AN ABSTRACT OF THE DISSERTATION OF

Lindsey D. Thiessen for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on May 20, 2016.

Title: Overwintering of *Erysiphe necator* and Inoculum Monitoring for Decision Aids

Abstract approved:

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Walter F. Mahaffee

Grape powdery mildew (*Erysiphe necator*) causes economic damages to grape worldwide due to the cost of management and injury to berries. Each region where European grapevine (*Vitis vinifera*) is grown experiences a unique epidemic, and disease prediction models that are based on empirical correlations of weather data to disease fail to predict disease accurately in regions with differing environmental conditions. Near real-time monitoring of grape powdery mildew epidemics using spore sampling and molecular detection techniques allowed grape growers to reduce fungicides applications by ~2.5 applications compared to their standard practice. Although spore sampling allowed growers to reduce fungicide applications, it is only a short-term solution to the lack of understanding of the factors surrounding initial inoculum availability. Additional factors affecting cleistothecia development and ascospore release conditions were also examined in order to improve current modeling efforts. This involved using controlled-environment studies to monitor cleistothecia initiation and development, and quantitative PCR was used to monitor

ascospore release. Several established ascospore release models were tested for prediction accuracy, and an improved ascospore release prediction model was developed. The results of this work show that a portion of cleistothecia are capable of releasing ascospores before leaf drop and continue to mature and release ascospores throughout grape dormancy and into the following growing season. The results also show that an improved understanding of inoculum availability may allow grapevine growers to reduce management costs while improving disease control.

Overwintering of *Erysiphe necator* and Inoculum Monitoring for Decision Aids
by
Lindsey D. Thiessen

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APPROVED:

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Lindsey D. Thiessen, Author

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

LIST OF TABLES (Continued)

Table Page

Overwintering of *Erysiphe necator* and Inoculum Monitoring for Decision Aids

1. Introduction

Erysiphe necator Schwein (syn. *Uncinula necator*) causes a polycyclic disease that causes damages to both the foliage and fruit of grapevine (*Vitis* spp.). Damages associated with infections by *E. necator* are mitigated through the use of fungicides in conjunction with cultural practices to delay the disease epidemic until fruit tissues exhibit ontogenic resistance (41, 47, 48, 56). Several disease prediction and risk assessment models have been developed to improve fungicide application timing through predicting early inoculum release (19, 21, 29, 86, 130); however, model predictions inaccurately predict inoculum release (117) or have yet to be assessed in the coastal viticulture regions of the U.S. (19, 29, 103). The overwintering cleistothecia house ascospores that function as the initial inoculum in most regions that grapevine is grown (37, 62, 113, 129). The release of ascospores has been previously characterized to occur with precipitation events of at least 2.5 mm and temperatures above 4 ºC (53). These conditions occur frequently throughout the dormancy of grapevine within the maritime regions of the Pacific Northwest. Despite the potential for depletion of initial inoculum prior to bud break, ascosporic infections are observed within the Willamette Valley of Oregon. In addition to environmental conditions that may affect the release of ascospores, the maturation of cleistothecia and ascospores may be dependent of the formation period of cleistothecia and influence the period of ascospore release and magnitude of ascospore release.

Initiation of cleistothecia production requires two mating types, Mat 1-1 and Mat 1-2 (52, 55), and sexual populations have been described in several regions within the U.S. (15). While cleistothecia initiation has been described in some regions as soon as both mating types are in contact (52), some regions describe cleistothecia formation toward the end of the growing season (17, 75, 86). Currently, the only factor that has been implicated in the initiation of cleistothecia formation is proximity to opposite mating type (52); however, detached leaves and tissue culture plants were utilized to assess the impact of environmental characteristics and cleistothecia development. The wounding to create detached leaves may cause host physiological changes that affect pathogen development, including upregulation of host defense compounds, such as jasmonic acid, and host senescence compounds, such as abscisic acid (87).

The primary goal of this research was to improve commercial vineyard disease management of grape powdery mildew through investigating the factors surrounding the development, overwintering, and dehiscence of cleistothecia, and subsequent disease development.

Ascospore release and germination initiates the disease epidemic and several ascospore release models have been developed for several regions that grapevine is grown (19, 29, 53, 104, 130), and many have not yet been tested in the Pacific Northwest U.S. (19, 29, 103), and inoculum detection techniques were used to monitor ascospore release from a naturally infested vineyard (Chapter 2). Additionally, the development of a binary ascospore release model for this region was developed by monitoring ascospore release from a known quantity of cleistothecia in

controlled environmental conditions, and was validated using ascospore release data collected in a naturally infested vineyard.

Due to the limited understanding of early season inoculum release, a grower conducted inoculum detection method using loop-mediated isothermal amplification (LAMP) assay was developed to initiate fungicide applications in the presence of airborne inoculum within a vineyard (Chapter 3). Three adjacent spore samplers were placed in commercial vineyards to sample airborne inoculum. One of the spore samplers was maintained by the grower, and the other two spore samplers were maintained by the lab. The LAMP assay was conducted by the grower and the lab, and the quantitative PCR assay was conducted by the lab. Growers were able to use the data from the grower-conducted LAMP assay, and approximately 3.3 fewer fungicide applications were made per season compared to the grower standard practice without compromising disease control (129).

An improved LAMP assay for the detection and quantification of *E. necator* DNA was developed (Chapter 4) because visually observing magnesium pyrophosphate in samples with low concentrations of DNA was difficult (Chapter 3). A FRET-based assimilating probe was used to assess DNA amplification of quantitative LAMP reactions (83), and hand-held LAMP devices were assessed for the utility of a grower-conducted quantitative LAMP. The qLAMP assay was developed that was not significantly different from a quantitative PCR assay; however, the qLAMP assay showed a loss of sensitivity to the *E. necator* DNA target during grower-conducted qLAMP testing. The cause for the loss of sensitivity has yet

to be determined, and field implementation of qLAMP as a decision aid for fungicide timing was not conducted.

To improve fungicide application interval timing, a quantitative PCR assay was used to detect and enumerate airborne *E. necator* inoculum, and a minimum spore quantity threshold was used to inform management decisions of fungicide interval lengths (Chapter 5). When the minimum 10 spore threshold was met, fungicide intervals were reduced to the shortest interval according to the fungicide label, and when spore detections were below the 10 spore threshold, the longest interval between fungicide applications according to the chemistry label was used. In commercial vineyards this method saved approximately 2 fungicides per season for commercial vineyards, while in a research vineyard it resulted in 3 more fungicide applications.

The initiation and development of cleistothecia at the end of the growing season is poorly understood in the coastal viticulture regions of the U.S. Cleistothecia form at the end of the growing season despite the presence of both mating types within the region. The distribution of mating types within a vineyard block, host senescence and drought stress, as well as temperature and photoperiod shifts on host plants grown from seed were assessed to determine the factors that affect the initiation of cleistothecia on *Vitis vinifera* L. (Chapter 6). Field surveys showed that both mating types were in close proximity throughout the growing season, but cleistothecia did not develop until after véraison. Field experiments on established *Vitis vinifera* cv. Chardonnay and growth chamber experiments using Chardonnay seedlings were unable to determine host derived or environmental factors associated

with the initiation of cleistothecia. There was, however, an association of temperature and photoperiod shift with the quantity of cleistothecia produced.

Despite not determining what factors influence the initiation of cleistothecia, cleistothecia are consistently formed at the end of the growing season, and interruption of cleistothecia development may be possible prior to leaf drop. Stylet oil was applied to Chardonnay grapevines at the end of the growing season to interrupt and reduce cleistothecia production (Chapter 7). Stylet oil was applied one time in each treatment, and treatments were applied after the first observation of cleistothecia primordia, 1 week, 2 weeks, and 3 weeks after that. Applying stylet oil reduced cleistothecia production significantly in 2014, independent of application timing. Although the reduction of cleistothecia was statistically different, cleistothecia were still produced in all treated plots.

The research presented herein may be used to improve current disease management recommendations and information regarding the development of overwintering inoculum. Inoculum detection may be used to monitor airborne *E. necator* inoculum within vineyard air to time fungicide applications in response to potential inoculum pressure. Additionally, environmental conditions associated with ascospore release events was used to generate an ascospore release model, which may be used by growers to initiate fungicide application programs.

2. Factors influencing *Erysiphe necator* ascocarp overwintering and ascospore release in the Willamette Valley of Oregon

2.1 Introduction

The regional variability of primary inoculum release of *Erysiphe necator* and subsequent development of the disease epidemic on *Vitis vinifera* (19, 53, 62, 129) may be related to the local adaption of the grapevine powdery mildew pathosystem. *Erysiphe necator* is thought to originate from the warm, temperate climate of southeastern North America (15), where the regional environment is characterized by hot wet summers and cool dry winters (79). European grapevine (*Vitis vinifera* L.) is thought to originate from southern Europe and the Mediterranean basin (145), which is characterized by dry-summer subtropical or temperate climate with wet winters with moderate temperatures (79). Under its native climatic conditions, *E. necator* evolved to overwinter in a cool, dry climate, and it is likely that evolutionary adaptations to this climate impacts the development of overwintering structures/ These adaptations may cause regional variability of ascospore release and disease development during the following growing season.

Ascospores released from cleistothecia are the primary inoculum source for most grape growing regions (38, 62, 113, 129). Cleistothecia (syn. chasmosthecia), the overwintering ascocarp, are formed in the previous growing season on green tissues and are dispersed from leaves to the exfoliating bark (52) where they overwinter until conducive conditions for release are met. Ascospore release in the field has been implicated to occur after ≥2.5 mm rain and temperatures are above 4 ºC

(53). In order to estimate the occurrence and severity of ascospore release, Thomas et al. (130) generated a modified Mills' apple scab table multiplied by $^{2}/_{3}$; however, Hall (66) demonstrated in the Willamette Valley that these conditions did not always predict disease onset.

Because management efforts are required before *E. necator* signs are readily detectable through scouting, a number of disease forecasting models have been developed to predict *E. necator* primary inoculum release as a function of environmental conditions to aid in management decisions (19, 62, 76, 103, 130). Several of the disease prediction models, however, inaccurately predict early disease onset for the Willamette Valley of Oregon (117), and others (19, 29, 103) have not been tested in the maritime climate of the western U.S. These models consist of empirical functions that are derived from environmental conditions that differ from those of the coastal viticulture regions of the U.S., and predictions may not accurately describe fungal overwintering, maturation, and ascospore release.

The degree day model described by Carisse et al. (29) for predicting ascospore release and growing season inoculum in the Quebec Province of Canada does not consider the loss of initial inoculum during grapevine dormancy in regions with warmer and wet climates, such as the maritime Western U.S.. The mechanistic model described by Caffi et al. (19) also ignores loss of inoculum during grapevine dormancy prior to January 1, and validated the model using simulated conidia data from Chellemi and Marois (35), ascospore release from a single temperature (53), or observed conidia germination data (40). Furthermore, this model predicts the proportion of ascospore available for release based on degree days from time that

primordia are observed in the previous growing season, which is not likely to be implemented by growers. The ascospore release model developed by Moyer et al. (103) accounts for loss of inoculum during overwintering; however, this model is based on induced ascospore release under optimal conditions in the laboratory from cleistothecia that have overwintered in field conditions. These limitations could affect the accuracy and sensitivity of these models to predict ascospore release and time disease management practices.

The purpose of this study was to assess the environmental factors affecting primary inoculum release in a region with temperate overwintering conditions and high precipitation. The specific objectives of this research were to 1) determine when ascospore release within the Willamette Valley or Oregon occurs, 2) examine the fit of previously developed ascospore release models to ascospore release observed within this region, and 3) determine the environmental factors associated with ascospore release in the Willamette Valley of Oregon.

2.2 Materials and Methods

2.2.1 Cleistothecia Collection

Leaves containing cleistothecia for all experiments were collected from a research vineyard and three different commercial vineyards at various locations in the Willamette Valley of Oregon in October (BBCH 92) prior to the first rain before leaf drop of each year. To prevent additional wetting and drying cycles, cleistothecia were

vacuumed from leaf surfaces into sterile, 15 ml Falcon tubes and stored at 4°C until placed in experimental conditions.

2.2.2 Ascospore Overwintering and Monitoring

An array of 8 open air boxes with top and bottom open and sides enclosed was created using twin wall polycarbonate greenhouse plastic (Fig 2.1A). In the center of each array box (Fig 2.1B), a custom impaction spore sampler suspended in the center of the box using 2 cm diameter PVC (Fig 2.1C) was placed and connected in parallel to a power control board similar to Thiessen et al (129). Artificial bark or grape trunk segments (15.2 cm length) were suspended 61 cm from the ground to mimic the height of a grapevine trunk head or cordon. The artificial bark pieces were generated using brown tweed-wool fabric covering 15.2 cm long × 3.2 cm diameter wooden dowels, which had similar wetting and drying properties to grape bark. Four replications of each treatment were placed in a randomized complete block design with each 8-box array as the block (Fig 2.1D).

Cleistothecia collected from vineyards were subsampled using an anti-static polypropylene 3-7 mg microscoop (Tradewinds Direct, Inc., Pleasant Prairie, WI) and suspended in 1 ml of deionized water, and the concentration of cleistothecia per microscoop estimated using a hemocytometer. Each artificial trunk piece, was inoculated with 10^3 cleistothecia by measuring dry-collected cleistothecia with a microscoop and then mechanically embedding the cleistothecia into the wool-tweed by gently rubbing cleistothecia into the wool. 15.3 cm long natural grapevine trunk pieces were also inoculated with 10^3 cleistothecia by depositing cleistothecia into

bark crevices with a microscoop. The artificial or natural trunk pieces were placed over custom impaction spore traps and placed into an array of open air clear boxes (Fig 2.1).

Boxes were tested for the presence of external inoculum contaminating ascospore release collections by placing an open air box with a spore sampler inside a greenhouse with *E. necator* infections. Ascospore arrays were placed outdoors away from known inoculum sources from the onset of precipitation events during overwintering in the Willamette Valley until the following spring when ascospore release was exhausted. Ascospore release from artificial bark was compared to grapevine trunk pieces to ensure that wetting and drying events of the artificial bark were consistent with the natural bark. Custom impaction spore samplers were run constantly and rods were collected on Mondays and Thursdays beginning in November and continuing until ascospores were no longer detected after wetting events. Spore rod samples were stored at -20 °C until processing.

An additional three custom impaction spore samplers (Chapter 3) were placed at the Botany and Plant Pathology Research vineyard adjacent to naturally infested grapevine trunks. Field traps were run continuously and collected on Mondays and Thursdays from leaf drop (BBCH 97, mid- to late November) until disease was observed in the following growing season. Collected spore rods were stored at -20 °C until processing.

2.2.3 Spore rod preparation

Stainless steel sampling rods (1.1 mm diameter × 36 mm long) were cut from 308LSI welding rods (Weldcote Metals) and soaked in hexane for 24 hours. Rods were rinsed with dishwashing detergent and water and then shaken in 10% Clorox bleach solution (0.83% NaOCl) for 15 minutes. The rods were then rinsed 3-4 times with deionized water to remove residual bleach. Next, sample rods were autoclaved for 30 minutes then dried in a laminar flow hood. Sample rods were transferred to a biocontainment hood and coated in a very thin layer of silicone vacuum grease (Dow Corning). Pairs of greased rods were placed into a small quantity of plumbers' putty fixed into the lid of sterile 14 ml Flacon snap-cap tubes (Corning Inc.).

A standard spore quantification curve and positive controls for each assay were produced by coating rods pairs with known quantities of conidia. The conidia suspensions of *E. necator* were created by suspending spores in 0.05% Tween 20 (Sigma Aldrich, St. Louis, MO, USA) and sterile deionized water solution. The spore suspension was pipetted onto rod pairs such that 100, 500, 1000, or 10,000 spores were present on the rods. To accurately produce 1 and 10 spore conidia concentrations, conidia were manually transferred to sterile rod pairs using an eyelash brush. The rods were allowed to air dry prior to processing or storage at -20°C.

2.2.4 Ascospore Enumeration

DNA from array samples and positive control rods was extracted by modifying a quick extraction technique. A set of rods inoculated with 500 *E. necator* conidia was included in each set of DNA extractions as an extraction efficiency control. Spore rods were placed into 2 ml tubes containing 200 µl 5% Chelex 100

(Sigma Aldrich, St. Louis, MO, USA) in DEPC treated water. Tubes were vortexed for 5 seconds then boiled in deionized water for 5 minutes for 2 cycles. Tubes were allowed to cool for 2 minutes prior to centrifugation at 16,000 g for 2 minutes. The rods were aseptically removed from the tubes and discarded. The supernatant was used as template for the qPCR reaction described below and stored at -20°C.

Quantitative PCR (qPCR) protocols were modified from that of Thiessen et al. (129) to accommodate the larger volume from the DNA extraction. Species specific primers (Uncin144 and Unc511) developed by Falacy et al. (44) were used in conjunction with the Unc TaqMan probe with minor groove binder (129). All qPCR reactions were conducted on an ABI StepOne Plus qPCR machine (Applied Biosystems, Grand Island, NY, USA). Each reaction included 7.5 µl of PerfeCTa qPCR ToughMix (Quanta Biosciences, Gaithersburg, MD, USA), and 400 nM final concentration of forward and reverse primers and TaqMan probe in a 15 µl reaction with 3 µl of DNA template. PCR conditions were 95°C for 15 seconds followed by 55 cycles of 95°C for 15 seconds and 65°C for 40 seconds.

Every reaction plate contained a 500 conidia extraction efficiency control, 100 and 10,000 conidia positive controls, and a template-free negative control. All reactions were performed in triplicate. Data collection and cycle threshold (C_T) analysis was conducted using the ABI StepOne software. The threshold value was manually set to 0.02 to allow for reaction plate relative comparison. The baseline was only manually manipulated when the automatic baseline value yielded abnormal amplification curves. Quantification of conidia was determined for each unknown by determining the average C_T value for each triplicate reaction and comparing to the

standard curve. The standard curve was generated by extracting 5 separate *E. necator* conidia dilution series prepared as described above, and the C_T value for each conidia quantity was determined. All unknown spore quantities were compared to the standard curve to determine spore quantity, and positive controls were compared to the standard curve to confirm the efficiency of the extraction and reaction.

2.2.5 Environmental Conditions Measurements

Relative humidity, temperature, rainfall, leaf wetness using capacitance (Decagon LWS, Pullman WA) and resistance (Campbell Scientific 237-L, Logan, UT) leaf wetness sensors, and bark wetness sensors (described below) were measured at 5-minute intervals throughout the duration of the experiment using Campbell Scientific CR10X dataloggers.

To test the comparability of an artificial bark and real grapevine bark, wetness measurements of brown tweed wool and bark from grapevine were compare using Pessl resistance leaf wetness sensors (IM521CD Pessl Instruments, Austria) where the filter paper was replaced with either a 5 cm × 2 cm bark piece or brown wool tweed, and resistance was measured every 5 minutes.

2.2.6 Ascospore Release Model Testing

All data were analyzed using R version 3.2.1. Models were tested using the weather data collected throughout the duration of grapevine dormancy in all years of this study. After predicted ascospore release was determined for each model,

predictions were summarized to the same time scale as the ascospore collection dates to compare predicted and observed ascospore release for each year.

2.2.6.1 UC Davis Risk Index

The UC Davis Risk Index describes ascospore release and germination using $^2/_3$ of the value from the Mills table for apple scab (64). Because the resulting risk index is binary, the true positive proportion, true negative proportion, accuracy, and Fisher's exact test was used to compare predicted and observed ascospore release.

2.2.6.2 New York Ascospore Release Model

The NY model describes ascospore release as a function of 2 mm of rain or irrigation with temperatures between 10°C and 27°C (53). Because the resulting risk index is binary, the true positive proportion, true negative proportion, accuracy, and Fisher's exact test was used to compare predicted and observed ascospore release.

2.2.6.3 Moyer Ascospore Release Model

The ascospore release model (Eq. 1) from Moyer et al. (103) describes percent ascospore release as a function of accumulated degree days with a base of 0 °C (DD_0) beginning January 1 and potential discharge events (PDE). PDE is defined as the number of days whereby rainfall is greater than or equal to 2.5 mm and daily average temperatures are above 0 °C.

$$Percent\ Release = 1 - exp[-exp(-3.335 + 0.00222\ (DD_0 + 0.150287 \cdot PDE)] \quad Eq.\ 1$$

2.2.6.4 Caffi Mechanistic Model

The mechanistic model described bv Caffi et al. (19) uses ascospore release and subsequent infection to predict the development of sporulating *E. necator* colonies on grape leaves. For this analysis, only the portions of the model t describing ascospore release (Eq. 2-4) were examined. This model also represents the error of model constants. For the purpose of testing the model, the error of the constants were ignored, and the constant means were used to test field collected ascospores. Although this model was developed to predict ascospore release after bud break (BBCH 08), the model was initiated at the start of field ascospore collection due to the potential for ascospore release prior to bud break.

The model describes ascospore release as a function of the proportion of ascospores ready for release (PAR_i, Eq. 2), the number of ascospores in cleistothecia (AIC, Eq. 3), ascospore maturation rate (AMR, Eq. 3), and the ascospore discharge rate (ADR). Degree days with a base of 10 °C (DD_i) are calculated by using the day of the year at bud break (DOY_{bb}) to assess the portion of ascospores ready for release (PAR_i).

$$PAR_i = exp[(-1.95 \pm 0.188) \cdot exp(((-1.91 \pm 0.293) \cdot DD_0) \div 100))] \qquad Eq.\ 2$$

$$DD_i = \sum_{DOY_{bb}}^{i}(T_i - 10); \text{ if T}i\text{-10} < 10, \text{ then } DDi = 0$$

The number of overwintering cleistothecia (OCH) is unknown, this number is set to 1 within the model and expressed as a percentage of total cleistothecia.

$$AIC = \Sigma_{DOY_{bb}}^{i}(AMR \cdot OCH) \qquad Eq.\ 3$$

$AMR = f'(PAR)$, where f'(PAR) is the first derivative of PAR

Ascospore release is described as a function of ascospores on leaves (AOL, Eq. 4). The ADR is a function based on the condition of 2 mm of precipitation (R_i) occurring on day I and temperature (T) between 4 and 30 ºC. If conditions for release are met, ascospore discharge depends on temperature and wetness duration (W, hours)

$$AOL_i = AIC_i \cdot ADR_i \qquad Eq.\ 4$$

If $R_i < 2$ mm or T<4 C or T>30 ˚C, $ADRi = 0$

When precipitation ≥ 2 mm or T≥4 ˚C or T≤30 ˚C,

$$ADR = 1 - 0.969 \pm 0.024 \times \exp(-0.0004 \pm 0.00003 \times T^2 \times W)$$

2.2.6.5 Carisse Degree Day Model

The degree day model developed by Carisse et al. (29) describes ascospore and subsequent conidia airborne inoculum concentration (P_{maxacc}) as a function of degree days between 6 and 30 ºC (Eq. 5).

$$P_{maxacc} = 1.0755(1 + e^{-0.0042DD})^{1/1-1.0169} \qquad Eq.\ 5$$

2.2.7 Model Development

Data analysis was conducted in the R version 3.2.1 environment using multiple linear regression. Percent ascospore release was analyzed in conjunction with the environmental data collected throughout the duration of the project to determine the impact of environmental factors on ascospore release. The environmental parameters that were tested were leaf wetness, cumulative leaf wetness duration, minimum temperature, average temperature, maximum temperature, dew point, duration of temperature over 4 ºC, precipitation, precipitation event duration, bark wetness, cumulative bark wetness duration, and relative humidity. Data transformations of ascospore release data, both raw ascospore quantity and percent of total ascospore release, were assessed, including the log, logit, first derivative, and arcsin transformations. Model and data transformations were chosen based on the correlation (R^2) to observed ascospore release. Model predictions of ascospore release events were assessed using Fisher's exact test comparing predicted results to field collected ascospore release data.

2.3 Results

2.3.1 Period of Ascospore Release

Ascospore release from both field and ascospore sampling array occurred from the onset of precipitation events in the fall through the start of the following growing season in all three overwintering periods (Fig 2.2). No differences in wetting or drying cycles were observed between artificial bark and natural trunk pieces, and

ascospore release was observed from both artificial and natural bark. No detections occurred in non-inoculated artificial or natural grapevine bark treatments.

2.3.2 Ascospore Release Model Testing

The UC Davis Risk Index ascospore release predictions were significantly correlated to observed ascospore release in all years ($P = 0.04$) (Table 2.1). The model predicted ascospore release had an 78% true negative proportion, but it over predicted ascospore release and had a 46% true positive proportion and 53% accuracy. The New York model was not correlated to observed ascospore release events in all years ($P = 0.34$) when the predicted ascospore release was compared to the observed ascospore release (Table 2.1). The model had 72% true positive proportion but over predicted ascospore release since it had a 37% true negative proportion, and 64% accuracy. Ascospore release predictions from the Moyer ascospore release model showed no relationship to observed ascospore release in 2012-2013 ($R^2 = 0$), 2013-2014 ($R^2 = 0$), and 2014-2015 ($R^2 = 0$) overwintering periods (Fig 2.3A). Predicted ascospore release from the Caffi model also showed no relationship to observed ascospore release in 2012-2013 ($R^2 = 0$), 2013-2014 ($R^2 = 0$), and in the 2014-2015 ($R^2 = 0$) overwintering periods (Fig 2.3B). The Carisse degree day model prediction had a poor relationship to observed ascospore release in the 2012-2013 ($R^2 = 0.05$), 2013-2014 ($R^2 = 0.01$), and 2014-2015 ($R^2 = 0.05$) overwintering periods (Fig 2.3C).

