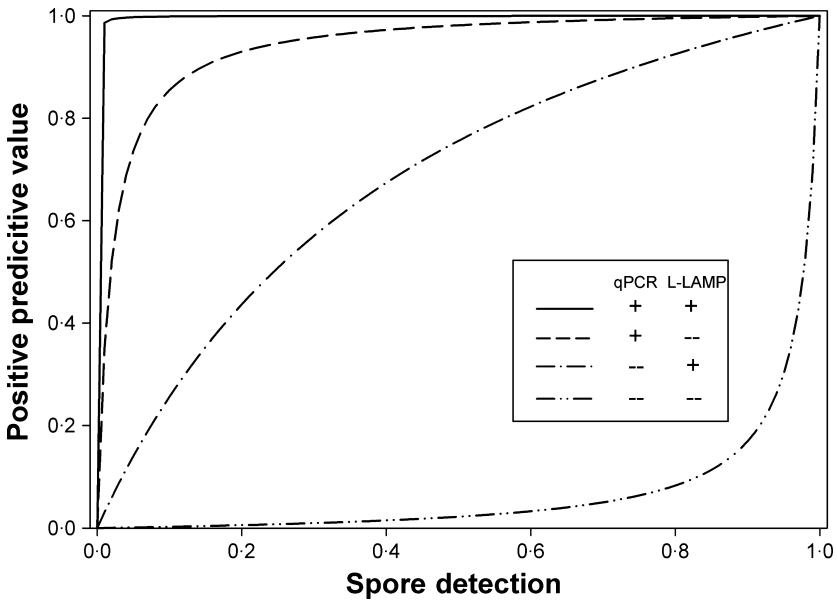


Figure 3 The posterior probability, or the positive predictive value (the probability of being truly positive given a specific set of test results) for the quantitative PCR (qPCR) and laboratory-conducted LAMP (L-LAMP) as determined by the 2-test latent class analysis using PROC LCA in SAS v. 9.3 (SAS Institute). Both axes represent the probability of a positive result, where the *x*-axis represents the probability of a positive detection and the *y*-axis represents the probability of a true positive detection for a given assay.

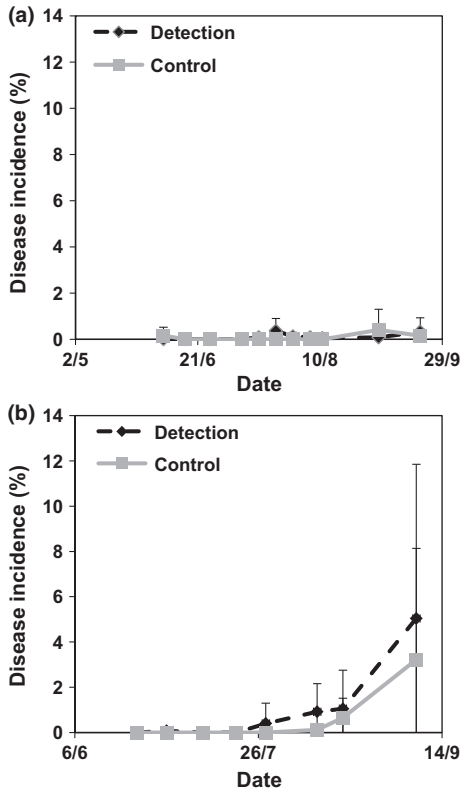


Figure 4 Disease progress curves for field disease incidence determined by field scouting in 2010 (a) for five commercial vineyards, and in 2011 (b) for five commercial vineyards. A sample of 500 leaves was assessed from a detection plot (fungicide programme initiation was delayed until disease detection) (dashed line) and a control plot (fungicide initiation followed the grower standard) (solid line). Error bars are based on the standard deviation for each data point. Area under disease progress curve (AUDPC) values were determined using average disease incidence (%). Detection and control AUDPC values were 9·3 ± 11·44 and 5·6 ± 5·8, respectively, in 2010 and were 94·1 ± 126·8 and 41·33 ± 30·8, respectively, in 2011. The control plots were not significantly different from the detection plots in 2010 ($P = 0{\cdot}30$) or in 2011 ($P = 0{\cdot}16$).