2.3.3 Environmental Factors Affecting Ascospore Release

Environmental factors observed in each growing season were used to generate an ascospore release severity model. The predicted percent ascospore release from the model developed using the ascospore release events collected in the ascospore sampling array (Eq. 6) showed a correlation ($R^2 = 0.41$) with precipitation (mm, P), cumulative precipitation duration (hours, P_D), and average temperature (°C, T) with no significant difference between predicted ascospore release and observed spore release from field collected ascospores ($P > 0.24$) (Fig 2.3D).

$$A = 4.13 \times 10^{-2} + 1.09 \times 10^{-2} \cdot P - 4.18 \cdot T - 8.94 \times 10^{-4} \cdot P_D - 2.07 \times 10^{-3} \cdot P \cdot T + 9.94 \times 10^{-6} \cdot P \cdot P_D + 1.38 \times 10^{-4} \cdot T \cdot P_D - 1.30 \times 10^{-6} \cdot T \cdot P \cdot P_D \quad \textit{Eq. 6}$$

The multiple linear regression model (Eq. 7) showed low correlation to field collected ascospore release in the 2012-2013 ($R^2 = 0.03$), 2013-2014 ($R^2 = 0.09$), and 2014-2015 ($R^2 = 0.05$) overwintering periods.

Due to the low correlation between predicted ascospore release magnitude and observed ascospore release, a daily binary ascospore release model (Eq. 7) was developed based on correlated weather variables described in described in section 3.2.8. Ascospore release (A), predicted as 1 (occurrence of release) or 0 (no release), is a function of whether within a 24-hour period, there was greater than 6 hours of cumulative leaf wetness during temperatures above 4 °C (W_{T4}), precipitation greater than 2.5 mm (P), and relative humidity above 80% (R), where the presence of each environmental condition is also represented as 1 and the absence as 0.

$$A = W_{T4} * P * R \quad \textit{Eq. 7}$$

The model was validated using field collected ascospore datasets, which predicted ascospore release with 66 % accuracy and significant agreement ($P = 0.02$) between observed and predicted ascospore release events (Table 2.1). The predicted values also showed a 68% true positive proportion and 56% true negative proportion compared to field observed values.

2.4 Discussion

Previously developed ascospore release models from dissimilar growing regions inaccurately predicted ascospore release in the Willamette Valley of Oregon (Fig 2.3). Each model frequently predicted no ascospore release when release was detected, indicating that the empirical models are not effective in discerning environmental conditions that were favorable for release. All of the ascospore release models predict ascospore release during grapevine dormancy and predict ascospore release more frequently in the Willamette Valley of Oregon than the regions in which they were developed (19, 28, 53, 103, 130). The ascospore release models (Eq. 6 and 7) developed in this study using data from the Willamette Valley also predict ascospore release during grapevine dormancy, and ascospore release continued into the growing season where susceptible host tissue was available for infection.

Ascospore release events occurred when leaf wetness occurred simultaneously with temperatures above 4 ºC, daily average relative humidity above 80%, and precipitation events ≥2.5 mm, and these conditions were used to develop a binary ascospore release model (Eq. 7). Other studies have implicated similar environmental variables including temperature, leaf wetness and precipitation (19, 29, 53, 75, 103,

120); however, the disease prediction models developed from the data derived in previous studies (19, 130) are based on daily summarized environmental variables and do not require that environmental variables occur concurrently. The New York model and the UC Davis Risk Index, both of which are based on daily average and cumulative moisture measurements, show a high proportion of false positives (Table 2.1). The binary model developed in this study also reduced the number of false positive results compared to the UC Davis Risk Index and New York models, which may be due in part to assessing leaf wetness assessments when temperatures were above 4 ºC.

Furthermore, the predictive values of ascospore release models may be influenced by ascospore collection methods used to develop models. Ascospore release assessment methods, such as lab-induced ascospore release (53, 103) or inspection of *E. necator* lesions caused by ascosporic infections (19, 130), may limit the accuracy of ascospore release predictions. Lab-induced ascospore release from cleistothecia that have been overwintered in environmental conditions (53, 103) may not accurately identify the environmental conditions required for ascospore release due to loss of inoculum within periods of conducive conditions during overwintering. Moreover, providing free moisture and temperatures between 22 and 25 ºC also excludes other environmental conditions that may influence ascospore release from model development and may cause premature release of ascospores that would not have occurred naturally. Ascospore release models developed from the physical inspection of *E. necator* ascosporic infections (19, 130) may inaccurately predict ascospore release because successful germination of ascospores to form visible

colonies is required. This assessment method likely underestimates ascospore release by not accounting for ascospore loss due to unsuitable conditions for infection and/or ascospores that did not land on susceptible host tissue. Examining the leaf tissue for ascosporic infections may also mischaracterize infections initiated by conidia, which may cause overestimation of ascospore release.

The improvement of ascospore release predictions in this study may be in part due to the sampling array system developed. The accuracy of the environmental variable driven ascospore release model developed in this study was somewhat improved (e.g. 66% accuracy) compared to previously developed models by sampling airborne inoculum and examining concurrent environmental variables that occurred during the sampling period. The sampling array was placed in a region without other known inoculum sources, and no contamination was observed between boxes. Inoculum sources were also located within 8.5 cm of the sampling arm to capture low magnitude ascospore release events. The qPCR assay is highly sensitive and capable of detecting DNA from one *E. necator* spore (Chapter 3), which allowed for detection of small magnitude ascospore release events. In addition to more sensitive ascospore sampling techniques, cleistothecia were monitored in environmental conditions on simulated bark that showed similar wetting and drying properties to natural bark, which likely better mimics naturally infested vineyards compared to overwintering on substrates like filter paper.

The ascospore release models developed in this study and other studies that predict the magnitude of ascospore release showed low correlation to ascospore release magnitude observed in field conditions (Fig 2.3), which may be due to the

asynchronous development of cleistothecia cohorts. The variability in the number of total cleistothecia present in a field and the range of ascospores produced within a cleistothecium may be due to environmental conditions (Chapter 6) and the duration of time *E. necator* isolates are within proximity to a compatible mating type (52). Additionally, cleistothecia have been shown to require a period of maturation to release ascospores capable of infection (53), which, in conjunction with long periods for development (e.g. between cessation of fungicide applications and leaf drop), may generate several cohorts that release ascospores at different times during the season. Cleistothecia production in regions with temperate overwintering conditions, such as the Willamette Valley of Oregon, occurs over a long period at the end of the growing season, and may allow for several cleistothecia maturation cohorts to develop. Observed cleistothecia production occurs at BBCH 85 in the Willamette Valley of Oregon and is inhibited by temperatures above 25 ºC (Chapter 6) or precipitation events (59). Models developed in other regions, such as New York (19, 52, 53, 103), were based on a shorter period for cleistothecia production that created fewer maturation cohorts, which may explain model prediction of ascospore release exhaustion prior to bud break in environmental conditions of the Willamette Valley. Complete depletion of inoculum through the release of ascospores was not observed despite the presence of conducive conditions.

In contrast to previous reports (53, 62, 75, 103), cyclical wetting was not required for ascospore release to occur, and release was observed at the first precipitation event in all years. The cleistothecia for this experiment were not exposed to moisture prior to placement in the ascospore sampling array; thus, the

immediate release of ascospores from the cleistothecia suggests that other factors, such as decreasing lipid content, may also impact cleistothecia rupturing. Hydrophobic lipid contents of cleistothecia decrease as cleistothecia mature (52), which may limit the uptake of water into less mature cleistothecia. Asynchronous lipid production and utilization may also prevent depletion of initial inoculum prior to the growing season, despite conducive conditions for release throughout the duration of grape dormancy. The asynchrony of cleistothecia production and lipid utilization may reduce the ability to predict ascospore release magnitude and depletion, and the employment of a binary ascospore release model that assumes the presence of viable cleistothecia may be more useful for field applications.

The binary ascospore release model developed in this study may be used to initiate fungicide programs based on the release and availability of ascospores when susceptible host tissue is available. Evaluating this model to initiate fungicide applications in a field environment is necessary to determine the impact on disease management. Additionally, it may be possible to use the binary ascospore release model developed in this study in conjunction with ascospore germination predictions and risk assessments (19, 20, 53, 54, 64, 130) to improve *E. necator* management strategies. Continued investigation of ascospore germination and subsequent disease development is required to determine the efficacy of using previously developed germination models in conjunction with the ascospore release conditions presented in this study.

Table 2.1. Contingency table representing model predicted ascospore release compared to observed ascospore release for all overwintering periods between 2013 and 2015.

		Observed [b]		Fisher's Exact Test (Probability)[c]
		Positive	Negative	
UC Davis Risk Index Predicted	Positive	21	50	0.04
	Negative	6	42	
NY Model Predicted	Positive	10	25	0.34
	Negative	17	67	
Oregon Predicted [a]	Positive	15	29	0.03
	Negative	12	63	

[a] "Positive" and "Negative" indicate the number of samples for which *E. necator* ascospore release was predicted to occur or not occur based on the binary ascospore release model as described in the text (Eq. 7).

[b] "Positive" and "Negative" indicate the number of samples for which *E. necator* DNA was detected and not detected, respectively using the qPCR assay based on TaqMan® probe with minor groove binder.

[c] Fisher's exact test was used to assess the null hypothesis that the observed ascospore release was not significantly different from the model predicted ascospore release.

Figure 2.1. Ascospore release spore trapping array design. Four separate ascospore sampling arrays (A) were arranged in a region with no grapevine nearby to avoid potential conidial contamination. Each array contained 7 separate units (B) whereby a spore sampler (C) was contained below surrogate bark or grape trunk segment (15.3 cm long) infested with cleistothecia. Surrogate bark consisted of 15.3 cm × 3.8 cm diameter wooden dowels covered in brown wool. Individual boxes were 30.5 cm × 30.5 cm × 91.5 cm, whereby surrogate bark pieces were suspended above the spore sampler in the center of the array box. Spore samplers were run constantly and sampled every 3 or 4 days. Each array box contained cleistothecia collected from vineyards within the Willamette Valley of Oregon. Each array also included non-inoculated bark or surrogate bark pieces to ensure ascospores or outside sources of inoculum were excluded from the system. All treatments were randomized within each array replicate.

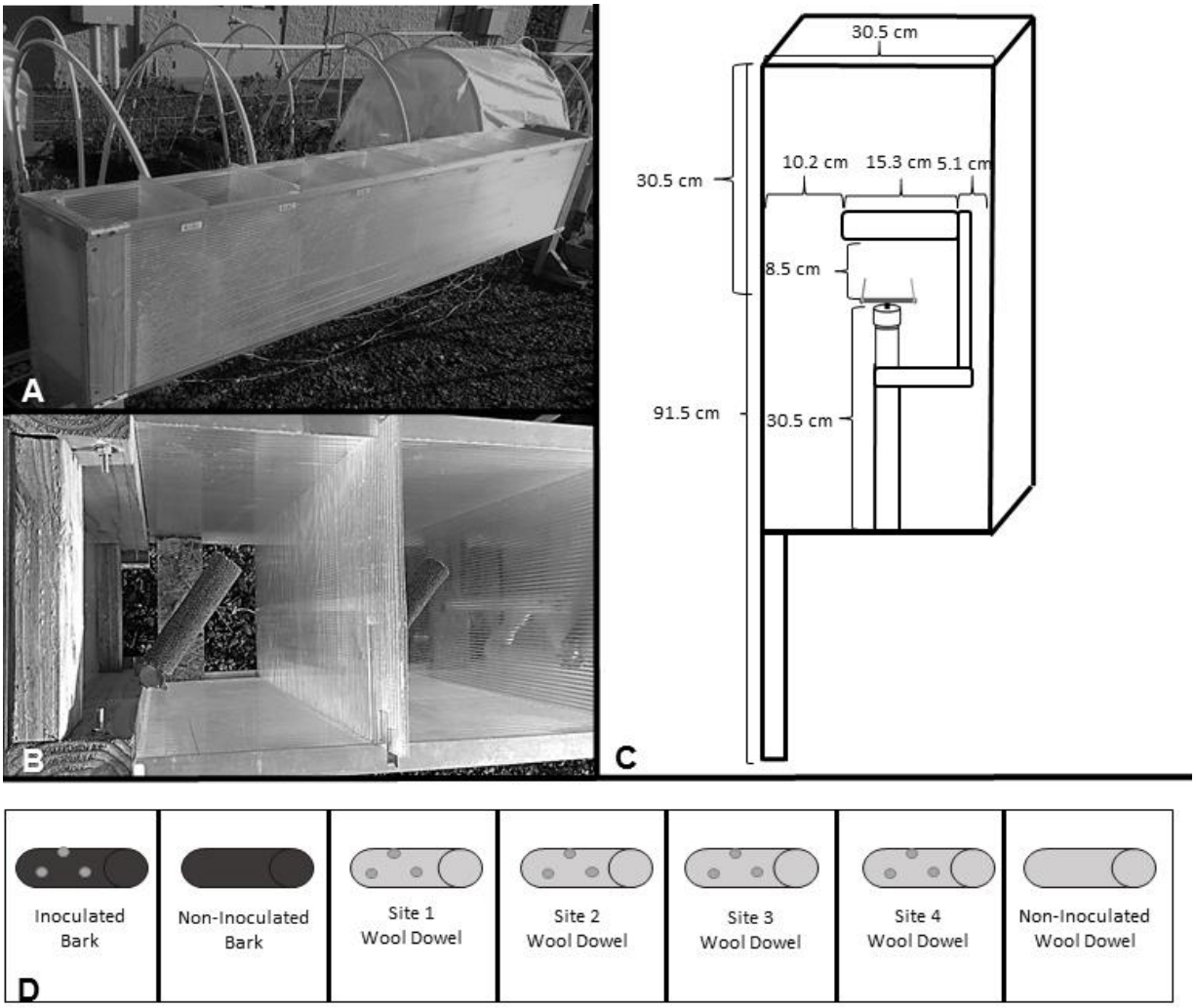
A
B
C
30.5 cm
10.2 cm
15.3 cm
5.1 cm
30.5 cm
8.5 cm
91.5 cm
30.5 cm
Inoculated Bark
Non-Inoculated Bark
Site 1 Wool Dowel
Site 2 Wool Dowel
Site 3 Wool Dowel
Site 4 Wool Dowel
Non-Inoculated Wool Dowel
D

Figure 2.1.

Fig. 2.2. Observed positive ascospore release events from the inoculated ascospore sampling array (black dots) and from the naturally infested research vineyard collected during 2012-2013 (A), 2013-2014 (B), and 2014-2015 grapevine dormancy seasons under environmental conditions. Ascospore samples were collected every 3 to 4 days, whereby DNA from samples was extracted using a Chelex 100 extraction (described in text) and enumerated using quantitative PCR.

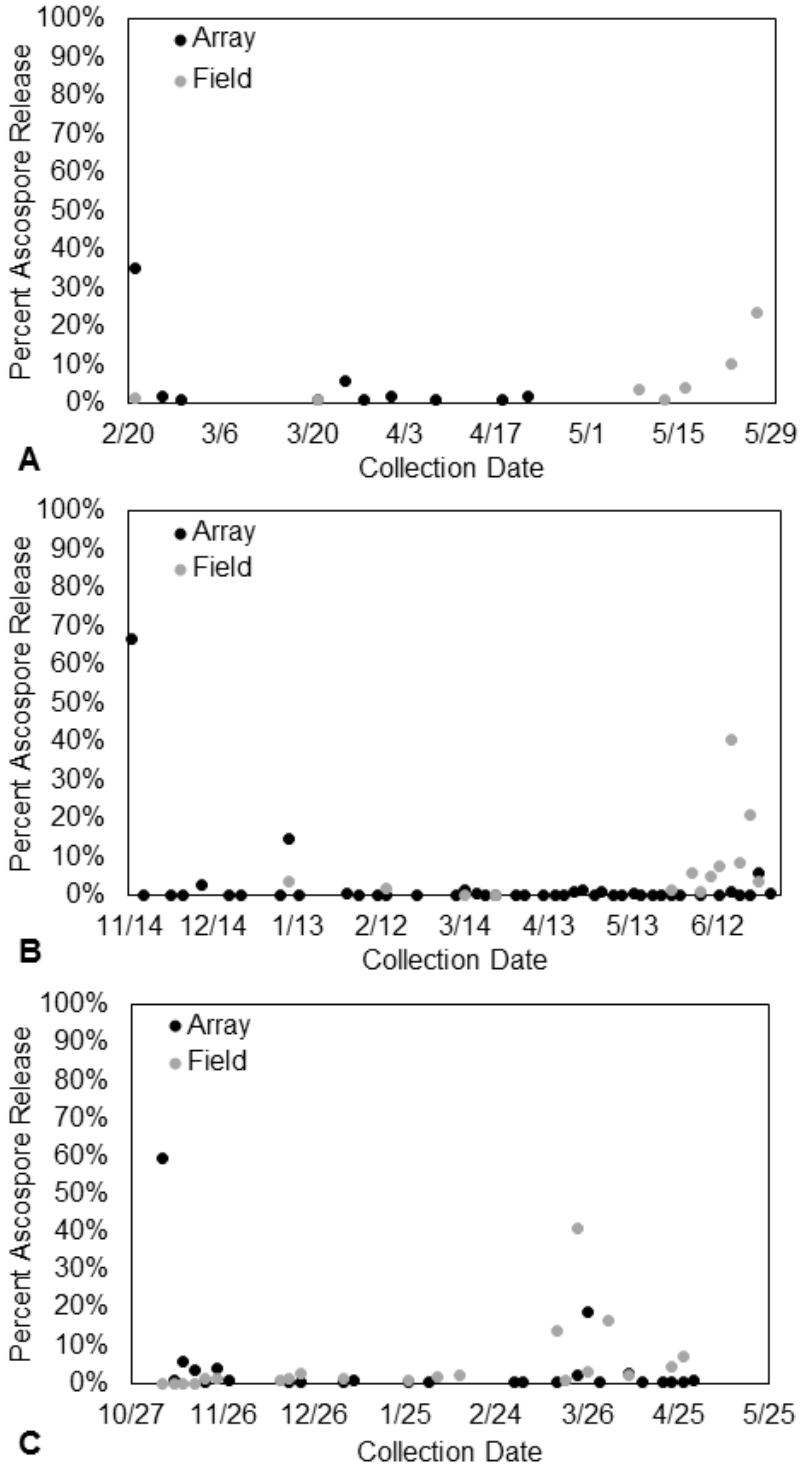
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percent Ascospore Release
Array
Field
2/20
3/6
3/20
4/3
4/17
5/1
5/15
5/29
Collection Date
A
11/14
12/14
1/13
2/12
3/14
4/13
5/13
6/12
Collection Date
B
10/27
11/26
12/26
1/25
2/24
3/26
4/25
5/25
Collection Date
C

Figure 2.2.

Figure 2.3. Observed ascospore release magnitude (Y-axis) compared to predicted ascospore release magnitude (X-axis). Predicted ascospore release magnitude was generated using environmental data collected from 2012-2103 (left column), 2013-2014 (middle column), and 2014-2015 (right column) grapevine dormancy seasons using the Moyer ascospore release model predicting percent ascospore release (top row), Caffi mechanistic model predicting ascospore quantity (second row), Carisse degree day model predicting percent ascospore release (third row), and the multiple linear regression model predicting percent ascospore release (bottom row) as described in text. In comparing observed versus predicted ascospore release, the Moyer model (A) showed no correlation ($R^2 = 0$) in each sampling period. The Caffi model (B) showed no correlation in the 2012-2013 sampling season ($R^2= 0$), 2013-2014 sampling season ($R^2 = 0$), and in the 2014-2015 sampling season ($R^2 = 0$). The Carisse model (C) showed low correlation in the 2012-2013 sampling season ($R^2 = 0.05$), 2013-2014 sampling season ($R^2 = 0.01$), and 2014-2015 sampling season ($R^2 = 0.05$). The multiple linear regression model developed from this study (D) showed low correlation to field collected ascospore release in the 2012-2013 sampling season ($R^2 = 0.03$), 2013-2014 sampling season ($R^2 = 0.09$), and 2014-2015 sampling season ($R^2 = 0.05$).

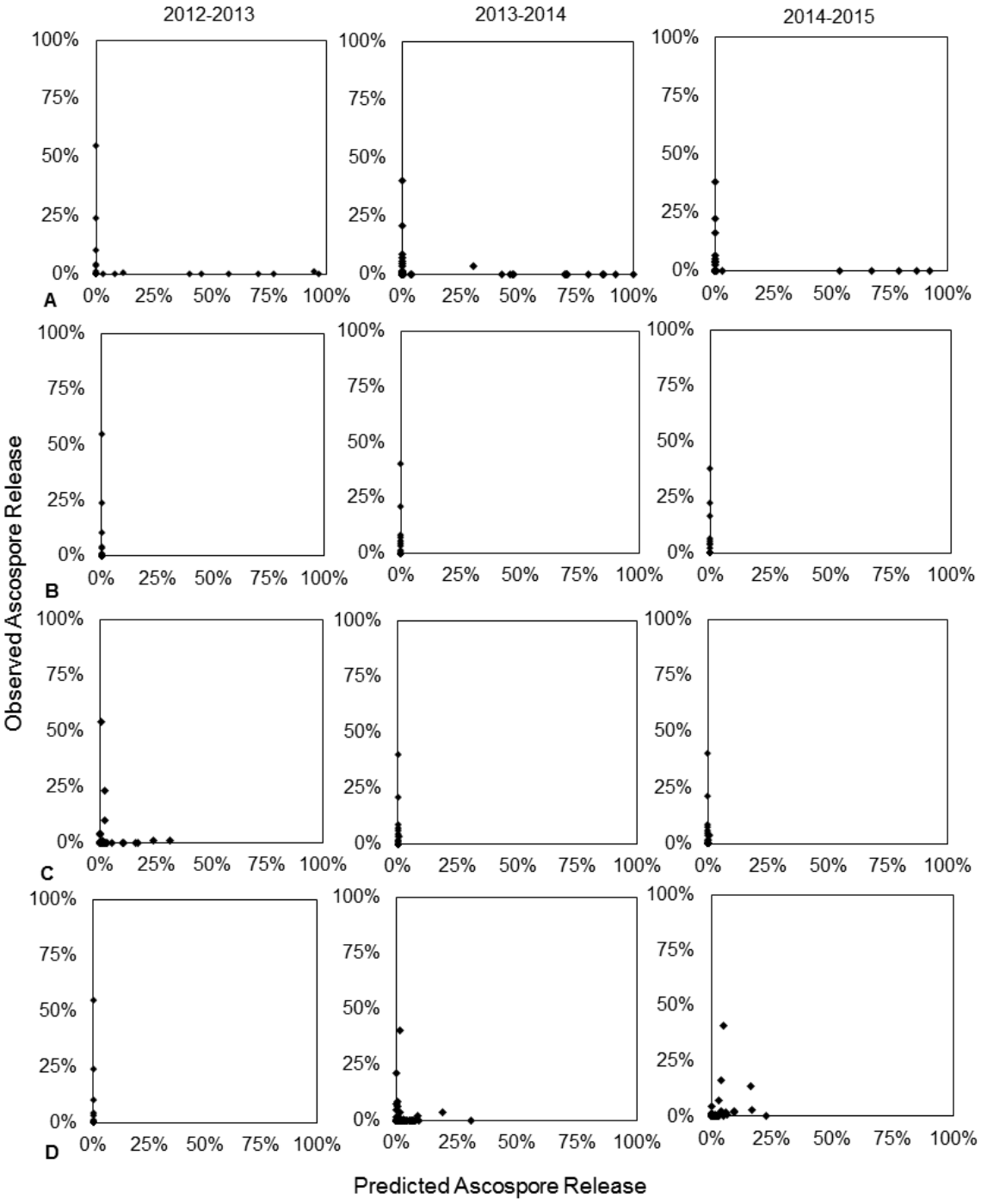
2012-2013
2013-2014
2014-2015
A
B
C
D
Observed Ascospore Release
Predicted Ascospore Release
0%
25%
50%
75%
100%

Figure 2.3.

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

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

3.1 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 (59). Both the foliage and fruit are affected, and as little as 3% incidence of fruit infection has been shown to cause off flavors in wine (109, 126). 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 (59, 112). 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* L., and pathogen do not have a long history of co-evolution (15, 145). Since the origin of *E. necator* is in the eastern United States (15) and the origin of *Vitis vinifera* is in southern Europe and the Mediterranean basin (145), each organism has evolved under very different climates, which may require different environmental conditions to break winter dormancy. Pscheidt et al. (117) and Hall (66) 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 the overwintering inoculum through the occurrence of ascospore release prior to bud break [BBCH stages 00 to 07] (20, 120), delayed pathogen development

compared to that of the host (66), or environmental stresses that impact pathogen development and rate of disease development (104).

Spore trapping and microscopy have been utilized in numerous pathosystems for the management of disease, including apple scab, downy mildew of hops, and sclerotinia stem rot of oilseed rape crops (4, 80, 128). For example, the use of visual detection and quantification of the sporangia of *Pseudoperonospora humuli* on hops in conjunction with weather monitoring (80, 122) is still used to guide fungicide programs 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 (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 (22, 28, 63, 88, 118). Falacy et al. (44) demonstrated that inoculum of *E. necator* can be monitored using molecular tools, specifically polymerase chain reaction (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 program to manage grape powdery mildew in the Yakima Valley of Washington State, USA (44). Similarly in Canada, it was shown that modeling could be improved, and action thresholds could be developed based on PCR detection of airborne *E. necator* (28). 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 (63, 139). Unfortunately, these assays must be performed in well-equipped laboratories with skilled staff (107, 139) 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 (136).

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 (10, 82, 107, 108, 128, 134). Because thermal cycling is not necessary, LAMP assays may be conducted using relatively inexpensive heat sources, such as a water bath or block heater (107). 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 (101). 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 (131). LAMP has also been shown to be less sensitive to PCR inhibitors, thereby requiring less DNA purification for high sensitivity (116, 131). 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: 1) 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*; 2) determine the sensitivity and specificity of the LAMP assay for detection of airborne *E. necator* inoculum in vineyards; and 3) test the implementation of a LAMP assay conducted by vineyard managers in commercial vineyards for the initiation of fungicide programs without compromising the level of disease control.

3.2 Materials and Methods

3.2.1 Sampling Rod Preparation

Stainless steel sampling rods 1.1 mm in diameter were cut to 36 mm lengths from 308LSI welding rods (Weldcote Metals, Kings Mountain, NC). Rods were first soaked in hexane for 24 hours, and then rinsed with dishwashing detergent and water. Next, the rods were shaken in 10% Chlorox™ bleach solution (0.83% NaClO) for 15 min, and then rinsed with deionized water in 3 to 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 bio-containment hood and coated with a very thin layer of silicon vacuum grease (Dow Corning, Midland, MI) 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 Incorporated, Tewksbury, MA).

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 *Vitis vinifera* cv. 'Chardonnay' vines in 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) in nuclease-free water then pipetted onto sterile prepped sampling rods. The concentration of the conidia suspension was estimated using a hemocytometer, and the suspension was pipetted onto pairs of coated stainless steel rods such that approximately 100, 500, 1000, or 10,000 conidia were present on the rods (depending on experiment). One and ten conidia concentrations were created by using an eyelash brush to hand 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.

3.2.2 Quantitative PCR Assay

DNA was extracted from rod pairs using the PowerSoil® DNA extraction kit (Mo Bio Laboratories, Inc. Carlsbad, CA) following the manufacturer protocol. A set of silicon vacuum grease-coated stainless steel rods containing approximately 500 *E. necator* conidia was included in each set of DNA extractions as a positive control for extraction efficiency. DNA samples were analyzed using qPCR the same day as processing, and then stored at -20 °C for subsequent analyses.

Species specific primers from Falacy et al. (44), which produce a 368bp PCR product, were paired with a TaqMan® probe with minor groove binder (Table 4.1). qPCR reactions were performed on an ABI StepOne Plus qPCR machine (Applied

Biosystems, Foster City, CA). Each 15 µl qPCR reaction included 7.5 µl Path-ID® qPCR Master Mix (Invitrogen, Life Technologies Corp., Carlsbad, CA), 400 nM final concentration of each *E. necator* forward and reverse primer and probe, and 1.5 µl extracted DNA. Two-step PCR conditions were 95 °C for 10 min, followed by 55 cycles of 95 °C for 15 sec and 65 °C for 40 sec.

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 (Ct) 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. 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 Ct 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* Ct 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 Ct values to conidia concentration.

Quantification of experimental samples was determined by comparing the Ct value of each unknown field sample to a standard curve. An *E. necator* standard curve was prepared by placing a ten-fold conidial dilution series on the stainless steel sampling rods 1 to 1×10^5 conidia 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 analyzed using qPCR as described. The standard curve was then generated by averaging the Ct values for each conidia quantity from the five independent DNA extractions.

3.2.3 LAMP Primer Development

A consensus sequence of the internal transcribed spacer regions 1 and 2 (ITS1and ITS2 respectively) and 5S ribosomal encoding region of the *E. necator* rDNA was derived from 25 *E. necator* isolates from Oregon, Washington, California, New York (16), and Europe (15). Sequences were used for primer design in the PrimerExplorer Software Version 3.0 (Eiken Chemical Co., Ltd). 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 4.1.

3.2.4 LAMP Assay

Pairs of prepared stainless steel sampling rods (described above) were collected from spore traps (described below) bi-weekly and transported in 14 ml Falcon™ tubes. For DNA extraction, rods were aseptically transferred to sterile 2 ml screw-cap tubes containing 200 µl of Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7.5) for DNA extraction. Extraction tubes were thoroughly mixed using a vortex at max speed for 5 seconds, centrifuged at 16,000 × g for 1 minute, boiled at 100 °C 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. (107) with some modification through the addition of BSA (Bovine Serum Albumin) 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 to 14 mM) and betaine (0.6 to 1.2 M). The final reagent mix contained ThermoPol buffer (1×; New England BioLabs, Beverly, MA), dNTP mix (1.4 mM), betaine (0.6 M), BSA (0.6 mg/ml), $MgSO_4$ (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 (1µM), *Bst* DNA polymerase (0.32 unit/µl; New England BioLabs, Ipswich, MA), 5 µL of extracted DNA, and Nuclease-free-DEPC-treated water (Growcells, Inc, Irvine, CA) for a final volume of 50 µl per reaction. Reactions were incubated in a 65 ºC 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 was caused by the precipitation of magnesium pyrophosphate from a positive LAMP reaction. If turbidity was observed, then the reaction was 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 were confirmed using restriction digest with the NruI enzyme (New England Bio Labs, Ipswich, MA) 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, pipettor, 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 1,000 spores periodically throughout the monitoring period, with each grower conducting at least 3 independent extractions of each concentration. Sample rods were prepared as described above and placed at -20 °C until used by growers.

3.2.5 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 4.2). Conidia from various Erysiphales species were manually collected from plant leaves using vacuum grease coated, stainless steel rods for specificity testing. *E. 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 (131) were used to amplify the ITS

region from these samples, and the products were then cloned into the TOPO 2.1 vector (Invitrogen, Grand Island, NY) according to the manufacturer protocols. Plasmid DNA was purified using the Wizard® Plus Minipreps DNA Purification System (Promega, Madison, WI). 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 were 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 biweekly through a growing season 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.

3.2.6 Field Inoculum Detection

Custom impaction spore traps (Fig. 3.1), similar to Rotorods (Sampling Technologies Inc., Minnetonka, MN), 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 fall. 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 PVC pipe extensions. Each trap was capable of sampling 48.3±1.2 L air/min by spinning two stainless steel rods (1.1×36 mm effective surface area) coated with vacuum grease at 1.05±0.03 m/s. Sampling rods were placed in traps every three to four days (bi-weekly), and traps were run continuously from April 26th to August 26th of 2010 and from April 21st to August 22nd of 2011.

Three traps were placed adjacent to one another at each location: one for G-LAMP, one for the 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 completely maintained and processed by lab 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, two traps were utilized; 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 amplicon from all initial positives for the L-LAMP and G-LAMP were confirmed using gel electrophoresis, as described above.

3.2.6.1 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 (45). The true positive proportion was calculated as 1 - (False Positive / Total Negative), and the true negative proportion 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.

3.2.6.2 Analysis of LAMP Performance Assuming No "Gold Standard"

The assumption that the qPCR results always correctly indicated *E. necator* presence is likely 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 procedure LCA (84,

85). PROC LCA is an add-on procedure available through the Pennsylvania State University Methodology Center for SAS version 9.3 (SAS Institute, Cary, NC). PROC LCA fits latent class models by treating the presence of spores as a two-class latent variable (135). 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 not) 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. (135). 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 8 possible comparison combinations as shown in Table 4). 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 (72), 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 the SAS LCA procedure, 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.

3.2.7 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 program (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 program was initiated, additional applications in both the control and detection plots were made using the grower's standard fungicide program. Detection plots were strategically placed in powdery mildew "hot spots" since 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) and the potential for inter-plot interference (26), 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 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 minute intervals at all field locations to assess the suitability of environmental conditions for disease development using the Gubler/Thomas index (64).

3.2.8 Vineyard Disease Monitoring

To monitor disease progress, each plot was visually scouted weekly for powdery mildew incidence starting June 16, 2010 and June 24, 2011 (BBCH 15-19, when leaf pubescence has decreased), for the respective years, until véraison by inspecting with a hand lens 10 arbitrarily selected leaves from each of 50 vines in each plot. 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 (109, 126), 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 (48). 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.

3.3 Results

3.3.1 Primer Specificity and Sensitivity

The LAMP primers did not cross-react with any of the Erysiphales species tested (Table 3.2), 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, 1×10^2, 1×10^3, 1×10^4, and 1×10^5 conidial quantities showing high primer sensitivity to low spore quantities (1-10 spores) (Fig. 3.2A and B). G-LAMP showed less sensitivity than L-LAMP in both years (Fig. 3.2A and B).

3.3.2 Field Inoculum Detection

3.3.2.1 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 ($P < 0.0001$) (Table 3). The L-LAMP had true positive 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 both 2010 and 2011.

3.3.2.2 Analysis LAMP Performance Assuming No "Gold Standard."

Considering only the laboratory results in Table 3.4 (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 ~63% and 62% of the time and with L-LAMP results ~60% and 87% of the time for 2010 and 2011, respectively (Table 3.4). Complete agreement among all three test results – either all positive or all negative – was found in approximately 56% and 59% of the cases in 2010 and 2011, respectively (Table 3.4). Both the 2-test and 3-test LCA (Table 3.5) 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 3.5). A positive result 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. 3.3).

3.3.3 Commercial Vineyard Test Sites

Of the 10 and 8 commercial test locations in 2010 and 2011, respectively, only 5 vineyard managers in each year kept the inoculation and control plots under independent management. Across both testing seasons, fungicide application savings were variable, approximately 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 4 fewer organic fungicide applications were utilized 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 5 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. 3.4). Nearby abandoned vineyards had 100% disease incidence by July 15 in both years (data not shown) and the Gubler/Thomas index (Gubler et al. 1999) remained mostly above 80 from June 15 to September 1 (data not shown). In addition, all vineyards had >50% leaf incidence on young leaves at harvest (30 to 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.

3.3.4 Vineyard Disease Monitoring

The leaf incidence AUDPC for 2010 (5 sites) detection treatment and commercial standard control were extremely low (9.3 ± 11.4 and 5.6 ± 5.8 respectively), and the control plots were not significantly different from the treatment plots (P = 0.30). The 2011 AUDPC values (5 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 were not significantly different from the treatment plots (P = 0.16). There were <0.1% visual symptoms of fruit infection by *E. necator* at all locations and treatments in both years, thus percent berry disease incidence was determined microscopically (described above). Berry disease incidence in 2010 (5 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 = 0.21). Berry disease incidence in 2011 (5 sites) detection treatment and commercial standard control were 30.8% ± 28.1 and 5.1% ± 4.5 respectively, and were not significantly different (P = 0.06). 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.

3.4 Discussion

Growers were able to effectively manage grape powdery mildew using inoculum detection (determined by performing the LAMP assay) to initiate fungicide applications, despite the less than optimal performance of the LAMP 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 disease control that is expected by the industry. Many growers in this region have since altered their standard fungicide program 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. 3.4) during our assessment period, even though nearby abandoned vineyards reached 100% leaf incidence by July 15 (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 since 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 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 (112). 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 (6, 8). 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.

Since 3% disease incidence (visually assessed) on berries affects wine quality (109, 126), 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 likely resulted in an inflated disease incidence compared to studies where only visual ratings were done (109, 126).

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. 3.2). 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. The 3-way LCA, however, 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 three-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 utilizing this assay, it is likely 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. 3.3), 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 (83). Growers sometimes also envisioned turbidity when no *E. necator* DNA was present. Upon subsequent examination of these samples in the lab using gel electrophoresis, the banding pattern of non-specific 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 (42, 100, 108, 134, 138) have been made available after 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 amplicon that have a complex tertiary structure that is highly stable and capable of self-replication (83), and is very difficult to clean up if spilled or aerosolized (Thiessen, personal observation). These dyes, while they may improve accuracy in determining DNA amplification, also present difficulty in discerning differences between 0 spore and low spore quantities without the aid of a spectrophotometer (102, 131). To reduce subjectivity of visual turbidity or dyed products inspections and allow for quantification of LAMP products, the use of a FRET-based probe (83) have been developed.

Since 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 likely 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. Daily spore samples were also collected at the research vineyard and compared side by side with the biweekly samples 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 bi-weekly sampling regime to guide management decisions. Under optimal conditions *E. necator* has a generation time of 5 days (40), 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 shown to be an effective management tool in several pathosystems (22, 30, 44, 88, 120), but relies on an inhibitor-free DNA source. The LAMP assay, however, does not require expensive technology or formal training for DNA extraction and the detection of inoculum (107), making it suitable for commercial use with grapevine growers conducting the detection analyses. This study, in conjunction with other studies (28, 44, 69), also demonstrates that there may be benefit to managing polycyclic diseases, at least those caused by other Erysphales, 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 3.3). 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 utilize a FRET-based assimilating probe (83) for quantitative LAMP is being conducted to adjust fungicide application intervals utilizing 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 (91).

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Table 3.1. Primers and probes used for detecting for the detection of *Erysiphe necator* ITS region.

	Primer/probe	Nucleotide Sequence (5' → 3')
LAMP[a]		
	FIP EN	ACCGCCACTGTCTTTAAGGGCCTTGTGGTGGCTTCGGTG
	BIP EN	GCGTGGGCTCTACGCGTAGTAGGTTCTGGCTGATCACGAG
	F3 EN	TCATAACACCCCCCTCAAGCTGCC
	B3 EN	AACCTGTCAATCCGGATGAC
	Forward Loop	
	EN	AAACTGCGACGAGCCCC
	Reverse Loop	
	EN	ACTTGTTCCTCGCGACAGAG
qPCR[b]		
	Unc144	
	Forward	CCGCCAGAGACCTCATCCAA
	Unc511	
	Reverse	TGGCTGATCACGAGCGTCAC
	Unc TM Probe[c]	6FAM-ACGTTGTCATGTAGTCTAA-MGBNFQ

[a] Primer concentrations in the reaction mix were 2.4 µM for FIP and BIP, 0.24 µM for F3 and B3, and 2.4 µM for Forward and Reverse Loop. Melting temperatures for the primers were between 64 and 99°C.

[b] Primer concentrations in the reaction mix were 400 nM for Unc144 Forward, Unc511 Reverse, and the TaqMan® Probe. Melting temperatures for the primers were 59.2 and 59.9°C, respectively.

[c] TaqMan® Probe contains 6-carboxyfluorescein (6FAM), Minor Groove Binder (MGB), and Non-fluorescing Quencher (NFQ).

Table 3.2. 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 found in the Pacific Northwest. All isolates were not detected using the LAMP primers specific to the ITS2 region of *E. necator*.

Pathogen Species	Host
Blumeria graminis (DC.) Speer	*Poa* sp.
Erysiphe aquilegiae var. *ranunculi* (Grev.) R. Y. Zheng & G. Q. Chen	*Aquilegia canadensis*
E. convolvuli DC	*Convolvulus arvensis*
E. chicoracearum DC var. *chicoracearum*	*Callistephus*
	Cirsium arvense
	Coreopsis sp.
	Lactuca serriola L.
	Mentha arvensis
	Rudbeckia laciniata L.
	Taraxacum officinale
E. magnicellulata var. *magnicellulata* U. Braun	*Phlox* sp.
E. pisi DC.	*Medicago sativa*
E. rhododendri Kapoor	*Rhododendron*
E. trifolii Grev.	*Trifolium pratense*
E. polygoni DC	*Beta vulgaris* L.
E. cruciferarum Opiz ex Junell	*Brassica rapa* L.
Leveillula taurica (Lev.) G. Arnaud	*Allium cepa* L.

Table 32 (Continued)

E. syringae Schewin. syn *Microsphaera syringae* (Schewin) H. Magn	*Caragana arborescens* Lam.
Microsphaera nemopanthis Peck	*Ilex verticillata* (L.) A. Gray
Sawadea sp.	*Acer* sp.
Podosphaera aphanis (Wallr.) U. Braun & S. Takamatsu formerly *Sphaerotheca macularis* (Wallr.) U. Braun	*Rubus ursinus*
	Rubus idaeus
Podosphaera aphanis (Wallr.) U. Braun & S. Takamatsu formerly *Sphaerotheca macularis* f. sp. *Fragariae* (Wallr.) U. Braun	*Fragaria* sp.
Podosphaera clandestina (Wallr.:Fr.) Lev.	*Prunus* sp.
P. delphinii (P. Karst U. Braun & S. Takamatsu formerly *S. delphinii* (P. Karst) S. Blumer	*Ranunculus abortivus* L.
P. fusca (Fr.) U. Braun & N. Shishkoff formerly *Sphaerotheca fusca* (Fr.) S. Bluer	*Cucurbita pepo* L.
P. macularis (Wallr.:Fr.) U. Braun & S. Takamatsu formerly *Sphaerotheca macularis* (Wallr.:Fr.) Lind	*Humulus lupulus* L.
P. leucotrica (Ell. & Ev.) Salmon	*Malus domestica* Borkh.
P.pannosa (Wallr.:Fr.) de Bary formerly *Sphaerotheca pannosa* (Wallr.:Fr.) Lev	*Prunus persica*
Uncinuliella flexuosa Peck	*Aesculus* sp.

Table 3.3. Contingency table representing grower-conducted LAMP assay and laboratory-conducted LAMP assay to quantitative PCR (qPCR) results for the presence of *Erysiphe necator* sampled from custom made impaction spore traps from both commercial vineyards and research plots at the Oregon State University Botany and Plant Pathology Research Vineyard.

			qPCR[b]		
			Positive	Negative	Fisher's Exact Test (Probability)[c]
2010	G-LAMP[a]	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 (31%)	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 and *n*=39 in 2011) and L-LAMP (*n*=266 in 2010 and *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 $P < 0.05$.

Table 3.4. Test response pattern used in the 3-test latent class analysis (LCA) where the number of samples testing positive (+) or negative (−) for the observed test response patterns possible of the quantitative PCR (qPCR), laboratory-conducted LAMP (L-LAMP), and grower-conducted LAMP (G-LAMP) resulting in 8 possible response patterns.

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 3.5. 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[a].

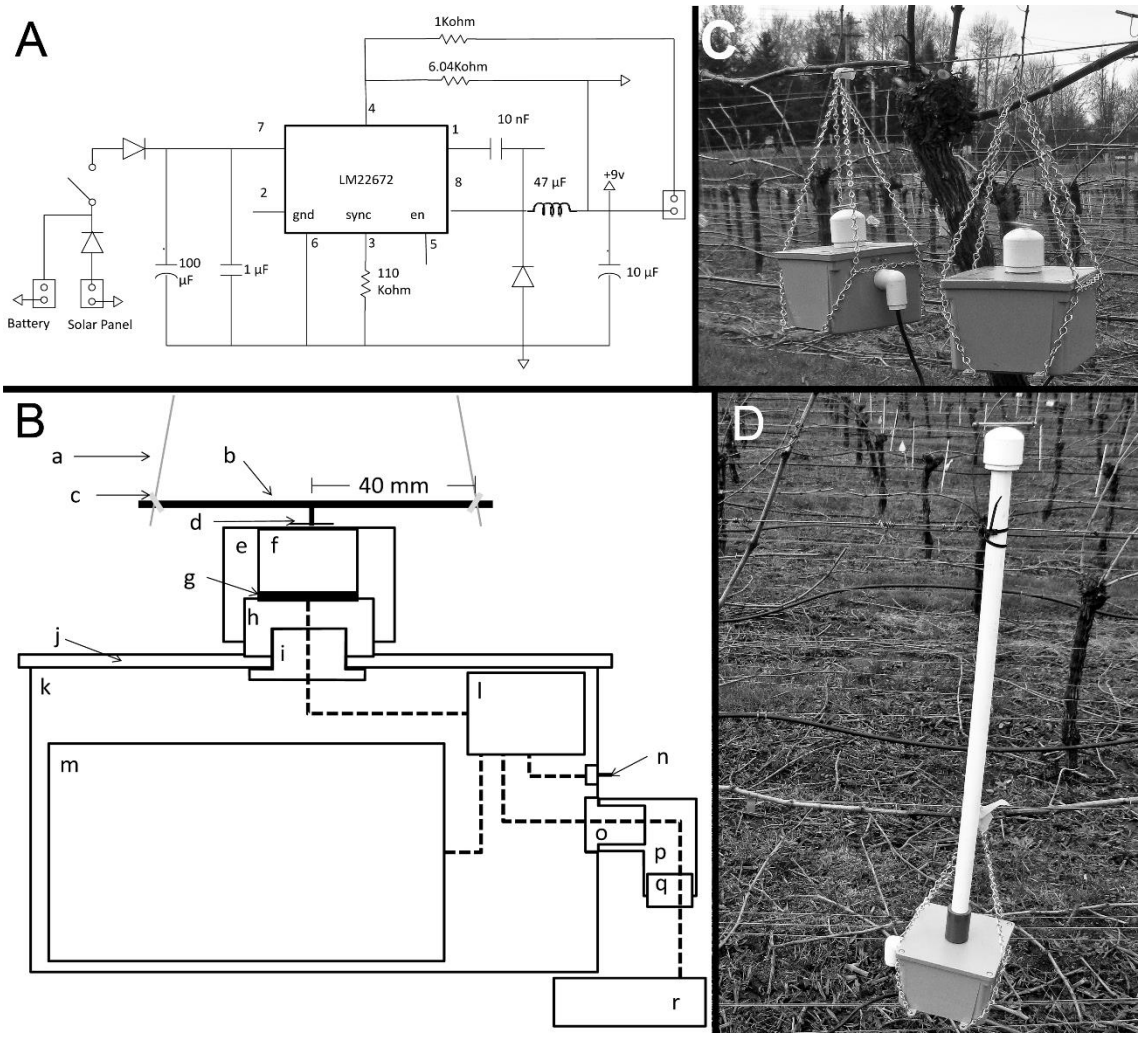
Test	2-test LCA		3-test LCA			
	2010/11		2010		2011	
	True Positive Proportion[b]	True Negative Proportion[c]	True Positive Proportion	True Negative Proportion	True Positive Proportion	True Negative Proportion
qPCR	0.9101	0.9957	0.9874	0.9573	0.8993	0.6372
L-LAMP	0.7551	0.9780	0.8074	0.9929	0.8474	0.9896
G-LAMP	—	—	0.4576	0.7857	0.6183	0.9924

[a] Latent class analysis (LCA) was conducted using PROC LCA in SAS version 9.3 (SAS Institute, Cary, NC)

[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.

Figure 3.1. 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 40mm sampling radius; (c) silicon o-ring [4.5mm ID]; (d) Teflon disk (6.5mm) with a 1.5mm hole in the center and vacuum grease placed underneath; (e) 32 mm [1.25 in] PVC endcap; (f) Mabuchi RF-500T-10750 DC motor; (g) silicon O-ring [24mm ID]; (h) 32 to 19 mm [1.25 to 0.75 in] PVC reducer; (i) 19 mm [0.75 in] PVC bushing; (j,k) 153 × 153 × 102 mm [6 × 6 × 4 in] PVC junction box with gasketed lid; (l) circuit board described in (Fig. 3.1 A); (m) 5 amph sealed lead acid battery; (n) toggle switch; (o) 13 mm [0.5 in] PVC bushing; (p) 13 mm [0.5 in] PVC elbow; (q) 13 mm [0.5 in] PVC plug; (r)18 watt 12volt solar panel [SunWise, Inc. Kingston, NY]. 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 [0.75 in] PVC pipe extension used to raise the sampling arm of the impaction spore trap above the grapevine canopy.


A
1Kohm
6.04Kohm
4
7
1
10 nF
LM22672
2
8
47 µF
+9v
gnd
sync
en
6
3
5
100 µF
1 µF
110 Kohm
10 µF
Battery
Solar Panel
C
B
a
b
40 mm
c
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j
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D

Figure 3.1.

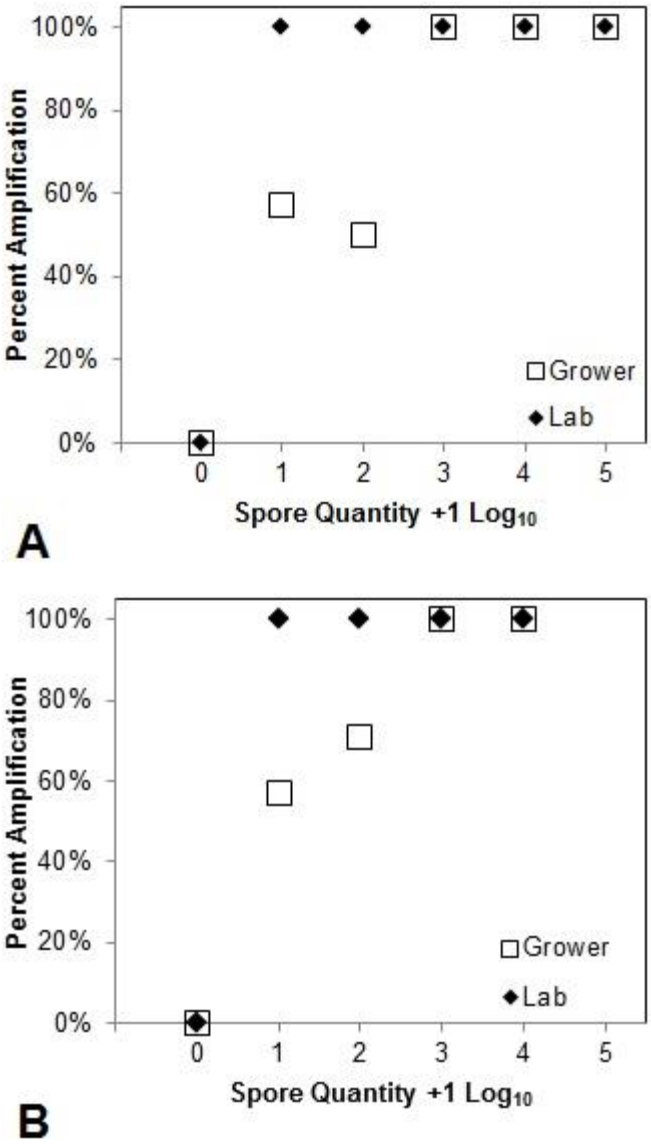

Figure 3.2. LAMP primer sensitivity to $\log_{10}$ conidial quantities +1 as tested by the laboratory (solid diamond) (n=53) and growers (square) (n = 42) in both (A) 2010 and (B) 2011 blind samples.