low (9·3 ± 11·4 and 5·6 ± 5·8, respectively), and the control plots were not significantly different from the treatment plots ($P = 0{\cdot}30$). The 2011 AUDPC values (five sites) were also very low but with significant variability (94·1 ± 126·8 for the detection treatment and 41·3 ± 30·8 for the commercial standard control) due to the focal nature of the disease incidence in the plots (data not shown). Because of this variability, leaf incidence in control plots was not significantly different from the treatment plots ($P = 0{\cdot}16$). There were <0·1% visual symptoms of fruit infection by *E. necator* at all locations and treatments in both years, thus percentage berry disease incidence was determined microscopically (described above). Berry disease incidence in 2010 (five sites) detection treatment and commercial standard control were 9·3 ± 16·7 and 1·6 ± 1·4%, respectively, and the detection treatment was not significantly different from the control ($P = 0{\cdot}21$). Berry disease incidence in 2011 (five sites) detection treatment and commercial standard control were 30·8 ± 28·1 and 5·1 ± 4·5%, respectively, and were not significantly different ($P = 0{\cdot}06$). In both years, one vineyard, where the detection plot was over a septic system, resulting in more succulent, highly susceptible tissue, was responsible for most of the variability in the leaf and fruit disease incidence between the detection and commercial standard plots. Removing this field from the data analysis causes the AUDPC to decrease to 6·2 ± 10·5 and 7 ± 5·6 for the detection plots and the control plots in 2010, respectively, and 39·9 ± 42·7 and 32 ± 26·3 for the detection plots and the control plots in 2011, respectively.

## Discussion

Growers were able to effectively manage grape powdery mildew using inoculum detection (determined by performing the G-LAMP assay) to initiate fungicide applications, despite the less than optimal performance of the assay compared to the qPCR assay. The G-LAMP assay performance could be less than optimal due to several factors, including freeze–thaw degradation of LAMP reaction components in poor on-site storage conditions, misinterpretation of LAMP turbidity at low inoculum concentrations, and samples containing different amounts of spores from the qPCR or L-LAMP traps. Despite the inaccuracies throughout the duration of the experiment, initiating fungicide applications based on a positive detection in the G-LAMP assay reduced fungicides required without compromising the disease control that is expected by the industry. Many growers in this region have since altered their standard fungicide programme practices by delaying their first fungicide application until inoculum has been detected, which, in this study, saved on average 3·3 fungicide applications per vineyard each year.

The disease scouting of grower plots conducted in 2010 and 2011 confirmed the utility of the G-LAMP assay for optimizing fungicide initiation. Disease incidence on leaf tissue did not exceed 1% in 2010 and the incidence in 2011 never surpassed 6% (Fig. 4) during the assessment period, even though nearby abandoned vineyards reached 100% leaf incidence by 15 July (data not shown). There were no significant differences between the grower standard and inoculum detection plot AUDPC values in either year. Delaying fungicide applications based on the detection of *E. necator* DNA did not result in economic damage because visual rating of fruit incidence was less than 0·1% for all clusters examined. This indicates that the G-LAMP assay is a useful tool for the implementation of inoculum detection in commercial vineyards.

While there was no statistically significant increase in the AUDPC between the detection-initiated plots and the control plots across all vineyards included in this study, there was one vineyard with considerably greater disease in the inoculum detection plot than the control plot. The detection plot in this vineyard was located over a septic

drain field, resulting in considerably more vigorous vines than the control plot vines, and therefore more conducive for disease development (Pearson, 1994). However, the disease levels in 2010 and 2011 in this vigorous block were substantially lower than the previous 6-year average from the same block (data not shown). An alternative explanation for the higher disease levels observed in the detection plots could be that the spore trap failed to detect the initial ascospore release. Infections may have occurred and initiated the secondary phase of the epidemic, but were not detected in these plots due to the differences in row spacing and canopy density (Bailey & Stoll, 2013; Bailey *et al.*, 2014). Large eddy simulations of particle dispersion in vineyards indicate that tight row spacing and increased canopy density reduced particle dispersion, which would also reduce spore movement and the ability to detect potential inoculum.

Because 3% disease incidence (visually assessed) on berries affects wine quality (Ough & Berg, 1979; Stummer *et al.*, 2003), and there were few visual disease symptoms on berries (less than 0·1%, data not shown), there was no economic damage associated with delaying fungicide applications until detection. However, berry incidence of powdery mildew was also measured microscopically to more accurately determine the establishment of *E. necator* on the fruit. A single necrotic spot associated with a germinated spore resulted in a berry being considered infected. This probably resulted in an inflated disease incidence compared to studies where only visual ratings were done (Ough & Berg, 1979; Stummer *et al.*, 2003).

The *E. necator* LAMP primers were highly specific and sensitive to their target, despite greater than 10 orders of magnitude greater background DNA from vineyard air biota and presence of other Erysiphales that commonly occur in and around vineyards in the Pacific Northwest (Fig. 2). In addition to the high sensitivity and specificity of the primers, both the qPCR and L-LAMP assays were shown to have true positive and true negative proportions above 80% when used for detection by both the LCA and 'gold standard' methods of analysis. The true positive and true negative proportions of the qPCR assay as shown by the LCA indicates that the assay could be useful as a 'gold standard' for developing other detection assays, such as the LAMP detection assay. However, the 3-way LCA indicated that the true positive and true negative proportions of the qPCR assay decreased in 2011 (90% and 64%, respectively) compared to 2010 (99% and 96%, respectively). This decrease may have been due to 3-way LCA analysis overestimating the influence of negative detection and agreement between the L-LAMP and G-LAMP assays when compared to 10 positive qPCR assay results. Based on the combination of the contingency table analysis and the LCA results, as well as experience using this assay, it is probable that qPCR assay results were correct.