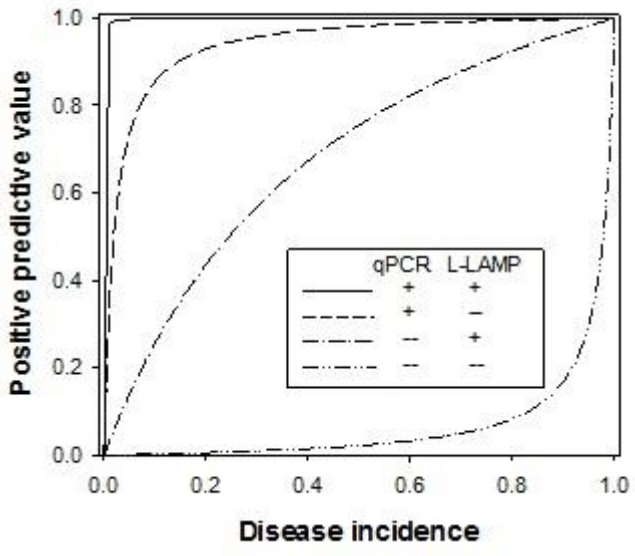

Figure 3.3. The posterior probability, or the positive predictive value (the probability of being truly positive given a specific set of test results) for the quantitative PCR (qPCR) and laboratory-conducted LAMP (L-LAMP) as determined by the 2-test latent class analysis using PROC LCA in SAS version 9.3 (SAS Institute, Cary, NC). 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 3.4. Disease progress curves for field disease incidence determined by field scouting in 2010 (A) for 5 commercial vineyards and in 2011 (B) for 5 commercial vineyards. A sample of 500 leaves were assessed from a detection plot (fungicide program 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 ($P = 0.30$) or in 2011 ($P = 0.16$).

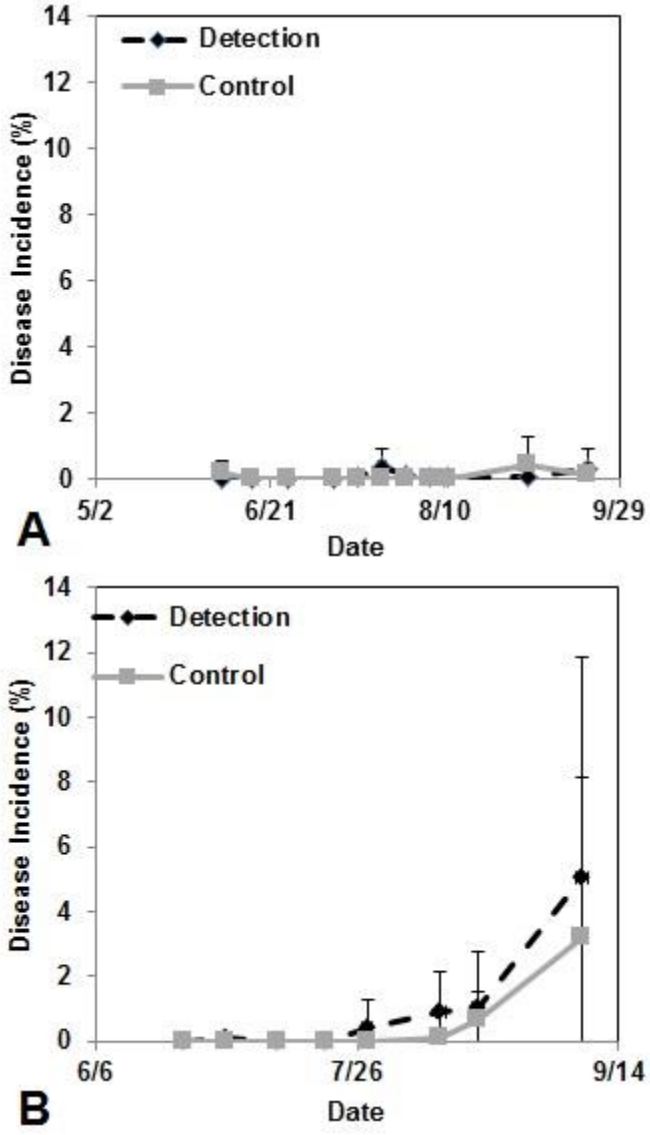
Detection
Control
Disease Incidence (%)
14
12
10
8
6
4
2
0
5/2
6/21
8/10
9/29
Date
A
Detection
Control
Disease Incidence (%)
14
12
10
8
6
4
2
0
6/6
7/26
9/14
Date
B

Figure 3.4.

4. Development of a Quantitative Loop-Mediated Isothermal Amplification Assay for the Field Detection of *Erysiphe necator*

4.1 Introduction

Molecular techniques, such as PCR or loop-mediated isothermal amplification (LAMP), are capable of being used to identify target organisms with high sensitivity and specificity (28, 30, 44, 129). The detection of pathogens have been improved through the development of quantitative PCR (qPCR) assays that allow for near real-time monitoring of pathogens through regular inoculum collections (30, 118, 128, 129). Despite the utility of qPCR to monitor pathogens, qPCR requires experienced laboratory staff and expensive equipment to accurately assess pathogen concentration (107, 139). LAMP assays, however, do not require thermal cycling, which allows for inexpensive, mobile equipment to be used for amplification in the field or remote facilities. The *Bst* polymerase used in LAMP reactions also tolerates reaction inhibitors better than PCR (83), which allows for quick, minimal DNA extraction protocols. These traits make LAMP an ideal assay for use in field detection assays (67, 82, 128, 131, 133).

LAMP has been developed for monitoring inoculum numerous plant pathosystems, including grape powdery mildew, fire blight of pear, and gray mold, but these methods do not allow for mobile quantification of samples (1, 67, 128, 129, 132, 133). Development of a quantitative LAMP assay may be utilized to improve field detection and real-time monitoring of plant disease inoculum to further optimize disease control methods. LAMP assays produce a magnesium pyrophosphate precipitate when DNA is amplified that can be detected with the human eye;

however, in low concentrations of target DNA, precipitate may be difficult to observe (78, 83, 129). Several dyes have been explored to improve detection including SYBR green (107), hydroxynaphthol blue (27), and other synthetic dyes (49), but the dyes have the potential to inhibit LAMP reactions or require the use of spectrophotometers, which increase labor and equipment costs. The use of a fluorescence resonance energy transfer (FRET) based probe, however, allows for specific detection of LAMP products and quantification of field samples without inhibiting amplification (83), and several portable fluorescence-reading LAMP devices have been made commercially available, such as the Genie (Optigene Ltd., West Sussex, UK) and Bioranger (Diagenetix Inc., Hawaii). Using a fluorescent probe also removes error from visual detection of LAMP products, which may improve the accuracy of pathogen detection.

Although a LAMP assay was successfully designed for field use in the grape powdery mildew pathosystem, false negatives or false positives caused by difficulty in perceiving the magnesium pyrophosphate precipitate reduced the predictive values of the LAMP assay (129). Kubota et al. (83) developed an assimilating probe, a fluorescent-labeled loop primer annealed to a quencher strand, that fluoresces when the quencher strand is displaced during DNA amplification. Incorporating a FRET-based assimilating probe and developing a qLAMP assay to quantify airborne spore samples in a field environment may improve on-site inoculum detection and management decisions based on detection.

The purpose of this research was to develop a quantitative molecular assay for detection of airborne *E. necator* inoculum for commercial implementation that can be

used by growers or vineyard consultants for the detection and quantification of airborne *E. necator* inoculum. The specific objectives of this project was to 1) develop a real-time, quantitative LAMP assay that was sensitive and specific to *E. necator,* and 2) test field use of a mobile, quantitative LAMP device by growers.

4.2 Materials and Methods

4.2.1 Sample Rod Preparation

Sample rods were prepared and sterilized according to Thiessen et al. (129). Standard curve and positive control spore rods were created by directly placing 1 or 10 individual *E. necator* conidia or pipetting a conidia suspension (0.05% Tween 20 (Sigma Aldrich, St. Louis, MO, USA) in sterile, deionized water solution resulting in rods with100, 500, 1000, or 10,000 spores. The rods were allowed to air dry prior to processing or storage at -20 °C.

4.2.2 Quantitative LAMP Assay

DNA for qLAMP analysis was extracted using a quick extraction method modified from Thiessen et al. (129). Spore rods were transferred to 2 ml screw-cap tubes containing 200 µl of 5% Chelex 100 (Sigma Aldrich) in molecular grade, DEPC-treated water. Tubes containing rods were vortexed for 5 seconds then placed in boiling water for 5 minutes. Tubes were removed from boiling water and vortexed another 5 seconds. The tubes were boiled for another 5 minutes, and then removed and allowed to cool at room temperature for 2 minutes. Samples were centrifuged at

16,000 × g for 2 minutes to collect the contents in the tube. Rods were aseptically removed prior to the sample being processed. After processing, samples were stored at -20 °C for further analyses.

The qLAMP reaction is a modified assay from Thiessen et al. (129) and Kubota et al. (83). A FRET-based probe was designed using the forward loop primer region with a FAM reporter (6-carboxyfluorescein) and a quencher strand (83). Each reaction was 25 µl and contained 14.8 µl of Isothermal Master Mix with no dye (OptiGene Ltd, West Sussex, UK), internal primers FIP EN and BIP EN (2.4 µM), external primers F3 EN and B3 EN (0.24 µM), forward loop primer FAM strand (FL-F, 0.08 µM, FAM–ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAAAACTGCGACGAGCCCC), and Quencher strand (Q-strand, 0.08 µM, TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT–BHQ). Lab-conducted qLAMP (L-qLAMP) reactions were carried on an ABI StepOne Plus qPCR machine (Applied Biosystems, Grand Island, NY, USA). Reaction conditions were 65°C for 45 minutes followed by 80°C.

Spore concentration standards of 1, 10, 100, 1,000, and 10,000 spores were created, as above, for the generation of the qLAMP standard curve. DNA was extracted using the Chelex extraction process described above, and qLAMP reactions were run in triplicate. The cross-threshold (C_T) values, measured in minutes, of the spore standards were averaged and used to create a log-linear standard curve to compare unknown samples to (Fig. 4.1). A log linear curve is required because LAMP amplification rate is faster than exponential due to concatenation of amplicon

(101). A 500 spore extraction control, 100 and 1,000 spore positive controls, as well as non-template controls were included in all reaction setups. Unknowns were compared to the standard curve to determine relative spore quantity. Positive control samples were also compared to the standard curve to determine extraction efficiency and amplification efficiency. Unknown sample C_T values were adjusted based on positive control C_T values if the positive controls showed poor alignment to the standard curve. To test the L-qLAMP sensitivity to target DNA, 10 separate spore concentration series were created and tested for positive amplification.

4.2.3 Grower Quantitative LAMP Assay

Growers were provided with all materials to conduct the DNA extraction and the qLAMP reaction described above. For the grower-conducted qLAMP assay (G-qLAMP), frozen aliquots of qLAMP master mix was stored in insulated cryoboxes (VWR North America, Radnor, PA) at -20 ˚C until reactions were conducted. All reactions were conducted in beta-version Smart-DART handheld LAMP reaction devices (most recently marketed as BioGuard, Diagenetix Inc., Honolulu, HI), which connected to Android 4.4 enabled, Bluetooth-capable Nexus 7 tablets for output (Google, Mountain View, CA). All G-qLAMP reactions were conducted in duplicate including 100 conidia positive controls and non-template controls. Reaction conditions followed protocol described above.

Smart-DART LAMP devices provided amplification curves and the C_T values associated with amplification curves. Growers were asked to determine if samples were positive, as indicated by the presence of a sigmoidal amplification curve, or

negative, no amplification observed, based on the output from the handheld LAMP device.

4.2.4 Field Spore Collection

Custom impaction spore samplers as described in chapter 3 (129), were placed at a research vineyard and commercial vineyards location within the Willamette Valley of Oregon. Spore samplers collected 45 L/min and were run continuously, and sample rods were replaced daily or every 3 to 4 days. At the research vineyard, paired spore samplers were collected by laboratory personnel and were assessed using qPCR and qLAMP assays. Three spore samplers were placed at 6 commercial vineyards that were collected by growers bi-weekly. The growers completely maintained one trap and processed all collections rods derived from that trap. Collection rods from the other two traps were transported by the growers to the lab for processing with the L-qLAMP assay. The third grower spore sampler was placed adjacent to the LAMP samplers, and samples were processed for qPCR analysis (Chapter 5).

Spore samplers were placed on April 15, 2013 and April 14, 2014 and were collected from until véraison (BBCH 83) for lab processing (Chapter 5). Spore samplers for grower processing were placed April 14, 2014 and were collected until July 1, 2014. Estimates of airborne inoculum concentration derived using qPCR and qLAMP were compared to assess the accuracy of the qLAMP procedure. The G-qLAMP assay detection results were compared to the L-qLAMP assay and qPCR detection data as described in Chapter 5.

4.2.5 Data Analysis

Data was analyzed using R 3.2.1. Detections from samples collected and quantified with L-qLAMP assay were compared to qPCR assay detections using a Student's T-test. The G-qLAMP detection results were compared to L-qLAMP detection results using a 2 × 2 contingency table whereby the L-qLAMP results were assumed correct. Both the L-qLAMP and G-qLAMP spore detections were evaluated using a 2 × 2 contingency table whereby the qPCR assay results were assumed correct. The G-qLAMP assay detection accuracy, true positive proportion, true negative proportion, and Fisher's Exact test were assessed comparing the G-qLAMP detection results to the L-qLAMP and qPCR detection results. The L-qLAMP assay detection accuracy, true positive proportion, true negative proportion, and Fisher's Exact test were assessed comparing the L-qLAMP detection results to the qPCR detection results.

4.3 Results

4.3.1 qLAMP Assay Sensitivity

The qLAMP assay showed high sensitivity to *E. necator* conidia DNA when 10 separate spore dilution series were tested (Fig. 4.1) with 88% of 1 conidia samples amplifying using the qLAMP assay. All other spore quantities tested showed 100% amplification when tested for sensitivity to the *E. necator* conidia DNA extractions.

4.3.2 qLAMP Quantification

The qLAMP assay standard curve development resulted in a standard curve ($R^2 = 0.99$) when fit with a log linear curve (Fig. 4.2). A log linear curve was fit to the log spore quantity to account for the number of primers used in the assay, and the amplicon produced concatenates resulting in greater than an exponential rate of amplification. This curve was used to quantify the L-qLAMP samples collected from the Botany and Plant Pathology Research Farm vineyard. The L-qLAMP spore quantification was significantly lower than the qPCR quantification when daily samples were collected in 2013 ($P < 0.001$) (Fig. 4.3A), but the biweekly L-qLAMP and qPCR sample quantification was not significantly different in 2013($P = 0.14$) (Fig. 4.3B). The L-qLAMP assay significantly underrepresented spore levels for both the daily collections ($P < 0.001$) (Fig. 4.4A) and the biweekly collections ($P = 0.01$) (Fig. 4.4B) compared to the qPCR assay in 2014.

4.3.3 Lab Conducted qLAMP Detection

Utilizing L-qLAMP for detection of *E. necator* showed similar results to qPCR assay detections in both 2013 and 2014 ($P < 0.001$) (Table 4.1). The L-qLAMP assay detection results were 83% and 70% accurate in 2013 and 2014, respectively compared to the qPCR assay detection results. The L-qLAMP assay detection results showed true positive proportions of 79% and 94% and true negative proportions of 76% and 37% in in 2013 and 2014, respectively.

4.3.4 Grower-Conducted qLAMP Assay

The software provided with the mobile LAMP device used auto-adjusting threshold values to account for noise of fluorescence readings which significantly reduced accurate quantification by growers. The G-qLAMP assay for the detection of *E. necator* was similar to L-qLAMP results ($P < 0.001$) (Table 4.2), but was not correlated to the qPCR detection results ($P = 0.22$) (Table 4.1). The G-qLAMP detection results showed 91% and 69% accuracy compared to the L-qLAMP and the qPCR assay results, respectively. The G-qLAMP detection results show true positive proportions of 93% and 94% for the L-qLAMP and qPCR detection results comparisons, respectively. The G-qLAMP detection results also showed true negative proportions of 50% and 18% compared to the L-qLAMP and qPCR detection results, respectively.

4.3.5 qLAMP Assay Troubleshooting

Due to loss of sensitivity of the qLAMP assay to *E. necator* observed during assay testing in 2014, troubleshooting was carried out during the sampling period to determine the cause of the loss of sensitivity of the qLAMP assay. Primer purification, polymerase used (*Bst* or OptiGene), master mix distributer, assimilating probe removal, primer and assimilating probe manufacturer, inhibitor removal compounds in the master mix, DNA extraction and clean up, adjustment of reaction temperature, and replacement of reagents and primers were all tested. Primer purification was tested prior to the implementation of the experiment, and during the

observed degradation of qLAMP sensitivity with no observable difference between reaction efficiency of HPLC or desalted primers.

Regardless of polymerase used, *Bst* or ISO-001 (Optigene Ltd, West Sussex, UK), reaction efficiency and sensitivity to *E. necator* DNA was reduced compared to assays conducted prior to implementation of field testing. Different distributers of the Optigene Isothermal Mastermix were also tested to determine if the decreased sensitivity was caused by storage or shipping errors; however, there was no difference among master mix vendors. It was not possible to test previous lots of the master mix prior to the observed decrease in sensitivity. The assimilating probe was removed and gel electrophoresis was used to compare with and without probe presence, and no difference was observed in amplification. There was also no difference between different primer and probe manufacturers, which also suggests that the primer and probe sequence was not manufactured incorrectly.

The concentrations of inhibitor removal compounds within the master mix were assessed, including PVP 40, EDTA, and BSA concentrations, to determine if inhibitor presence was causing decreased reaction efficiency, and no differences were observed for inhibitor removal compounds. In addition to testing master mix removal of inhibitors, the DNA extraction was also tested for inhibitor removal. To test this, three DNA extraction methods [extractions with pH 7.5, 10mM Tris-0.1mM EDTA buffer (Affymetrix, Santa Clara, CA, USA), 2% polyvinylpyrrolidone (PVP) 40 (Sigma Aldrich, St. Louis, MO, USA) in DEPC-treated water, and PowerSoil® DNA extraction kit (Mo Bio Laboratories, Inc. Carlsbad, CA)] were assessed with separate

field collected spore samples. No differences were observed in amplification time or efficiency when testing each extraction method.

To test the optimal reaction temperature of the polymerase, temperatures between 60 and 70°C were examined to find the optimal reaction temperature. Lower spore quantities amplified at 62°C. This is likely a result of lower specificity rather than optimal reaction temperature since amplification curves showed less variability at 65°C. A last effort to determine if the effect was due to degradation of reagents of primers during the growing season, all reagents, primers, and probe were replaced; however, the decreased sensitivity to *E. necator* DNA was still observed. Despite targeting various portions of the reaction and extraction, the cause for loss of qLAMP assay sensitivity remains undetermined.

4.4 Discussion

A highly sensitive qLAMP assay was successfully developed using a simple DNA extraction method for use by growers or crop consultants. The qLAMP assay developed for the assay was sensitive to *E. necator* DNA with one spore amplifying 88% (n = 10) using the simplified DNA extraction. This sensitivity indicated that the assay should be suitable to detect inoculum (i.e. ascospores) at low concentrations and be suitable to aid management decisions. However, the qLAMP assay consistently underrepresented spore quantities later in the growing season compared to the qPCR assay, which may be due to the high presence of potential inhibitors (such as pollen, humic acids from soil, spider webs, etc.) found in air samples (143) that may not have been removed by the rapid Chelex DNA extraction. The

PowerSoil® extracted DNA showed more consistent amplification of field samples than the other extraction methods; however, the PowerSoil® DNA extraction kit requires a larger time commitment and several steps that may not be feasible for in-field DNA extractions. The LAMP assay has been widely described to tolerate more inhibitors than qPCR, but it appears that the LAMP assay tolerates different inhibitors than the qPCR assay (105). The inhibition of the qLAMP assay may indicate that the assay shows more utility as a qualitative inoculum detection tool as opposed to quantitative.

The G-qLAMP results were similar to the L-qLAMP assay detection ($P < 0.001$) (Table 4.1), but showed differences to the qPCR detection results ($P = 0.22$) (Table 4.2). This may be due to difficulty in assessing positive detections from the output of the mobile device. The curve smoothing algorithm used by the device application often produced curves that drifted linearly and had C_T values reported from reactions that had no detectable amplification using gel electrophoresis. The insignificant exact test results comparing the results of the G-qLAMP assay and the qPCR assay results is due to the limited number of detection results from the G-qLAMP assay testing. In addition, the disease pressure in 2014 was very limited and not suitable for accurate assessment of the G-qLAMP assay.

The L-qLAMP assay detection results were similar to qPCR assay detection results in both 2013 and 2014 (Table 4.2), but true positive and true negative proportions were variable between years, which may be due to the presence of inhibitors. In 2013, the source of stainless steel rod material was changed from previous testing, and significant inhibition of DNA amplification was observed. After

troubleshooting various rod cleaning processes and DNA extraction techniques, a hexane soak was added to the steel rod cleaning protocol to remove oils prior to sterilization and 5% Chelex 100 was used as the extraction buffer. After the hexane wash step addition, the accuracy of samples was improved to 85%, and the misclassification rate was reduced from 17% to 14%. In addition to inhibitors from the rods, the variability of inhibitors from field collections caused inconsistencies in qLAMP assay detection results compared to the qPCR assay detection results. The results from the qLAMP development showed lower true positive proportions and true negative proportions than that of turbidimetric LAMP previously developed (129). These reductions may be due to other factors besides amplification inhibitors, such as manufacturer differences, degradation of polymerase, or qLAMP reaction buffering (36, 121).

Using qLAMP assay for field detection and quantification of fungal pathogens may not be as feasible as previously thought due to the random loss of assay sensitivity and potential inhibition of polymerase activity by environmental contaminants. Redesigning primers was another potential approach to the loss of sensitivity; however, the primer set used here was the result of two previous redesigns during development and testing. In addition, if periodic primer redesign is required, it indicates that LAMP assays might not be robust enough for commercial implementation and would significantly increase cost due to added requirement for monitoring potential issues and primer redesign. Additionally, the LAMP assay quantification was also affected by numerous inhibitors, such as soil, pollen, or insect debris, found in field collected samples despite being previously described to better

tolerate inhibitors than traditional PCR assays (51). LAMP is capable of tolerating some inhibitors that affect PCR assays; however, to determine the extent that LAMP assays are capable of tolerating inhibitors, each potential inhibitor should be tested (105). Other LAMP assays developed have utilized more complex DNA extractions to reduce the effect of inhibitors on amplification for quantitation of DNA (67, 83, 102); however, more steps during DNA extraction may allow for opportunities for contamination.

The LAMP assay developed was utilized due to reports of high sensitivity and specificity to target DNA, tolerance of the reaction to the presence of reaction inhibitors, and the potential for use by growers or crop consultants using hand-held LAMP devices such as the BioRanger (Diagenetix, Inc., Hawaii, USA) or the Genie II and III (Optigene Ltd, West Sussex, UK) (82, 83, 101, 102, 107, 128, 133); however, the development of the qLAMP assay revealed the degradation of the sensitivity of the assay to the target DNA. Since the inception of this project, other DNA amplification techniques have become more accessible for field use (94, 115), including qPCR (BioMeme, Inc., Philadelphia, PA, USA) and Recombinase Polymerase Amplification (RPA) (Agdia, Inc., Elkhart, IN). These assays require minimal DNA preparation, are capable of real-time data, and may be more easily adapted to fungal pathosystems than the qLAMP assay developed here. The qLAMP assay may still be a useful tool for field inoculum detection, but further analysis of the system is required to determine the specific cause of the degradation of the assay.

Table 4.1. Contingency table representing the lab quantitative LAMP assay (L-qLAMP) and grower quantitative LAMP assay (G-qLAMP) compared to quantitative PCR (qPCR) detection results for the presence of *Erysiphe necator* sampled from custom made impaction spore samplers from both commercial vineyards and research plots at the Oregon State University Botany and Plant Pathology Research Vineyard.

			qPCR[c]		Fisher's Exact Test (Probability)[d]
			Positive	Negative	
L-qLAMP[a]	2013	Positive	146 (46%)	13 (4%)	< 0.0001*
		Negative	42 (13%)	115 (37%)	
	2014	Positive	36 (16%)	8 (3%)	< 0.0001*
		Negative	61 (27%)	123 (54%)	
G-qLAMP[b]	2014	Positive	2 (3%)	4 (5%)	0.22*
		Negative	9 (13%)	58 (79%)	

[a] "Positive" and "Negative" indicate the number of samples for which *E. necator* DNA was detected and not detected, respectively as tested by L-qLAMP (*n*=316 in 2013 and *n*=228 in 2014) assays as described in the text.

[b] G-qLAMP (*n* = 73 in 2014) assessed by growers using mobile qLAMP devices (Diagenetix Inc., Honolulu, HI) as described in the text.

[c] 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. qPCR detection data based on quantitative data from Chapter 5.

[d] 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 $P < 0.05$.

Table 4.2. Contingency table representing the grower quantitative LAMP assay (G-qLAMP) compared to lab quantitative LAMP assay (L-qLAMP) detection results for the presence of *Erysiphe necator* sampled from custom made impaction spore samplers from both commercial vineyards and 2013 and 2014.

		L-qLAMP		
		Positive	Negative	Fisher's Exact Test (Probability)[b]
G-qLAMP[a]	Positive	1	5	
	Negative	1	63	< 0.0001*

[a] "Positive" and "Negative" indicate the number of samples for which *E. necator* DNA was detected and not detected, respectively as tested by G-qLAMP assay (n = 70) as described in the text.

[b] 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 $P < 0.05$.

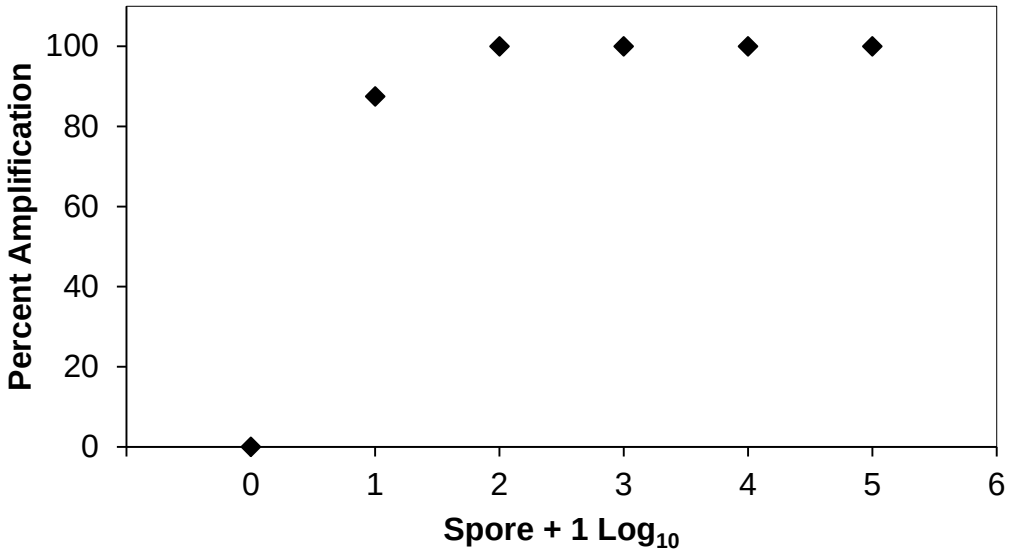

Figure 4.1. Sensitivity of qLAMP assay to *Erysiphe necator* as a function of percent amplification (y-axis) and spore + 1 $\log_{10}$ concentrations (x-axis). Each point represents the average of 10 separate extractions created from different *E. necator* conidia dilution series (10^2, 10^3, and 10^4 conidia concentrations), 1 and 10 conidia eyelash transferred spore rods, and conidia-free spore rods (n = 10).

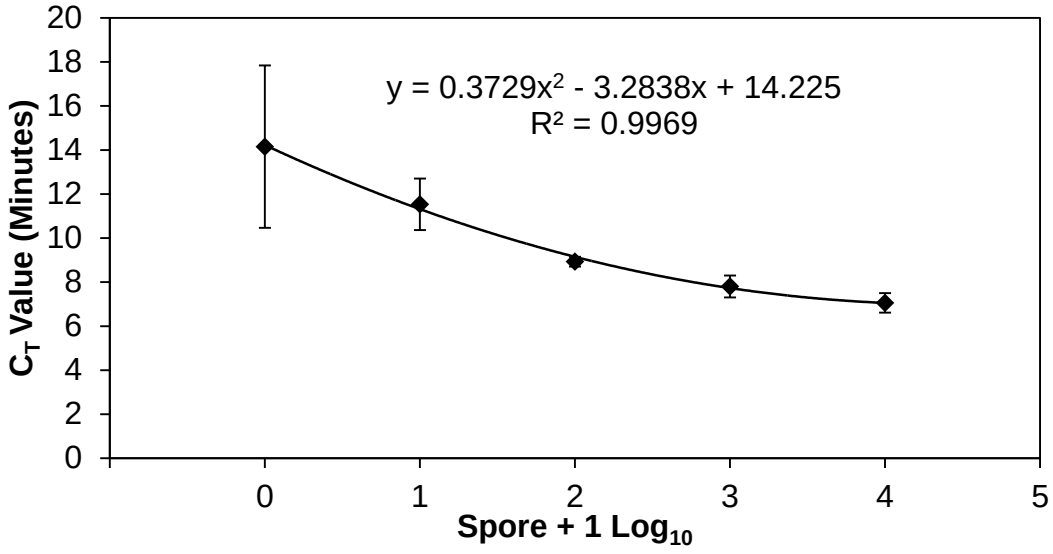

Figure 4.2. qLAMP standard curve developed from 6 separate *Erysiphe necator* spore dilution series comparing the spore + 1 $\log_{10}$ quantity to the cross-threshold (C_T) value (minutes). The threshold was set to 1100 to assess quantification across reaction setups. The average C_T value was used to determine the spore quantities of unknown samples.

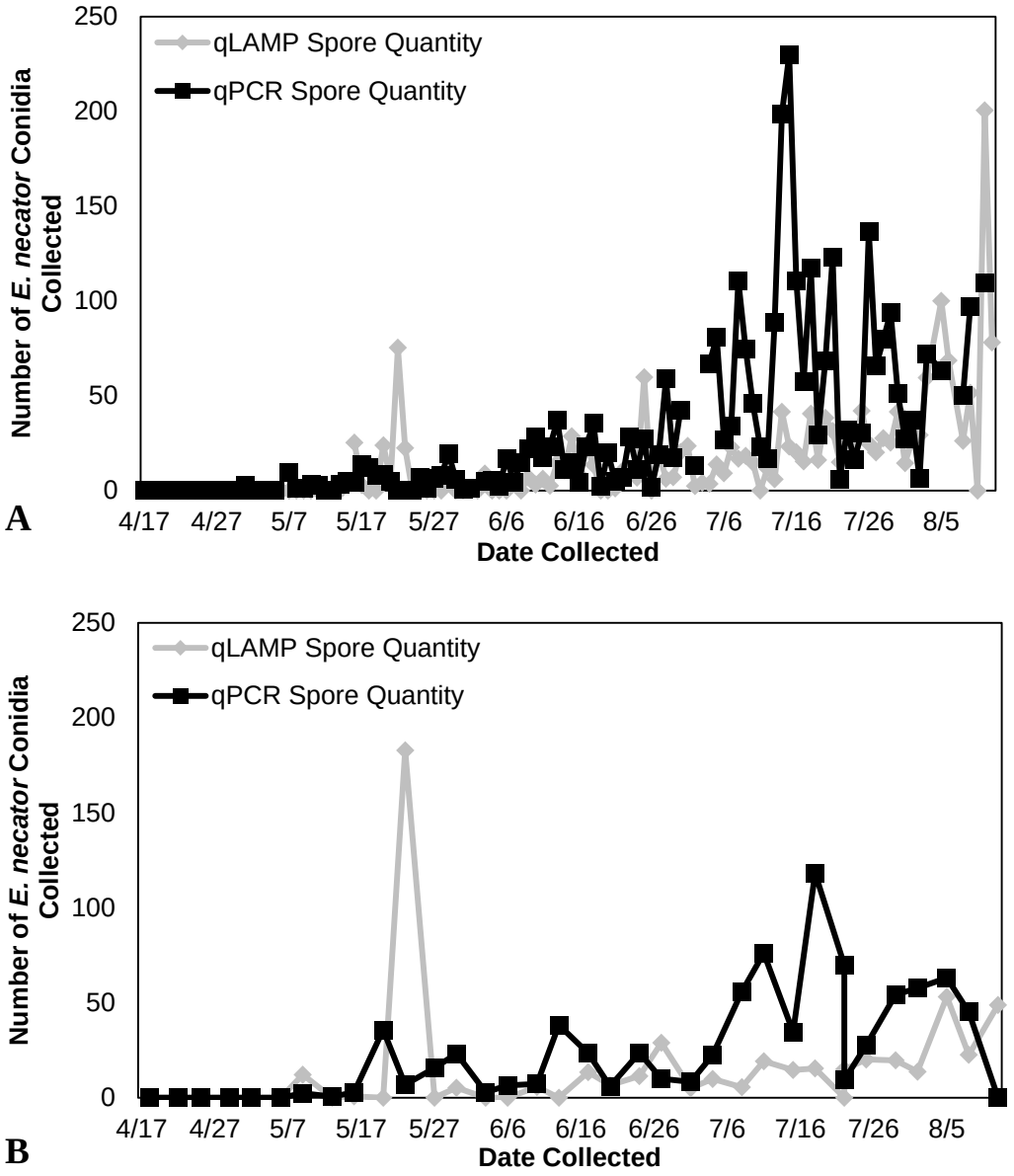

Figure 4.3. *Erysiphe necator* spore enumeration determined by qLAMP (gray diamond) and qPCR (black square) assays collected daily (A) and biweekly (B) from the Botany and Plant Pathology Research Farm vineyard (Corvallis, OR) during the 2013 growing season. The qLAMP spore quantification was significantly lower than the qPCR daily samples ($P < 0.001$), but the biweekly qLAMP and qPCR sample quantification was not significantly different in ($P = 0.14$).

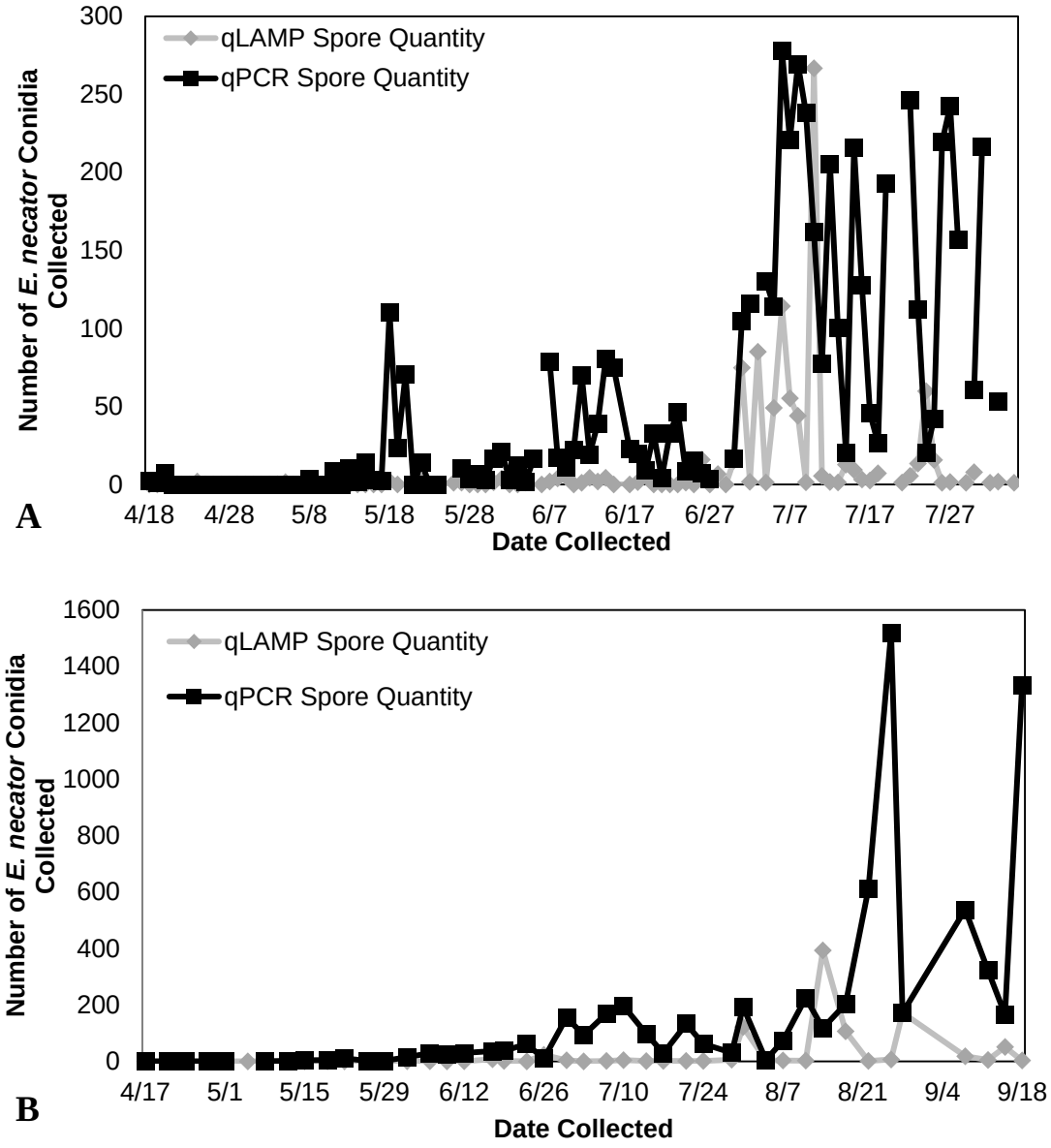

Figure 4.4. *Erysiphe necator* spore enumeration determined by qLAMP (gray diamond) and qPCR (black square) assays collected daily (A) and biweekly (B) from the Botany and Plant Pathology Research Farm vineyard (Corvallis, OR) during the 2014 growing season. The qLAMP assay significantly underrepresented spore levels for both the daily collections ($P < 0.001$) and the biweekly collections ($P = 0.01$) compared to the qPCR assay.

5. Optimization of fungicide application intervals based on airborne *Erysiphe necator* concentrations

5.1 Introduction

Erysiphe necator, the causal agent of grape powdery mildew, causes damages to the foliage and fruit of grapevine wherever susceptible varieties of grape are grown, and causes economic losses worldwide (24, 38, 59, 62, 77). To maintain disease-free fruit, vineyard managers often employ numerous fungicide applications throughout the growing season in conjunction with other cultural practices (41, 47). However, over the past several years viticulturists have encountered economic and political pressures to reduce fungicide use while continuing to produce high quality fruit, and a changing climate that may adversely affect disease dynamics (32-34, 50, 111). Additionally, marketing pressures to employ management practices that are considered more environmentally friendly (e.g. organic, biodynamic) are also causing changes in pest management that have led to the increased utilization of fungicides with limited mobility and duration, such as sulfur and mineral oil. The increased use of fungicides with limited mobility further necessitates improved application timing throughout the growing season (89). Despite the apparent incompatibility of reducing fungicide usage and utilizing immobile fungicide chemistries while still maintaining the high level of fruit quality demanded by the industry, there is still further opportunity to optimize fungicide application timing.

Historically, managers have followed a calendar-based fungicide application program or have recently begun to utilize disease forecasting models, particularly the UC Davis Risk Index (64), in order to time fungicide applications. There have been

numerous efforts to improve on existing disease forecast models to continue to reduce the frequency of fungicide application (19, 20, 23, 25, 103, 104, 120). These approaches, however, make various assumptions about overwintering inoculum maturity and availability, which causes inconsistency in predictions in dissimilar environments. These assumptions are also inconsistent with spore sampling data collected in commercial vineyards in the Pacific Northwest (44, 129). Because of the inconsistency in predicting the onset of the disease epidemic, unnecessary or untimely fungicide applications may be made to mitigate disease pressure. Near real-time inoculum detection methods to manage grape powdery mildew epidemics may further reduce the reliance on fungicides by directing application timing based on actual inoculum quantity.

Inoculum detection has been used to manage disease in various pathosystems, including hop downy mildew, grape powdery mildew, and Botrytis leaf blight of onion (28, 30, 44, 80, 122). Because visual identification of spores is difficult, DNA technologies have been pursued to identify airborne inoculum (30, 44, 118, 129). By utilizing air sampling methods and DNA technologies, airborne inoculum with high quantities of background DNA can be detected and subsequently used as a decision tool for the management of disease (92). Molecular techniques, such as PCR or loop-mediated isothermal amplification (LAMP), have been developed to further optimize airborne inoculum detection by utilizing specific DNA sequences to identify target organisms and modify fungicide programs based on detection for multiple pathosystems, such as onion, grape, and oilseed rape (22, 30, 44, 118, 129, 139). In the grape powdery mildew pathosystem, delaying fungicide application until

inoculum detection reduced fungicide applications by about 2.5 applications per growing season (129). These methods, while sensitive to low inoculum concentrations, are binary and do not assess relative disease risk due to varying amounts of inoculum concentration.

Highly accurate and sensitive quantitative molecular assays, such as quantitative PCR (qPCR), have been developed for real-time detection and quantification of numerous plant pathogens (30, 93, 118, 129, 144). Utilizing an impaction spore sampler to collect airborne inoculum in conjunction with qPCR analysis allows for direct enumeration of inoculum levels in air samples (92). This near real-time information could allow for an inoculum concentration threshold to be utilized as risk estimation for potential spore deposition and subsequent germination (28, 30), and to adjust fungicide application intervals. This approach could also reduce untimely and potentially unnecessary fungicide applications.

The purpose of this project was to improve fungicide application timing for the management of grape powdery mildew by monitoring airborne inoculum to initiate and subsequently adjust fungicide applications based on an inoculum concentration threshold. To accomplish this, a highly specific and sensitive quantitative PCR assay was utilized to enumerate spore quantities collected in vineyard air samples. The inoculum levels were then used to adjust fungicide application intervals based on a spore quantity threshold.

5.2 Materials and Methods

5.2.1 Inoculum Detection

Stainless steel welding rods (1.1 mm in diameter) were cut to 36 mm lengths, washed, sterilized, and coated in vacuum grease according to procedures outlined by Thiessen et al. (129). Rod pairs were stored at room temperature until placement into impaction spore samplers. Custom impaction spore samplers (129) were placed at 6 commercial vineyards within the Willamette Valley of Oregon and at the Oregon State University Botany and Plant Pathology Research Vineyard (Corvallis, OR) in both 2013 and 2014. Sample rods were placed and collected by vineyard managers and transported to the lab the same day as collection for DNA extraction and qPCR analysis as described by Thiessen et al (129).

Vineyard managers were directed to place impaction spore samplers in vineyard blocks with perennially severe disease or where disease levels were observed to be highest in the previous fall, confirmed by cane scarring observations. Samplers were to be placed directly adjacent to the trunk such that the sampling arm of the sampler was within 10 cm of the trunk or cordon until shoot growth reached 30 cm, whereby the sampling arm was extended above the canopy for the rest of the growing season (129). Vineyard managers collected samples and transported them to collection points for delivery to the lab, whereby samples were processed and analyzed. Rod pairs were collected and replaced every three to four days (bi-weekly). Samplers were run continuously from bud break (BBCH 08) to véraison (BBCH 81), April 17 to August 12, 2013 and April 17 to August 11, 2014. Additionally, at the

research vineyard, a paired spore sampler was placed adjacent to the bi-weekly collected spore sampler, which was collected daily from April 17 to August 12, 2013 and April 17 to August 11, 2014 to monitor the daily fluctuations of airborne inoculum concentrations.

5.2.2 Quantitative PCR Assay

DNA was extracted from the collected rod pairs using the PowerSoil® DNA extraction kit (Mo Bio Laboratories, Inc. Carlsbad, CA) following the manufacturer's protocols. In addition to field samples, a set of prepared rods containing 500 *E. necator* conidia was included as an extraction efficiency control. *Erysiphe necator* primers developed by Falacy et al. (44) were paired with a TaqMan® probe with a minor groove binder (129). 15 µl qPCR reactions contained 7.5 µl PerfeCTa® qPCR ToughMix® (Quanta Biosciences, Gaithersburg, MD), 400 nM final concentrations of each *E. necator* forward and reverse primers and probe, and 1.5 µl extracted sample DNA. Reactions were carried out using an ABI StepOne Plus qPCR machine (Applied Biosystems, Foster City, CA).

All qPCR reactions were performed in triplicate and each reaction plate contained 500 conidia extraction control, 100 and 10,000 conidia positive reaction controls, and template-free negative controls. Positive controls and standard curve known samples were generated by suspending *E. necator* spores from *Vitis vinifera* cv. 'Chardonnay' vines in 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) in nuclease-free water then pipetted onto sterile prepped sampling rods. A hemocytometer was used to estimate the concentration of the conidia suspension,

which was then pipetted onto pairs of coated stainless steel rods such that 100, 500, 1000, or 10,000 conidia were present on the rods. An eyelash brush was used to hand transfer conidia to create one and ten conidia concentrations. The rods were allowed to air dry, and were either processed, as above, or stored at -20 °C until processing.

Cycle threshold (C_T) analysis was conducted using ABI StepOne™ software. 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. Conidia concentration 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 C_T values of 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 Ct values to conidia concentration. An *E. necator* standard curve was developed to determine the relative concentration of unknown samples. The standard curve was generated by creating five separate ten-fold conidial dilution series on the stainless steel sampling rods 1 to 1×10^5 conidia as described above. DNA extractions were conducted using the PowerSoil® DNA extraction kit. The standard curve was then generated by averaging the C_T values for each conidia quantity from the five independent DNA extractions.

5.2.3 Commercial and Research Vineyard Test Sites

Each vineyard, both the commercial sites and the research site, contained paired treatment plots consisting of their standard management program (control plot) and an adjusted fungicide interval plot (interval plot). All plots were placed in Pinot noir blocks on rootstock with various management practices (Table 6.1). Plots were located in the vineyard at the vineyard manager's choosing, which did not need to be collocated with the impaction spore sampler; however, these plots were often placed in the same location as the sampler. Plot size varied from six 30 m rows to 1 ha, depending on vineyard site. Fungicide programs in the interval plots were initiated at first detection of *E. necator* spores or at the onset of bloom (BBCH 61), whichever occurred first. Subsequent fungicide intervals were maintained at the longest application interval labeled for the fungicide chemistry used by the grower until the spore sampling sample had greater than or equal to 10 spores detected. The subsequent fungicide application would then be applied at a shorter application interval. This occurred until spore samples were less than or equal to 10 spores, whereby fungicide intervals were lengthened to the longest interval according to the label of the fungicide last applied to the plot. A 10 spore threshold was used because the broader representation of the sampler within a field is unknown (92), and, in previous spore sampling experiments, 10 spore detections were observed in fields with less than 1% leaf incidence (Chapter 3). It is also possible that over the 3 to 4 day sampling period that spores sampled early in the deployment period are no longer detectable. All management decisions were made by vineyard managers in their grower standard practice plots (Table 6.1), and fungicides were applied in the interval

adjustment plots based on spore concentrations detected via spore sampling. Non-treated control plots for each vineyard were not possible due to the high crop value of wine grapes in Oregon and the potential for plot-plot interference (26). Non-treated controls are also not required for experimental comparisons because the objective of this project was to develop a fungicide application program that is equal to or superior to current industry practices. Environmental conditions, including relative humidity, leaf wetness, rainfall, and temperature, were recorded at 15 minute intervals at field locations to assess the suitability for disease development.

5.2.4 Disease Monitoring

Each plot was scouted weekly for foliar powdery mildew incidence from May 30 to August 1 in 2013 and May 27 to August 11 in 2014 to monitor for disease progress. Incidence was determined by inspecting 10 randomly chosen leaves from each of 50 vines in each treatment plot. Disease severity was not assessed in commercial vineyards due to the low disease incidence observed on foliar tissue before véraison. In 2014, one final visual assessment was made post-harvest after all fungicide applications had stopped to assess natural disease pressure in the vineyards and account for the low disease observed during the growing season.

Fruit infection incidence was determined by destructively sampling one grape cluster per vine from 50 vines per plot at the onset of véraison. After collection, clusters were frozen until assessment at -20 °C. Clusters were visually assessed for powdery mildew presence both microscopically (40 × magnification) and visually unassisted to account for diffuse colonies and industry standard disease assessments

(48, 56). For microscopic analysis, frozen berries were removed from the rachis, and 25 berries were arbitrarily chosen to be assessed. The number of infected berries out of 25 was recorded; a single penetration site observed on a berry was classified as positive for disease.

Each sampler, and consequent vineyard, was treated as the experimental unit because the independent impaction spore sampler represented the unit for a management decision in this experiment. Leaf disease incidence from both interval and control plots were compared using the area under disease progress curve (AUDPC) for each set of paired plots, and were subsequently compared using a one-tailed Student's *t*-test. Berry incidence from paired plots was also compared using a one-tailed Student's *t*-test. Fungicide application records were used to compare the number of fungicides used in adjusted interval plots and grower-standard control plots.

5.3 Results

5.3.1 Field Inoculum and Disease Monitoring

In both 2013 and 2014, *E. necator* spores were sampled in all sites tested (Fig. 5.1). There was less inoculum concentration observed in 2014 (Fig. 5.1A) compared to 2013 (Fig. 5.1B). Inoculum was detected on May 23, 2013 and May 1, 2014 in commercial vineyards, and disease incidence was not observed until May 30 in commercial vineyards in 2013 and not until after véraison in 2014. In 2014, a research vineyard was included in the study to observe the effect of higher inoculum

pressure, and disease incidence was first observed on June 12, 2014. Bi-weekly spore sampler collections were not additive of daily sample collections in either 2013 (Fig. 5.2A) or 2014 (Fig. 5.2B), and the daily and bi-weekly collections showed similar concentration fluctuations during the growing season.

Both years showed low leaf disease incidence, less than 4%, in all vineyards and treatments (Fig. 5.2). AUDPC values of adjusted interval treatments were 25.08 ± 11.51 and control treatments were 22.72 ± 9.40 in 2013 (Fig. 5.3 A). AUDPC values of adjusted interval treatments were 3.03 ± 3.28 and control treatments were 1.41 ± 1.52 in 2014 (Fig. 5.3 B). Adjusted interval and control leaf disease incidence AUDPC were not significantly different in both 2013 ($P = 0.44$) and 2014 ($P = 0.31$). Because 2014 disease observations were almost 0% incidence in commercial vineyards throughout the duration of the growing season, an end of season (BBCH 89) disease assessment was conducted to determine any differences in disease after fungicide applications ceased. The late season disease assessment showed leaf disease incidence of 47.20% ± 11.39 in adjusted interval treatments and 63.45% ± 13.50 in control treatments, with disease observed in adjusted interval treatments after véraison being significantly different from the control ($P = 0.04$).