The 2-test LCA indicated that the L-LAMP assay showed a high true negative proportion when compared to the qPCR assay (Fig. 3), but the 3-test LCA indicated that the L-LAMP assay showed lower true positive and true negative proportions than the qPCR assay. It also indicated that the G-LAMP assay had lower true positive and true negative proportions compared to the L-LAMP assay. These differences may be due to the difficulty of assessing turbidity at very low concentrations of target DNA (Kubota *et al.*, 2011). Growers sometimes also envisioned turbidity when no *E. necator* DNA was present. Upon subsequent examination of these samples in the laboratory using gel electrophoresis, the banding pattern of nonspecific reactions or the banding pattern for *E. necator* DNA were not observed in these false positive samples (data not shown). Various dyes such as PicoGreen or hydroxynaphthol blue dye (Ohtsuka *et al.*, 2005; Dukes *et al.*, 2006; Mori & Notomi, 2009; Tsai *et al.*, 2009; Ward & Harper, 2012) are now available, since the onset of this project, for improving the visual inspection of LAMP products; however, these dyes are often added post-reaction and opening a LAMP reaction tube will increase the chance of contaminating future reactions. LAMP reactions produce large quantities of amplicons that have a complex tertiary structure which is highly stable and capable of self-replication (Kubota *et al.*, 2011), and is very difficult to clean up if spilled or aerosolized (L. D. Thiessen, personal observation). These dyes, while they may improve accuracy in determining DNA amplification, also present difficulty in discerning differences between zero spores and low spore quantities without the aid of a spectrophotometer (Mori *et al.*, 2004; Tomlinson *et al.*, 2007). To reduce subjectivity of visual turbidity or dyed product inspection and to allow for quantification of LAMP products, the use of a FRET-based probe (Kubota *et al.*, 2008) has been developed.

Because independent sets of sample rods were used for each assay and each assay had different DNA extraction procedures, differences in assay results could be the result of differences in the quantity of *E. necator* DNA present on sample rods or the amount of inhibitors present on the sample rods processed for qPCR. Inhibitor removal efficacy of the DNA extraction protocol probably varies between the qPCR and LAMP assays. It is possible that not all inhibitors were removed sufficiently by the LAMP extraction process for amplification to occur. Spore samples that were collected daily at the research vineyard were compared side by side with the samples collected twice a week, with no significant difference in positive and negative detections in the L-LAMP assay and in the qPCR assay (data not shown). The potential for misclassification, either by false negative reactions or failure of available spores to be retained on spore rods, would also be a reason to use a twice-a-week sampling regime to guide management decisions. Under optimal conditions *E. necator* has a generation time of 5 days (Delp, 1954); thus, there would be a minimum of two samples every generation time. This approach could reduce the impact of a false negative on management decisions.

Inoculum detection by PCR has been shown to be an effective management tool in several pathosystems

(Calderon *et al.*, 2002; Falacy *et al.*, 2007; Luo *et al.*, 2007; Carisse *et al.*, 2009a; Rossi *et al.*, 2010), but relies on an inhibitor-free DNA source. However, the LAMP assay does not require expensive technology or formal training for DNA extraction and the detection of inoculum (Notomi *et al.*, 2000), making it suitable for commercial use with grapevine growers conducting the detection analyses. This study, in conjunction with other studies (Falacy *et al.*, 2007; Carisse *et al.*, 2009b, 2012; Van der Heyden *et al.*, 2014), also demonstrates that there may be benefit to managing polycyclic diseases, at least those caused by other Erysiphales, using airborne inoculum detection assays. In both sampling years, the L-LAMP detection results were not significantly different from that of the qPCR detection results, indicating that the extraction assay was sufficient for detection (Table 3). G-LAMP detection results were significantly different from the qPCR detection results in 2010 but were not significantly different in 2011, which may be due to growers improving their ability to assess the turbidity of the LAMP reactions from the first year of the project to the second or due to changes in storing the master mix. The results presented here indicate that the LAMP assay may be useful for the management of grape powdery mildew and is feasible in the absence of a laboratory, but would benefit from further refinement of the procedure. Presently, work to use a FRET-based assimilating probe (Kubota *et al.*, 2011) for quantitative LAMP is being conducted to adjust fungicide application intervals using a minimum spore action threshold and to further optimize LAMP inoculum detection. Further study is needed to assess the utility of the quantitative measure of inoculum provided by qPCR versus the convenience and reduced cost of a quantitative LAMP detection assay (Mahaffee *et al.*, 2011).

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