5.3.2 Berry Disease Assessment

In commercial vineyards, microscopic berry disease incidence in adjusted interval treatments and in control treatments was 3.62% ± 1.47 and 1.84% ± 1.07, respectively, in 2013 and 0.01% ± 0.01 and 0.27% ± 0.22, respectively in 2014. Berry disease incidence was not significantly different between adjusted interval and control

treatments in 2013 (P = 0.11) or 2014 (P = 0.15). In the research vineyard, where disease pressure much higher than in a commercial vineyard setting, berry incidence was significantly higher (P = 0.02) in the control treatment (50.58% ± 5.67) than in the adjusted interval treatment (33.22 ± 4.02).

5.3.3 Fungicide Applications

Commercial vineyards had 2.4 fewer fungicide application in 2013 and 1.6 in 2014 in adjusted interval plots (Table 6.1). Although only adjusted fungicide application interval plots should have an adjusted number of applications in commercial fields, fungicide applications were reduced in both adjusted interval plots and control plots in commercial vineyards compared to calendar-based fungicide application intervals. In addition, the number of fungicide applications in the grower standard program was also reduced from previous years according to fungicide application records provided by growers (data not shown). Disease and inoculum pressure in the research vineyard was higher than commercial vineyards (Fig. 5.1), which resulted in three more organic fungicide applications in the adjusted interval plots relative to the control plots (Table 6.1).

5.4 Discussion

Through the use of real-time inoculum monitoring and an inoculum threshold, growers were able to reduce the number of fungicide applications made within a growing season and maintain the level of disease control by adjusting fungicide application intervals based on the concentration of airborne *E. necator* inoculum

detected. Field scouting confirmed that leaf disease incidence was maintained below 3% visual incidence in both years, and microscopically observed fruit disease incidence was maintained below 4% in commercial vineyards in both control and adjusted interval fungicide application treatments. All commercial growers that participated in this study reduced the number of fungicide applications while maintaining the level of disease control, which suggests that fungicide applications can be reduced or more optimally timed without decreasing the level of disease control. Because the research vineyard was under heavy disease pressure from adjacent grapevines not managed with fungicides, the number of fungicide applications in adjusted interval plots increased compared to the calendar-based application interval standard practice. Although fungicide applications were not reduced, disease control was significantly improved on fruit in the research vineyard when the site was included in the study in 2014 ($P = 0.02$). This may indicate that utilizing spray programs that focus on calendar-based fungicide applications are not optimally timed for field specific climatic conditions (2, 137) that affect fungal development and may be improved through adjusting fungicide application intervals based on spore concentration threshold.

The area of the vineyard that is represented by the spore sampler within the vineyard, and the subsequent effect on the captured spore concentration is unknown (92), which may affect the spore concentration action threshold. The spore concentration action threshold was chosen conservatively in this study due to the potential for spore degradation and PCR inhibitor presence on spore sampler rod pairs. Comparison of daily collections of spore samplers from the research vineyard

show that biweekly samples collected from later in the growing season are not additive (Fig. 5.2), which may indicate that DNA from spores is degraded when exposed to environmental conditions (i.e. sunlight), and a biweekly sample detection of 10 spores may represent a higher concentration of conidia. Based on the low disease observed in commercial vineyards, it may be possible to utilize a less conservative threshold. A higher threshold may further reduce fungicide applications without reducing disease control. The loss of quantifiable spores also needs to be taken into consideration when deciding on the sampling interval employed (Fig. 5.2). Commercial implementation of this technology would be more economically viable if samples could be retrieved every 7 days; however, there could be a significant risk of inaccurately determining if the inoculum concentration threshold was met due to loss of detectable spores.

The number of spores collected may also be impacted by the placement of the impaction spore sampler within the vineyard due to microclimate and environmental influences on spore release and dispersal. Fungal spore dispersal, and subsequent airborne concentration, is dependent on several factors, including spore production and release rate (57), colony age (141), and environmental factors such as wind, temperature, humidity, and precipitation (95, 141). *Erysiphe necator* conidia appear to utilize passive dispersal mechanisms to escape leaf surfaces, such as wind (2.3 ms^{-1} on stationary leaves), leaf movement, intense rainfall, or by turbulence and leaf movement created by high pressure sprayers (140, 141). Once spores have become liberated, they travel within the turbulent airflows until they deposit (74). An increased understanding of how turbulent structures are created by complex canopies, such as in vineyards, may

improve placement of spore samplers by better predicting inoculum dispersal patterns. Unique turbulence structures within vineyard canopies form eddies and sweeps that influence the direction of particle dispersal (6, 9, 98). These air turbulence structures cause air to channel along the row, regardless of wind direction, with few escapes into the laminar flow layer (98). Placing spore samplers within perennial disease "hotspots" or in the direction of turbulent airflow channeling from the "hotspot" in the vineyard may allow for more airborne inoculum to be sampled.

The utility of spore sampling and use of spore concentration to time fungicide applications is heavily reliant on spore sampler placement because inoculum release at the onset of the epidemic may go unnoticed if spore samplers are placed in a portion of the vineyard that does not contain overwintering inoculum. Spore samplers in this study were placed in regions with heavy cane scarring or perennial "hotspots," where primary inoculum is most likely to overwinter, in order to sample early spring spore release from cleistothecia or bud perennation. Misplacement of the spore sampler may allow for epidemic initiation to go unnoticed due to the rapid production of *E. necator* condiophores and conidia. In 2013, one vineyard sampler was not placed according to the protocol, but rather for convenience of location instead of the region most likely to have overwintering inoculum. It was located 4500 m to southwest on the other side of a ridge from the managed blocks in a region of the vineyard that was sheltered from the prevailing wind direction, adjacent to the winery. Although, this vineyard was removed from the 2013 analysis for failure to follow protocol, the data indicates that monitoring site location is critical to using inoculum monitoring as a decision aid. In this field, berry disease incidence in the

adjusted fungicide application interval plot was 26.73 % compared to 0.32% incidence in the control plot, and the leaf incidence AUDPC was 191.45 and 0 in the adjusted interval plot and control plot respectively. Inoculum is less likely to be detected the farther the spore sampler is placed from the source of inoculum due to the dilution of spore concentration as distance increases from the inoculum source and terrain (99). Placing the spore sampler nearest to site with the highest concentration of initial inoculum, primarily cleistothecia within the bark, increases the likelihood that the spore sampler will detect early release events that initiate the epidemic. Placement of spore sampler outside a disease focus may cause underestimation of field-wide spore concentration and disease, whereas placement within a disease focus may potentially overestimate field-wide spore concentration and disease.

Most vineyards that participated in this study placed fungicide timing plots in the same location as spore samplers, however, one field, V6, placed their adjusted interval and control treatment plots approximately 1 km from the spore trap. This field showed no leaf disease incidence in either adjusted interval or control plots (AUDPC = 0) until after véraison. Including a late leaf disease incidence inspection (BBCH 89), leaf disease incidence AUDPC was 6.1 and 2.65 in adjusted interval and control plots, respectively. Additionally, berry incidence was not affected by placing the adjusted interval plots away from the spore sampler, and no disease incidence was observed on berries within either control or adjusted interval plots for the V6 vineyard. While placement of the spore sampler is imperative for early detection of airborne fungal inoculum, it appears that the spore sampler may be informative to

adjusted fungicide application intervals in vineyard blocks within close proximity to the spore sampler. Further exploration of spore dispersal and subsequent deposition within complex canopy architectures may improve the placement of spore samplers and potentially provide information as to how far away from the spore sampler the quantitative spore concentration data may be applied.

Table 5.1. Vineyard location general practices and fungicide applications for both 2013 and 2014 growing seasons.

	Fungicide Applications	
	2013	2014

Field	Training System	Fungicides [a]	Location of samplers	Grower Standard Control	Adjusted Interval	Grower Standard Control	Adjusted Interval
V1	VSP [b]	Conventional	Inside Adjusted Interval Treatment	9	5	7	3
V2	VSP	Conventional	Inside Adjusted Interval Treatment	7	4	6	4
V3	VSP	Organic (2013), Conventional (2014) [d]	Inside Adjusted Interval Treatment	16	13	6	5
V4	GDC [c]	Conventional	Inside Adjusted Interval Treatment	6	5	5	4
BPP	VSP	Organic	Inside Standard Control	— e	—	6	9
V5	VSP	Organic	Inside Adjusted Interval Treatment	11	10	—	—
V6	VSP	Conventional	~1 km NW of Plots	—	—	4	4

[a] Vineyards were not directed to use specific fungicide chemistries, and both organic and conventional pesticide vineyards were assessed.

[b] Vertical shoot positioning

[c] Geneva double curtain

[d] Vineyard managers changed from 2013 to 2014, and the vineyard that was participating in the experiment changed from organic pesticide management to conventional pesticides.

[e] Vineyards that only participated in one year of the study did not provide fungicide application records for the growing season indicated (—)

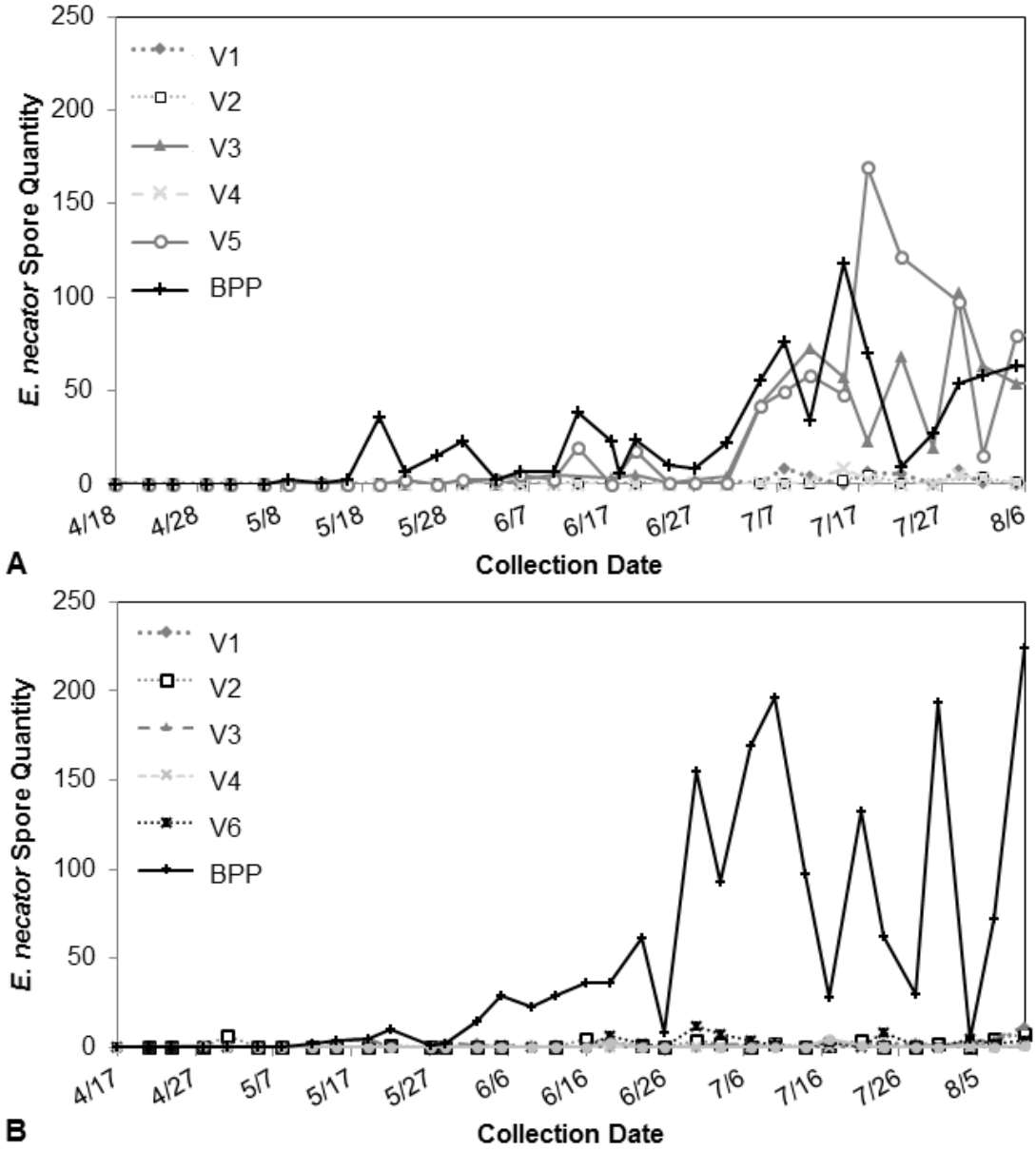

Figure 5.1. *Erysiphe necator* spore concentrations from commercial vineyards in (A) 2013 (B) 2014. The Botany and Plant Pathology Research vineyard (black diamond) was also included in the 2014 study. Vineyards were managed organically (solid lines) or with conventional pesticides (dashed lines).

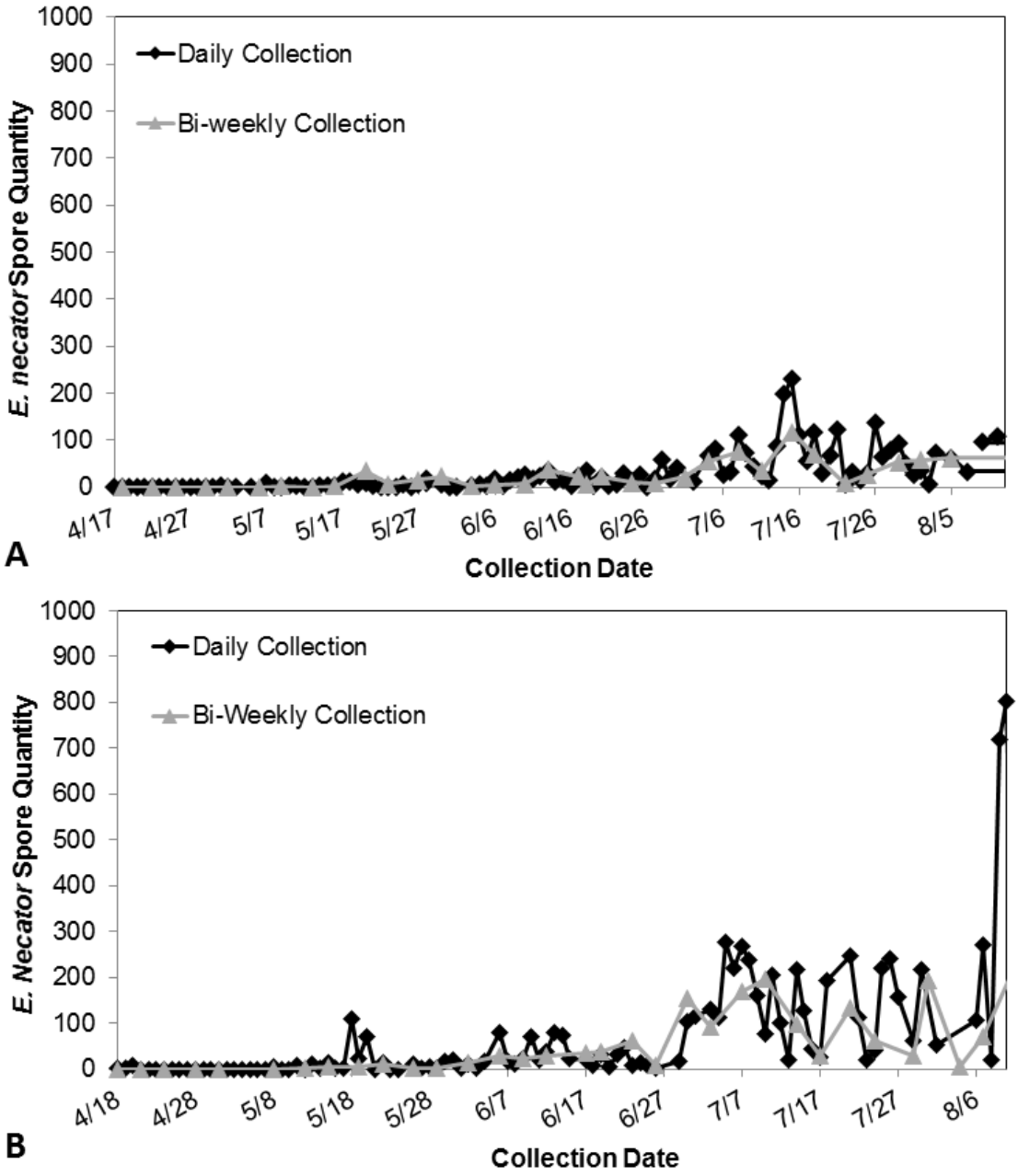

Figure 5.2. *Erysiphe necator* spore concentrations collected from the Botany and Plant Pathology Research Vineyard observed in (A) 2013 and (B) 2014. Daily (black diamond) and bi-weekly (gray triangle) spore concentrations were monitored from bud break (BBCH 08) to véraison (BBCH 81).

Figure 5.3. Grapevine leaf disease percent incidence observed from budbreak (BBCH 16) to véraison (BBCH 81) from 5 commercial vineyards in 2013 (A) and 6 commercial vineyards and 1 research vineyard in 2014 (B). A sample of 500 leaves was assessed from the control (solid gray line) or adjusted interval treatment (dashed black line) plot. Error bars are based on the standard error for each data point. Area under disease progress curve (AUDPC) values were determined using the average percent disease incidence observed in a plot. The AUDPC for the adjusted interval plots was 25.08 ± 11.51 and 3.03 ± 3.28 in 2013 and 2014, respectively. The AUDPC for the control plots was 22.72 ± 9.40 and 1.41 ± 1.52 in 2013 and 2014, respectively. Control and adjusted interval plots were not significantly different in either 2013 (P = 0.44) or 2014 (P = 0.32).

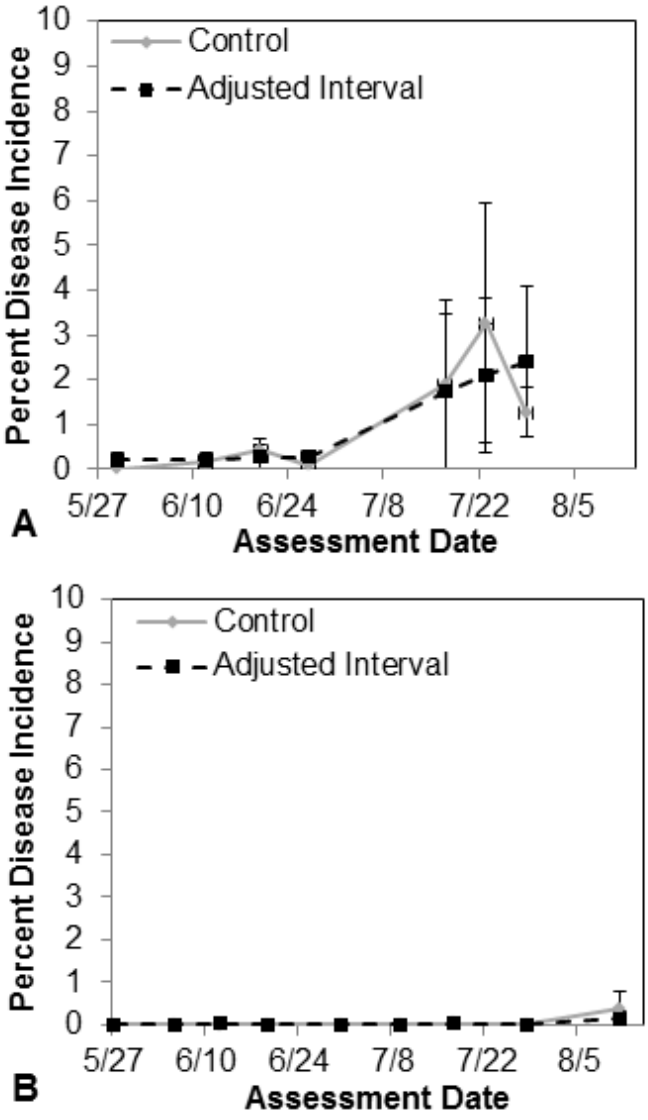
Control
Adjusted Interval
Percent Disease Incidence
10
9
8
7
6
5
4
3
2
1
0
5/27
6/10
6/24
7/8
7/22
8/5
Assessment Date
A
Control
Adjusted Interval
Percent Disease Incidence
Assessment Date
B

Figure 5.3.

6. Cleistothecia Initiation

6.1 Introduction

The primary inoculum of *Erysiphe necator*, ascospores that are contained within cleistothecia, is released when conducive conditions, such as free moisture and temperatures above 4 ºC (53), are present. These conditions are common throughout the dormancy of grapevine in the maritime regions of the Western U.S. Despite potential depletion of inoculum during grapevine dormancy, ascospore release continues to occur throughout host dormancy and into the growing season (Chapter 2). Several models have been developed to predict ascospore release and magnitude of release to generate management decision aids (19, 29, 53, 103, 130); however, these models inaccurately predict ascospore release in the Willamette Valley of Oregon (Chapter 2). These inaccuracies may be due to the effect of regional climatic conditions on cleistothecia and ascospore maturation.

Initiation of cleistothecia production requires hyphal fusion of opposite mating types, Mat 1-1 and Mat 1-2 (52, 55), and the production of cleistothecia has been described in some regions as soon as both mating types are in contact (52) or, in other regions, forming toward the end of the growing season (17, 75). Several factors may influence the initiation of cleistothecia, including the proximity to a compatible mating type, environmental conditions (i.e. temperature or photoperiod shifts), or host stress responses (i.e. drought or senescence stresses) that occur in response to environmental conditions. Proximity to mating type is presently described as the sole factor for initiation of *E. necator* cleistothecia (52) and suggested as an explanation

for the late formation of cleistothecia in other heterothallic species (18, 52, 125). Thus, it is important understand the proximity to mating types throughout the duration of the growing season and at the initiation of cleistothecia primordia.

The initiation of cleistothecia may also be influenced by environmental factors. Temperature, humidity, and photoperiod, have not been correlated with the initiation of cleistothecia formation on tissue culture plants and detached leaves (52). However, tissue culture and detached leaves require wounding plant tissues that could induce physiological changes in the plant tissue and impact development of cleistothecia (87). Evans et al. (43) observed delayed development of cleistothecia on tissue culture plantlets on MS media amended with hormones, but cleistothecia developed within 10 days of inoculation on detached leaves. Gadoury and Pearson (52) observed immediate cleistothecia production on detached leaves and plantelets on cultural media without hormones. Because *E. necator* has closely evolved with grapevine, the host likely has a strong influence on the formation of cleistothecia. By utilizing detached leaf assays, the pathogen may form overwintering structures in response to host stress or senescence. Injury of plant tissues for tissue culture or detached leaf assays may cause an upregulation of host defense compounds that are not observed when testing whole plants (12), causing differences in growth of *E. necator*. Similarly, growth regulatory hormone signaling found during stressful conditions, such as drought conditions (11, 124), may be differentially expressed in vines found in field environments compared to tissue culture or detached leaves used in laboratory experiments, which may affect the development of *E. necator* cleistothecia. Host signaling during stress or host senescence has been previously

suggested as a factor in the initiation of ascocarp formation of some powdery mildew species (71), and may provide an explanation for the late development of cleistothecia in the Pacific Northwest.

To understand the factors influencing cleistothecia initiation, such as mating type proximity, environmental signals, and host stress factors were studied using field and growth chamber studies to isolate environmental and host influences. The specific objectives for this study were to 1) identify the growth stage and temporal stage of grapevine development that cleistothecia are produced within the Willamette Valley of Oregon, 2) determine if the initiation of cleistothecia is dependent on environmental cues when whole plants are inoculated, and 3) examine potential stress effects or host signaling on cleistothecia initiation in both field and growth chamber trials.

6.2 Materials and Methods

6.2.1 Mating Type Field Survey

To determine if cleistothecia development in the field is a result of proximity to mating type, *E. necator* lesions were collected in a stratified sampling by using flame sterilized forceps to place and remove a ~70.9 mm^2 Tough-Spots® microcentrifuge tube label (Diversified Biotech, Dedham, MA) from *E. necator* lesions, such that individual colonies were collected. The sample was placed in a sterile 2 ml microcentrifuge tube and kept at -20°C until processing. At each sampling time the canopy was divided into equal thirds (upper, middle and lower) and 20 samples were collected from each area from three rows of grape vines at three

sampling date (Table 7.1). All samples were collected from treated Pinot Noir vines (Corvallis, OR) and repeated for three years.

DNA extraction of samples was modified from methods by Brewer, et al. (15). After samples were frozen, 200 µl of Chelex 100 (Sigma Aldrich, St. Louis, MO, USA) in DEPC treated water was added to each sample tube. Samples were vortexed horizontally for 5 minutes, and then centrifuged briefly at maximum speed to collect the contents at the bottom of the tube. Tubes were incubated at 95°C for 10 minutes. Samples were vortexed for five seconds, and centrifuged to collect contents. The tubes were incubated again at 95°C for 10 minutes. After the second incubation, tubes were allowed to cool to room temperature for about 20 minutes. Tubes were centrifuged for 2 minutes at 10,000 x g to collect the contents. The liquid was transferred from the extraction tube to a new, sterile 1.5 ml microcentrifuge tube before running the PCR analysis.

Multiplex PCR of field samples utilized the EnαF2, EnαR3, EnHMGF1, and EnHMGR1 primers developed by Brewer et al. (16). PCR reactions were conducted in a total volume of 25 µl, using Accustart II PCR ToughMix (Quanta Biosciences, Gaithersburg, MD, USA), nuclease-free DEPC-treated water (Growcells), EnαF2 (0.4 µM), EnαR3 (0.4 µM), EnHMGF1 (0.4 µM), and EnHMGR1 (0.4 µM). Cycling conditions included an initial denaturation at 94°C for 2 minutes followed by 40 cycles of a template denaturation step at 94°C for 30 seconds, an annealing step at 55°C for 30 seconds, and an extension step at 68°C for 30 seconds. Following a final extension at 68°C for 7 minutes, reactions were kept at 10°C until processed with gel electrophoresis. 10 µl of each PCR product with loading dye was analyzed using

electrophoresis through a 2% (w/v) agarose/TAE gel containing 10% ethidium bromide. As a control to check that the DNA amplified from colony collections was from *E. necator* and not from a closely related powdery mildew species, a subsample of the amplicons from MAT 1-1 and MAT 1-2 primer sets were sequenced using Sanger sequencing at the Oregon State University Center for Genome Research and Biocomputing Core Laboratory (Corvallis, OR). After mating types were determined from colony samples, the number of samples of each mating type was compared with a two-tailed Student's T-test.

6.2.2 Effect of Plant Stress on Cleistothecia Initiation

To assay the development of cleistothecia in the field, a research vineyard containing bilateral vertical shoot-positioning, cane-pruned CH-76 Chardonnay vines on 101-14 rootstock was used for all field testing. To ensure that both mating types were available throughout the duration of the growing season, a liquid suspension containing both mating types was applied at growth stage BBCH 19 using a Preval Aerosol Sprayer (Chicago, IL). The conidia were mechanically separated from leaves infested into 0.05% Tween 20 (Sigma Aldrich, St. Louis, MO, USA) and sterile deionized water solution and mixed at 1:1 ratio of each mating type with 2 $x10^3$ conidia/ml. The conidia suspension was applied to field plants within 1h.

To test host stress induction of cleistothecia, vines were either girdled or sprayed with abscisic acid solution (ABA) at two different time periods (BBCH 75 and BBCH 79). Girdling was done by removing a 1 cm wide section of vascular cambium from the circumference of both fruiting canes. A 150 $mg \cdot l^{-1}$ ABA solution

was applied using a Preval Aerosol Sprayer to cover the surface of all green tissues of vines within a plot. Non-treated control plots were used to compare natural cleistothecia development to stress-induction treatments. All treatments were applied to three replications using a randomized complete block design. Each replication consisted of 5 Chardonnay vines (5 x 6 spacing) with 101-14 rootstock vines separating plots.

Disease was monitored through weekly scouting for visual signs on leaf and fruit tissues beginning one week after field inoculation occurred. Leaves were collected as soon as cleistothecia primordia were observed in the field (BBCH 91) in each year, which occurred August 23, 2013 and August 28, 2014 and July 28, 2015. Ten leaves were randomly sampled from the middle of the canopy regardless of leaf age, and stored at -20°C until cleistothecia developmental stages (primordia, immature, and mature) (Fig. 6.1) could be enumerated. Cleistothecia were classified as primordia if they formed small white spheres without lipid accumulation; immature if they were yellow to orange, signifying that lipids were accumulating; and mature if they were brown to dark brown-black. Cleistothecia quantities were compared using GLM procedure in R version 3.2.1.

6.2.3 Environmental Conditions on Seedlings

Because detached leaf assays and tissue culture plants may have different effects on obligate fungal growth, growth chamber experiments to determine the

effect of environmental conditions on whole plants grown from seed. Chardonnay seeds were collected from crush pomace, cleaned, and stored at 4°C until used. Prior to planting, seeds were scarified by soaking seeds in 9% H_2O_2 for 48 hours at room temperature, rinsed with sterilized deionized water, and re-suspended in sterilized deionized water for 48 hours at room temperature. Seeds were rinsed again with sterilized deionized water and soaked in 10% Abound fungicide solution (Syngenta, Greensboro, NC) for 10 minutes. Seeds were spread on moistened filter paper and stored in the dark at 20°C. After one week, germinated seed were planted into potting mix within 10 cm pots and grown in the growth chamber until use.

Plants with 3 true leaves were inoculated with both Mat 1-1 and Mat 1-2 *E. necator* isolates by spreading conidia over two fully expanded leaf surfaces with sterile paint brushes. Plants were placed into growth chambers programmed for photoperiod, humidity, and temperature as described below. Plants were watered and fertilized regularly to maintain optimal growth.

6.2.3.1 Effect of Temperature

Growth chambers for inoculated seedlings were held at 60% humidity and 16 hour days with 8 hour dark periods. The temperature in each chamber were set to 5, 10, 15, 20, 25, or 30°C. The growth chamber with 3 subsamples (seedlings) served as the experimental unit and the temperature trial was replicated in time three times. Leaves were monitored for disease progression and cleistothecia development daily for 45 days post-inoculation. Treatment differences were compared by paired T-tests

of the area under cleistothecia development curve (AUCDC) values in R version 3.2.1.

6.2.3.2 Effect of Photoperiod

To determine if change in the photoperiod length affected cleistothecia formation, four photoperiod shift regimes were used. Plants were grown for 14 days at either short day (8h of light and 16h darkness) or long day (16h of light and 8 h of darkness) photoperiods before inoculations as above. Plants were then inoculated and incubated for 14 days at the initial photoperiod before being switched to a long day or short day photoperiods. This design resulted in 4 treatments: long day to long day, long day to short day, short day to short day, and short day to long day. All growth chambers were programed for 60% humidity and 20°C. An experimental unit consisted of 3 vines per light photoperiod shift regime. The entire experiment was replicated in time three times. Leaves were monitored daily until 35 days post-inoculation for disease progression and cleistothecia development. Treatment differences were compared by paired T-tests of the AUCDC values in R version 3.2.1.

6.3 Results

6.3.1 Mating Type Survey

In all three years, both Mat 1-1 and 1-2 amplicons were found within the same 70.9 mm^2 at all sampling times (Table 6.1). However, cleistothecia primordia were

not observed until August 23, 2013 and August 28, 2014 and July 28, 2015. Sequencing of sample amplicons yield sequences identical to *E. necator* DNA, thus indicating that the amplicons where derived from *E. necator* Mat 1-1 and Mat 1-2 mating types. Mat 1-2 was significantly greater in representation of isolates collected in 2013 ($P = 0.04$) compared to Mat 1-1 samples (Table 6.1). Mating type proportions of Mat 1-1 to Mat 1-2 varied between 0.5 to 0.9 with no significant differences in the proportion of Mat 1-1 to Mat 1-2 in 2014 or 2015 ($P > 0.7$) (Table 6.1). There was no significant difference in mating type proportions in collections from different canopy levels ($P > 0.1$) (Table 6.1).

6.3.2 Effect of Plant Stress on Cleistothecia Initiation

All treatments produced cleistothecia by late August (BBCH 85) in both 2013 (Fig. 6.2A) and 2014 (Fig. 6.2B). Once mature cleistothecia were produced in the field, there was no significant difference in the number of primordia, immature, or mature cleistothecia produced ($P > 0.05$). There were no significant differences between cleistothecia induction treatments in either 2013 or 2014 ($P > 0.5$) (Fig. 6.2). Grapevine fruiting canes showed signs of healing and recovery from girdling treatments approximately one month after treatments were made. Premature leaf drop was also not observed in ABA treatments.

6.3.3 Effect of Environmental Conditions on Cleistothecia Primordia Development

6.3.3.1 Effect of Temperature

Treatments between 10 and 25 °C all produced cleistothecia within three weeks of inoculation, and cleistothecia were not produced at 5, 30, or 35 °C (Fig. 6.3). The AUCPC of the 10 °C temperature treatment was 19.08 ± 19.08, the 15 °C was 153.22 ± 107.78, the 20 °C was 289.93 ± 5.19, and the 25 °C was 5.19 ± 5.19. Pairwise comparisons showed that the number of cleistothecia produced at 20 °C was significantly different than 5, 10, 25, 30, and 35 °C ($P = 0.05$), and the number of cleistothecia produced at 15°C was not significantly different from 20 °C ($P = 0.31$).

6.3.3.2 Effect of Photoperiod

All photoperiod shift treatments produced cleistothecia within three weeks of initial inoculation (Fig. 6.4). The AUCPC of the short photoperiod treatment was 157.28 ± 51.40, the short to long photoperiod shift treatment was 577.08 ± 56.68, the long to short photoperiod shift treatment was 110.81 ± 41.67, and the maintained long-long photoperiod treatment was 283.78 ± 176.80. The short to long photoperiod and long to short photoperiod treatment AUCPC were significantly different ($P = 0.02$), and no significant differences between other photoperiods treatments ($P > 0.05$) was observed.

6.4 Discussion

In contrast to previous work conducted on cleistothecia initiation (43, 52), cleistothecia were not observed to initiate under field conditions until BBCH 85 each year (Fig. 6.2) despite the presence of both mating types throughout the growing season within the same 70.9 mm^2 (Table 7.1). Late season development of cleistothecia of *E. necator* cleistothecia has been described in other regions (17, 75, 86) and in other powdery mildew systems (77); however, the cause for cleistothecia initiation has yet to be determined. Proximity to mating type has been suggested as a potential mechanism for cleistothecia initiation of grape powdery mildew (52) and other powdery mildews (18, 125), but the presence of both mating types of *E. necator* within a 70.9 mm^2 sampling area suggests that proximity is not the sole limiting factor in cleistothecia initiation.

Field collections of *E. necator* colonies showed that both Mat 1-1 and Mat 1-2 were present within a single vineyard block, on the same leaf, and within a single sample throughout the growing season over three growing seasons (Table 3.1), which would indicate that additional conditions are required for the initiation of cleistothecia. Several samples collected during the 2013 growing season in the upper canopy did not amplify with mating type primers (Table 3.1), which may be due to the health of colonies collected. Colonies from the upper portion of the canopy were small and may have been affected by increased UV exposure (142) and increased leaf surface temperature (5, 7, 114). Consequently, these colonies may have been dead and not suitable for DNA extraction and amplification. Despite reduced samples in the upper canopy 2013, both mating types were still found within the same 70.9 mm^2

sample (Table 3.1). Mating type proximity has been suggested as an explanation for the late formation of cleistothecia in several heterothallic powdery mildew species (18, 52, 125); however, evidence of the presence of both mating types within the same region (16, 96) and in the same field indicate that additional factors are required for cleistothecia production.

A potential mechanism for the induction of cleistothecia of powdery mildews is host senescence (71) or drought stress that also occurs at the end of the growing season. Inducing a drought stress response or senescence stress response in the host vines was not achieved in the field trial, and despite girdling and ABA treatments, cleistothecia developed at the same time in all plots including non-treated control plots. This may be due to the recovery of the host to girdling treatments, which was observed within 30 days after treatments were made. The high vigor of grapevine plants at the research vineyard may also have allowed the vine to cope with damaged xylem tissue from the girdling treatment. Host senescence was not observed the ABA treatments, which may be due to the inhibition of ABA by auxin production prior to the end of the growing season (39, 127). While host senescence or drought stress have not been implicated in this study, host senescence or other host stresses may still be a factor in the initiation of cleistothecia.

The effect of temperature and photoperiod shift was assessed on seedlings to limit host physiological differences between field and growth chamber plants, but cleistothecia were observed within 3 weeks of inoculation regardless of treatment (Fig. 6.3 and 7.4). These results are similar to those found by Gadoury et al. (52) using tissue culture or detached leaf assays, which may be due to the rapid abscission

and death of inoculated lower leaves of the potted vines at the start of the growth chamber study. This could also be due to physiological differences in seedlings and mature vines associated with ontogenic resistance (47, 48, 56, 61), hormone regulation (11, 39, 127), or carbohydrate source or sink relationships (97). Plant stresses, such as tissue age, temperature stress, water stress, or nutrient stress, have also been correlated with hormone regulation and carbohydrate metabolism (65, 110), and potted seedlings may be affected different stresses than established grapevines in the field. The use of 10 cm pots may have caused grapevine roots to become restricted and caused stress to the plant that would not be observed in plants without restricted roots, which has been observed in other plant systems resulting in decreased plant growth and accumulation of abscisic acid (31, 81). Several plants in this study also died as a result of *Cylindrocarpon* root rot, and may have expressed physiological differences from healthy vines (87).

There was no significant effect of temperature or photoperiod shift on the initiation of cleistothecia; however, temperature and photoperiod affected the number of cleistothecia produced (Fig. 6.3 and 7.4). Cleistothecia were produced between 10 and 25 ºC with the greatest number of cleistothecia produced at 15 and 20 ºC, which is consistent with previous report of cleistothecia development (52, 86). Cleistothecia were produced in all photoperiod shift regimes with the most cleistothecia produced from short to long photoperiods. Short photoperiods induce dormancy responses in grapevine, and longer photoperiod in grapevine maintains growth and inhibits periderm development (46). The transition from short to long days may have allowed

continued to growth of the grapevine after the onset of cleistothecia development that may have supported further production of cleistothecia.

Proximity of opposite mating types and environmental conditions, such as temperature and shifts in photoperiod length, were not correlated to the initiation of cleistothecia, suggesting that other factors influence the late development of cleistothecia. Host chemical signaling, either such as host senescence hormone signaling or hormonal shifts, may provide an explanation for the late development of grape powdery mildew. Induction of rose powdery mildew (*Podosphaera pannosa*, syn. *Sphaerotheca pannosa*) has been previously hypothesized to be a result of hormonal shifts due to the development of cleistothecia on fruit or prickles of rose (60). Assessment of hormonal influence on *E. necator* cleistothecia initiation, however, may be difficult to assess on grapevine due to the inability to grow the fungus on artificial media and exclude other host processes that may influence fungal growth and development. Carbohydrate reallocation may also influence cleistothecia initiation. Carbohydrate resources have been previously implicated in the severity of *E. necator* infection (97), and reallocation of resources from leaf tissue to other portions of the plant may influence the production of cleistothecia. During berry ripening, carbohydrates rapidly increase in fruit (73), and carbohydrate reallocation from leaves may not occur in established vines until véraison (BBCH 83) in the Willamette Valley of Oregon due to high vigor of vines. In regions with greater stress on vines due to environmental conditions, carbohydrates may shift in response to stress conditions (119), which may cause cleistothecia to develop sooner than what is observed in more temperate environments. Further investigation of the host-derived

factors influencing cleistothecia initiation is required to better predict development of cleistothecia across various regions in differing environmental conditions.

Table 6.1. Percentage of *Erysiphe necator* lesions (70.9 mm^2) collected from the upper (n = 180), middle (n = 180), and lower portion (n = 180) of the vineyard canopy at three different sampling points (early, middle, and late) of three growing seasons (2013, 2014, and 2015).

		Sample Date	Lower Canopy [a]			Middle Canopy			Upper Canopy		
			Mat 1-1	Mat 1-2	Both Mat	Mat 1-1	Mat 1-2	Both Mat	Mat 1-1	Mat 1-2	Both Mat
2013[b]	Early	6/21/13	10	5	85	9	6	85	8	0	20
	Middle	8/8/13	15	17	68	29	11	60	23	23	53
	Late	9/4/13	1	70	29	1	49	50	8	41	51
2014	Early	7/1/14	14	56	31	20	54	25	33	18	49
	Middle	7/30/14	3	45	52	0	56	44	2	62	36
	Late	9/1/14	10	60	30	7	49	44	8	52	40
2015	Early	6/18/15	20	20	60	25	23	52	29	18	53
	Middle	7/15/15	12	65	23	23	48	29	13	68	20
	Late	8/12/15	21	46	32	14	49	37	8	66	25

[a] There was no difference in mating type proportions in collections from different canopy levels ($P > 0.1$)

[b] Mat 1-2 was significantly greater in representation of isolates collected in 2013 ($P = 0.04$) compared to Mat 1-1 samples; however, both Mat 1-1 and Mat 1-2 were found in single samples (70.9 mm^2) collected for all sampling dates.

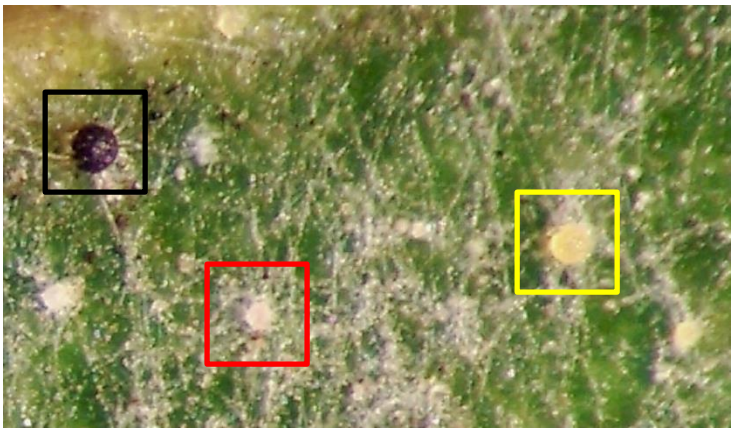

Figure 6.1. Cleistothecia formed adjacent to the midvein of a *Vitis vinifera* leaf heavily infested with *Erisyphe necator*. Three different cleistothecia age classes were described: primordia (red box), immature (yellow box), and mature cleistothecia (black box).

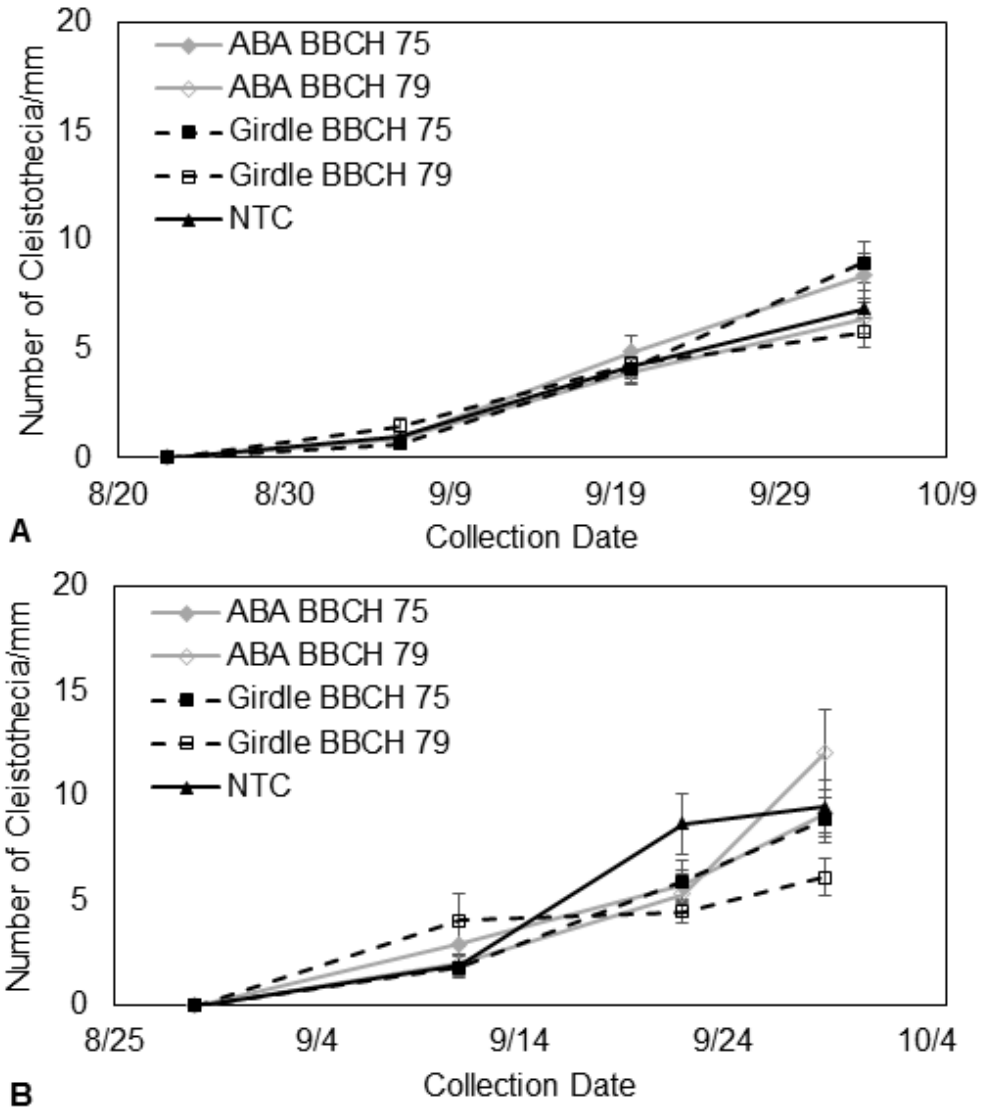

Figure 6.2 Cleistothecia production curves observed in 2013 (A) and 2014 (B). Host senescence induction treatments with abscisic acid (ABA, gray solid lines) and girdling treatments (black dashed lines) applied at two time points (BBCH 75, solid marker; BBCH 79, outlined marker) showed no significant differences in the development of cleistothecia.

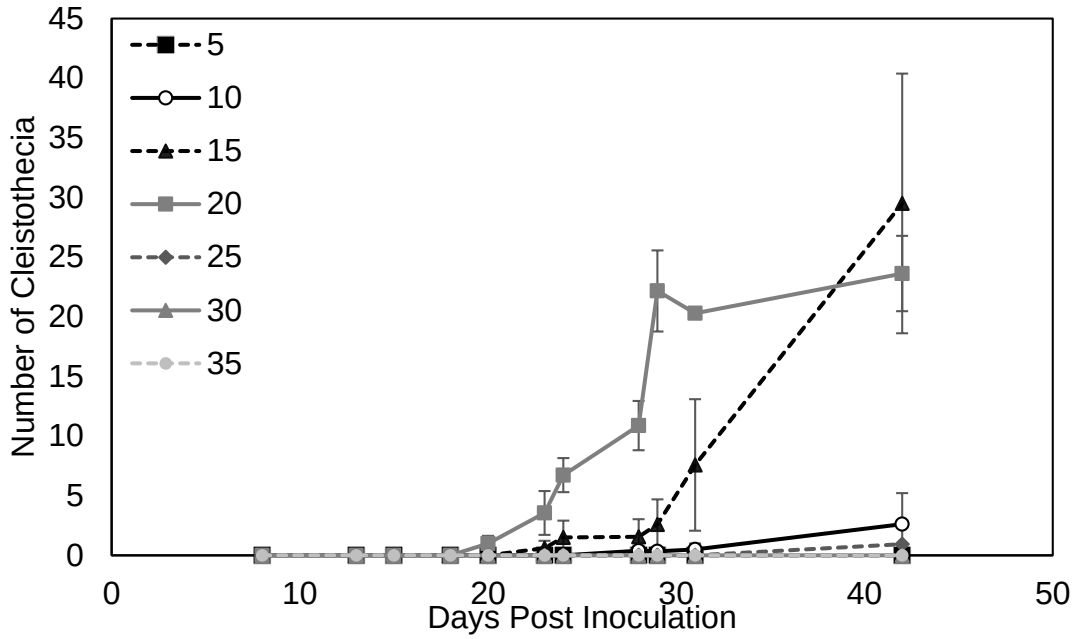

Figure 6.3. Cleistothecia initiation and production on Chardonnay grapevine seedlings inoculated with Mat 1-1 and Mat 1-2 of *Erysiphe necator* and incubated in growth chambers under different constant temperature treatments (5 - 35 °C) at 60 % relative humidity and 16 hour day lengths. No cleistothecia were produced at 5, 30, or 35 °C. The area under cleistothecia progress curves (AUCPC) of the 10 °C temperature treatment was 19.08 ± 19.08, the 15 °C was 153.22 ± 107.78, the 20 °C was 289.93 ± 5.19, and the 25 °C was 5.19 ± 5.19. The AUCPC at 20 °C was significantly different than 5, 10, 25, 30, and 35 °C ($P = 0.05$), but not significantly different from 15 °C ($P = 0.31$)

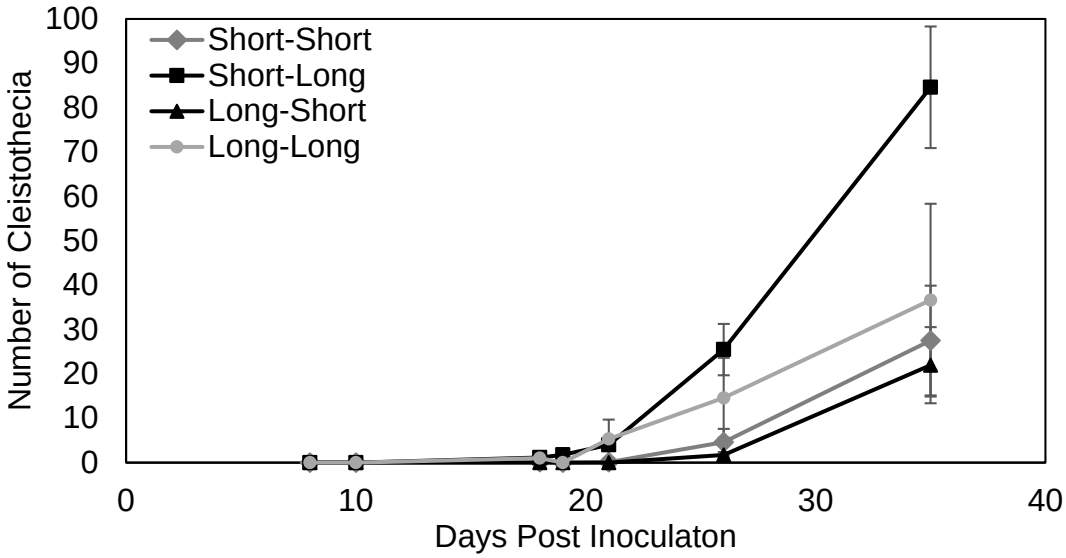

Figure 6.4. Cleistothecia initiation and production on Chardonnay grapevine seedlings inoculated with Mat 1-1 and Mat 1-2 of *Erysiphe necator* and incubated in growth chambers at 60 % relative humidity and 20 ºC under different photoperiod change treatments: short day (8 h light/16 h dark) to short day, short day to long day (16 h light/8 h dark), long day to short day, and long day to long day. The area under cleistothecia production curve (AUCPC) of the short day to short day photoperiod treatment was 157.28 ± 51.40, the short to long day treatment was 577.08 ± 56.68, the long to short day treatment was 110.81 ± 41.67, and the long to long day treatment was 283.78 ± 176.80. The AUCPC of the short to long day and long to short day photoperiod treatments were significantly different ($P = 0.02$), and no significant differences between other photoperiods treatments were observed ($P > 0.05$).

7. Interruption and reduction of *Erysiphe necator* cleistothecia development utilizing fungicidal oil

7.1 Introduction

The disease cycle of grape powdery mildew, caused by *Erysiphe necator*, begins by the release of ascospores from overwintering cleistothecia (syn. chasmothecia (13)) that germinate on susceptible leaf tissue. The resulting colonies produced by ascosporic infections produce asexual spores, conidia, which spread rapidly and cause exponential growth of disease epidemic during the grapevine growing season (54). Management of this pathogen is focused on delaying the exponential increase of conidia production during the grape growing season (90) with fungicide applications initiated with the presence of susceptible tissue to prevent ascosporic infections. Fungicide applications cease at the development of grape cluster ontogenic resistance at véraison (BBCH 81). Cessation of fungicide applications at véraison, however, allows for exponential growth of the pathogen and significant production of overwintering inoculum in the form of cleistothecia.

Cleistothecia serve as the primary overwintering structure and source of initial inoculum for *E. necator* in most regions where grape is grown (37, 59, 113). The formation of *E. necator* cleistothecia requires both Mat 1-1 and Mat 1-2 isolates for sexual reproduction (55). External environmental factors for initiation of cleistothecia production have not been demonstrated (Chapter 6); however, proximity to opposite mating type is thought to be the main driving factor in cleistothecia development (52). Within the Willamette Valley of Oregon, cleistothecia are only formed at the end of

the growing season (BBCH 89-91) (Chapter 6), despite the presence of both mating types throughout the vineyard and within close proximity early in the growing season. Because cleistothecia formation occurs near harvest, it may be possible to interrupt cleistothecia production after harvest to reduce cleistothecia production and potential primary inoculum for the following growing season without damaging fruit at harvest.

Reduced ascosporic inoculum through eradication of overwintering cleistothecia have been shown to reduce ascosporic infections (58); however, dormant lime sulfur applications may be cost-prohibitive for incorporation into a vineyard management program due to the large quantities of lime sulfur solution needed to drench grapevine trunks. The use of horticultural oils at the end of the growing season, post-harvest, to interrupt *E. necator* reproduction may reduce cleistothecia production prior to deposition within the bark. Horticultural oils act as a curative by smothering the mildew lesions (106), and may be incorporated into organic or conventional management programs. By interrupting cleistothecia development at the end of the growing season, fewer cleistothecia may be available to overwinter within the bark and subsequently release early season inoculum, which may also reduce early season fungicide applications.

The purpose of this study was to test the efficacy and use of applying a curative fungicide, stylet oil, on interrupting cleistothecia development. The specific objectives of this study were to: 1) determine if cleistothecia development may be interrupted by applying fungicidal stylet oil, and 2) determine the optimal application time of stylet oil to reduce the number of cleistothecia produced.

7.2 Materials and Methods

7.2.1 Stylet Oil Application

A research vineyard (Corvallis, OR) containing bilateral vertical shoot-positioning, cane-pruned CH-76 Chardonnay vines on 101-14 rootstock was used to test fungicidal oil application and timing to potentially interrupt and reduce cleistothecia production. Each plot consisted of 5 Chardonnay vines (5 x 6 spacing) with 101-14 rootstock vines separating plots. Throughout the growing season, vines were pruned to manage vigor, but no external irrigation or fertilizer was applied to the vines. Sulfur fungicide applications were utilized minimally (3 to 4 times within the growing season prior to véraison) to allow *E. necator* to be found throughout the vineyard at 100% incidence, but prevent significant damage to the vines.

Cleistothecia primordia were observed on leaf tissue at BBCH 85, on 8/28/14 and 7/28/15, of each growing season. At the onset of cleistothecia production, vines were treated once with Organic JMS Stylet Oil (Vero Beach, FL) at a rate of 10 ml/L. Stylet oil applications were made using an air-assisted backpack sprayer and applied to run-off. In separate field plots, stylet oil application times were separated by one week, unless weather conditions prevented fungicide applications, to determine an optimum application time (Table 7.1). Non-treated control plots were included to determine baseline production of cleistothecia without fungicidal intervention. Treatments were replicated 3 times and blocked across grape vigor differences to reduce the effect of vine vigor on cleistothecia production.

7.2.2 Cleistothecia Enumeration

Because stylet oil applications were made to leaf tissues and the difficulty of consistently enumerating cleistothecia from bark (70), cleistothecia production on leaves was used to examine fungicide application differences. Production was enumerated from 10 leaves in each treatment plot. Leaves were collected approximately 1.4 meters from the ground in the region of high density of lateral shoot development, selecting leaves with nearly 100% area covered by powdery mildew to ensure cleistothecia were likely to be present. Leaves were stored at -20°C until cleistothecia could be enumerated. Leaf collections began as soon as cleistothecia primordia were observed on the leaves and weekly thereafter until the onset of heavy rains in October of each year.

Cleistothecia were counted along a transect that was adjacent to the mid-vein of each leaf, measured using a digital caliper. Cleistothecia were additionally classified into an age class: primordia, immature, and mature cleistothecia (Fig. 7.1). Cleistothecia were counted as primordia if they formed small spheres without lipid accumulation. Cleistothecia were counted as immature if they had begun to accumulate lipids, appearing yellow, and had not yet turned dark brown. Cleistothecia were classified at mature if they were dark brown-black. Cleistothecia quantities per cm were compared using a GLM procedure in R version 3.1.1 to determine the effect of stylet oil application and application timing.

7.3 Results

Cleistothecia were produced at the end of the grape growing season (BBCH 85) in both years of testing. There was no significant difference between the number of primordia, immature, or mature cleistothecia produced for each application time, and these were combined for all subsequent analyses. There was a significant difference in cleistothecia produced between 2014 and 2015, so the two years were analyzed separately ($P < 0.001$) (Fig. 7.1).

Applying stylet oil significantly reduced the total number of cleistothecia produced in 2014 ($P = 0.04$) (Fig. 7.1A), but not in 2015 ($P = 0.06$) (Fig. 7.1B). There was no effect of stylet oil application timing on the number of cleistothecia produced in any plot in either 2014 ($P = 0.47$) or 2015 ($P = 0.57$). Date of leaf collection was significant in both 2014 ($P = 0.03$) or 2015 ($P = 0.01$), and cleistothecia production increased in all plots.

7.4 Discussion

Cleistothecia production was significantly reduced when an application of stylet oil was made at the end of the growing season in 2014 ($P = 0.04$); however, cleistothecia were not significantly reduced in the 2015 replication of this trial ($P = 0.06$). This result suggests that applying stylet oil may not sufficiently interrupt the production of cleistothecia at the end of the growing season to reduce disease during the following epidemic. Cleistothecia also continued to be produced in all treatment plots, regardless of the application of stylet oil, which may indicate a limited economic return in commercial vineyards. The cost of diesel and labor limits the

number of fungicide applications that may be made at the end of the growing season if fungicide applications during the growing season are not equally reduced, which has also been described in other studies examining the removal of overwintering inoculum (52, 58, 123). Additionally, the continued production of cleistothecia after stylet oil applications may also indicate that the stylet oil may not have provided adequate curative activity, either due to poor coverage or limited curative ability of the stylet oil. While stylet oil is considered to have curative activity of grape powdery mildew, it has limited preventative activity for new infections (106). This may allow for proliferation and production of cleistothecia post stylet oil applications. Nearly complete eradication of cleistothecia would be required to improve management in the following growing season without being economically disadvantageous.

Furthermore, significantly more cleistothecia were produced in 2014 than in 2015 ($P < 0.001$), which may have allowed for greater resolution of treatment differences. The environmental conditions in 2015 were warmer and drier than in 2014; the average precipitation in 2014 was 930 mm and 859 mm in 2015, and 8.1 degree days above 30 ºC during cleistothecia production in 2014 and 19.5 degree days in 2015. Higher temperatures appear to inhibit cleistothecia production (52, 86) and reduce the quantity of cleistothecia production (Chapter 6), which may have contributed to the decreased production of cleistothecia in 2015. Water stress associated with 2015 may also have impacted the grapevine causing a stress response, upregulating abscisic acid, affecting carbohydrate production, and reducing new tissue production (11, 14, 68), consequently impacting the development of cleistothecia (Chapter 6). Assessing stylet oil use in the vineyard in a year with cooler

end-of-growing-season temperatures may show similar results to cleistothecia reduction as 2014.

In addition to incomplete eradication of cleistothecia, end-of-season fungicides would be difficult to apply in regions where harvest coincides with the onset of cleistothecia production. Fungicides cannot be applied during harvest in hand-harvested vineyards due to labor constraints, and cleistothecia may be produced abundantly from the initiation of cleistothecia, with mature cleistothecia being produced 25 days after inoculation at 20 ºC in controlled experiments (52). The delay of applying stylet oil during the period of cleistothecia formation may allow for a large accumulation of inoculum that would not be eradicated with a late application of stylet oil. No difference was observed in fungicide application time in either 2014 ($P = 0.47$) or 2015 ($P = 0.57$). For the Willamette Valley of Oregon, fungicide applications would not be possible outside the time period used in this study due to narrow time window between harvest and the onset of heavy precipitation. In regions that continue to experience dry conditions post-harvest, it may be beneficial to determine an ideal time period prior to leaf drop for curative applications to rejuvenate abandoned or ill-managed vineyards. However, more arid regions may find greater benefit in utilizing strategies that reduce inoculum from the bark, such as applying a water drench prior to bud break (BBCH 08) to release inoculum (Gubler, personal communication), or monitoring the disease epidemic via disease prediction or risk assessment models.

Table 7.1. Application dates of Organic JMS Stylet Oil for each timing treatment in both 2014 and 2015 field trials. Cleistothecia were observed on 08/28/14 in 2014 and 8/26/15 in 2015.

Application Timing Treatment	2014 Application Date	2015 Application Date
0	Non-treated Control	Non-treated Control
1	9/15/14	8/31/15
2	9/22/14	9/8/15
3	10/3/14	9/15/15
4	10/14/14	9/22/15

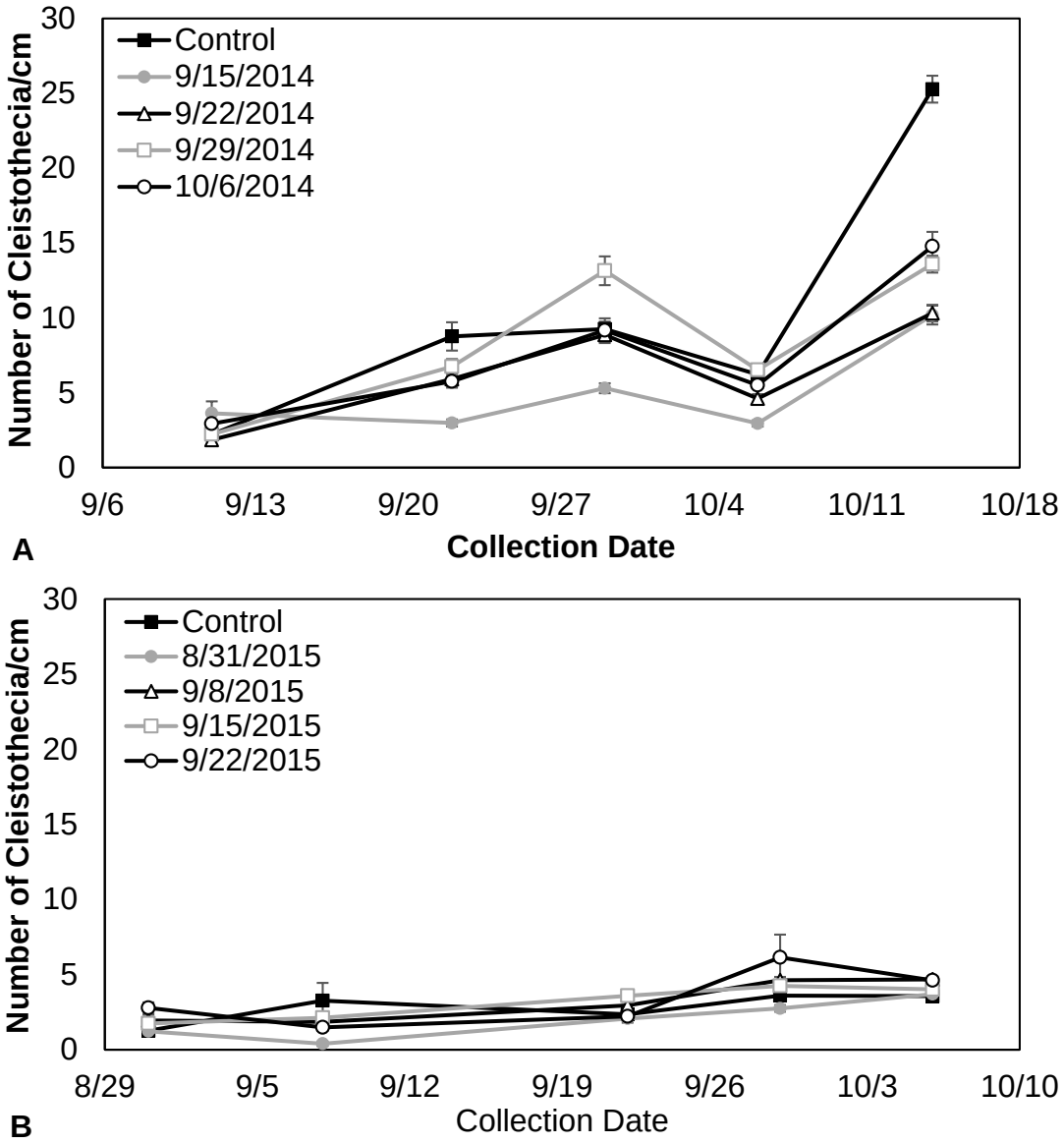

Figure 7.1. Total number of cleistothecia enumerated in both (A) 2014 and (B) 2015 along leaf transects collected during application timing treatments. Fungicide treatments were compared by subsampling 10 leaves from each treatment plot, and enumerating the number of cleistothecia along a mid-vein transect.

8. Conclusion

This research provides management tools and an improved understanding of inoculum overwintering of *Erysiphe necator* and availability in response to the increasing calls to reduce fungicide use in wine grape production while maintaining disease control. Inoculum detection was used to inform ascospore release model development and provide information on airborne inoculum concentration to time fungicide applications. Additionally, this research showed that cleistothecia initiation is affected by factors other than proximity to mating type, and the development of cleistothecia may be interrupted with the application of stylet oil.

A previous approach on ascospore release indicated that ≥ 2.5 mm of precipitation and average temperature above 4 ºC within 24 h period induced ascospore release of *E. necator* (53). However, the research conducted in Oregon showed that in addition to temperature, cumulative wetness duration above 6 hours during temperatures above 4 ºC with daily average relative humidity ≥80% and cumulative precipitation ≥2.5 mm improved accuracy of predictions (Chapter 2). Ascospore release models developed in other regions showed low correlation between predicted ascospore release events based on the environmental conditions of maritime Willamette Valley compared to observed ascospore release. Additionally, ascospore release magnitude appears to not be a function of environmental conditions, and could not be predicted using multiple linear regression methods. The false positives observed using binary ascospore release models and the inability to predict the

magnitude of ascospore release may be associated with the period of cleistothecia initiation and rate of maturation during overwintering.

This research attempted to determine the factors surrounding cleistothecia initiation (Chapter 6). Both mating types were found within a vineyard block and within a 70.9 mm^2 sampling area throughout the growing season with cleistothecia formation not observed until the end of the growing season (BBCH 85). These results suggest that other factors are important in the initiation of cleistothecia. This study did not show an effect of host-related factors, such as senescence or drought stress, or environmental factors, such as temperature or photoperiod, associated with the initiation of cleistothecia primordia. Environmental factors do, however, appear to affect the number of cleistothecia produced, with the greatest quantity of cleistothecia produced at 15 and 20 ºC compared to other temperatures ($P = 0.05$), with no cleistothecia produced above 25 ºC (Chapter 6). The most cleistothecia were also produced at photoperiod shifts from short to long day lengths compared to other photoperiod shift regimes ($P = 0.02$) (Chapter 6). Shaded leaves show lower surface temperatures than exposed leaf surfaces, and disease is more severe in shaded leaves (3). Photoperiod also decreases at the end of the growing season, which may reduce solar radiation of leaf surfaces and decreasing leaf surface temperature at the end of the growing season. At the end of the growing season, temperatures decrease below 25 ºC; however, cleistothecia are not found in exposed or shaded areas of the canopy prior to BBCH 85. It was not possible to determine why environmental factors influenced the number of cleistothecia produced and not the initiation of cleistothecia in this study of cleistothecia development. Further investigation of factors influencing

the induction of cleistothecia is needed to predict initiation and magnitude of cleistothecia production in vineyards, which may influence the magnitude of ascospore release.

While specific factors for the development of cleistothecia could not be determined, cleistothecia formed consistently at the end of the growing season, and a method to interrupt the production of cleistothecia prior to leaf drop was assessed (Chapter 7). Cleistothecia were significantly reduced when stylet oil was applied compared to not applying stylet oil in 2014 ($P = 0.04$); however stylet oil did not reduce cleistothecia significantly at $\alpha_{0.05}$ in 2015 ($P = 0.06$). Because cleistothecia were not completely eradicated or significantly delayed in their maturation, there appears to be limited utility of this method for established vineyard management.

Inoculum detection methods were shown to be useful as a decision aid to time fungicide applications and maintain a high level of disease control without understanding all of the factors influencing early inoculum release. In addition, the turbidimetric, grower-conducted loop-mediated isothermal amplification (LAMP) detection results were not significantly different from qPCR and lab-conducted LAMP assays. This research showed that initiating fungicide applications based on detection of airborne inoculum saved approximately 3 fungicides for commercial vineyards (Chapter 3). Further optimization of fungicide application intervals was achieved by using quantitative PCR to monitor inoculum concentrations during the growing season. Approximately 2 fungicide applications were saved by commercial vineyard managers and the research vineyard increased 3 fungicide applications by adjusting fungicide intervals based on a 10 spore concentration threshold, with

improved disease control in both commercial and research vineyards (Chapter 5). A quantitative LAMP (qLAMP) procedure was developed for use by growers to conduct in-field inoculum concentration monitoring; however, accurate quantification was not achieved when performed by growers (Chapter 4).

While a grower-conducted assay was developed for inoculum detection within commercial vineyards, access to LAMP assay materials and an *E. necator*-free workspace to generate master mix limits the ability of small vineyard operations to conduct in-house inoculum detection. Inoculum detection as a commercial service for monitoring airborne *E. necator* inoculum may allow for more vineyards to utilize inoculum detection. Several limitations of inoculum detection methods may limit the utility of spore sampling in commercial disease management practices. The representation of the spore sampler within the vineyard is presently unknown; however, work to understand the movement of airborne inoculum within complex, trellised canopies (9, 92, 99) may help describe the flow of particles with relation to the spore sampler. Widespread commercial implementation of inoculum detection appears is also a limitation of using inoculum detection to time fungicide applications, but this technology has been incorporated by several commercial consultant companies in California and Oregon.

Other than proximity to opposite mating type, the factors influencing the initiation of cleistothecia primordia have still not been determined. The initiation of cleistothecia production may affect the level of maturation of cleistothecia, and subsequently affect the magnitude of ascospore release during conducive conditions. Future assessment of host responses, including host growth regulating signals or

carbohydrate source-sink shifts, on cleistothecia initiation is still needed to determine their influence on cleistothecia initiation. Immature cleistothecia have previously been shown to not release ascospore naturally, and ascospores are less capable of causing infection when ascospores are forcibly discharged from immature cleistothecia (52). The level of cleistothecia maturation, which has been previously assessed by visual inspection of ascocarps, may also impact ascospore release and following disease epidemic. Measuring the level of glycogen utilization and lipid utilization may allow for more accurate assessments of cleistothecia maturity to improve ascospore release and disease prediction models. An improved ascospore release prediction model was developed, and may be used in conjunction with ascospore germination conditions to predict early disease within vineyards. Previous investigations of ascospore germination have been conducted under controlled conditions (54, 75), but conditions in controlled experiments may not accurately represent ascospore germination in field conditions. Further investigation of ascospore germination under field conditions is necessary to determine the conditions for lesion development after ascospore release has occurred.

Bibliography

1. Abd-Elsalam, K., Bahkali, A., and Moslem, M. 2011. An Optimized Protocol for DNA Extraction from Wheat Seeds and Loop-Mediated Isothermal Amplification (LAMP) to Detect *Fusarium graminearum* Contamination of Wheat Grain. International Journal of Molecular Sciences 12:3459-3472
2. Aust, H., and Hoyningen-Huene, J. V. 1986. Microclimate in relation to epidemics of powdery mildew. Annual Review of Phytopathology 24:491-510.
3. Austin, C. N., and Wilcox, W. F. 2012. Effects of sunlight exposure on grapevine powdery mildew development. Phytopathology 102:857-866.
4. Aylor, D. E. 1995. Vertical Variation of Aerial Concentration of *Venturia inaequalis*. Phytopathology 85:175-181.
5. Backup, P. 2009. Effects of high temperature on grape powdery mildew (*Erysiphe necator*). University of California, Davis.
6. Bailey, B., and Stoll, R. 2013. Turbulence in Sparse, Organized Vegetative Canopies: A Large-Eddy Simulation Study. Boundary-Layer Meteorology 147:369-400.
7. Bailey, B., Stoll, R., Pardyjak, E., Halverson, S., and Willemsen, P. 2012. Development of a Ray-Tracing Based Canopy Radiation Model. Pages Session 2.6. in: 30th Conference on Agricultural and Forest Meteorology/ 1st Conference on Atmospheric Biogeosciences. Boston, MA.
8. Bailey, B., Overby, M., Willemsen, P., Pardyjak, E., Mahaffee, W., and Stoll, R. 2014. A scalable plant-resolving radiative transfer model based on optimized GPU ray tracing. Agricultural and Forest Meteorology 198:192-208.
9. Bailey, B. N., Stoll, R., Pardyjak, E. R., and Mahaffee, W. F. 2014. Effect of vegetative canopy architecture on vertical transport of massless particles. Atmospheric Environment 95:480-489.
10. Boehme, C. C., Nabeta, P., Henostroza, G., Raqib, R., Rahim, Z., Gerhardt, M., Sanga, E., Hoelscher, M., Notomi, T., Hase, T., and Perkins, M. D. 2007. Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. Journal of Clinical Microbiology 45:1936-40.
11. Boneh, U., Biton, I., Schwartz, A., and Ben-Ari, G. 2012. Characterization of the ABA signal transduction pathway in *Vitis vinifera*. Plant Science 187:89-96.
12. Bowles, D. J. 1990. Defense-related proteins in higher plants. Annual review of biochemistry 59(1):873-907.
13. Braun, U., Cook, R. T. A., Inman, A. J., and Shin, H. D. 2002. The taxonomy of the powdery mildew fungi. Pages 13–55. in: The Powdery Mildews: A Comprehensive Treatise B. R. R., B. W. R., D. A. J., and C. T. L. W., eds. APS Press, St Paul, MN, USA.
14. Bray, E. A. 1997. Plant responses to water deficit. Trends in Plant Science 2:48-54.

15. Brewer, M. T., and Milgroom, M. G. 2010. Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator,* from diverse *Vitis species.* BMC Evolutionary Biology 10:268.
16. Brewer, M. T., Cadle-Davidson, L., Cortesi, P., Spanu, P. D., and Milgroom, M. G. 2011. Identification and structure of the mating-type locus and development of PCR-based markers for mating type in powdery mildew fungi. Fungal Genetics and Biology 48:704-713.
17. Bulit, J., and Lafon, R. 1978. Powdery Mildew of the Vine. in: The Powdery Mildews D. M. Spencer, ed. Academic Press Inc.
18. Byford, W., and Bentley, K. 1976. Cleistothecia of *Erysiphe betae* in England. Transactions of the British Mycological Society 67:544-545.
19. Caffi, T., Rossi, V., Legler, S. E., and Bugiani, R. 2011. A mechanistic model simulating ascosporic infections by *Erysiphe necator*, the powdery mildew fungus of grapevine. Plant Pathology 60:522-531.
20. Caffi, T., Legler, S. E., Rossi, V., and Bugiani, R. 2012. Evaluation of a Warning System for Early-Season Control of Grapevine Powdery Mildew. Plant Disease 96:104-110.
21. Caffi, T., Legler, S. E., Bugiani, R., and Rossi, V. 2013. Combining sanitation and disease modelling for control of grapevine powdery mildew. European Journal of Plant Pathology 135:817-829.
22. Calderon, C., Ward, E., Freeman, J., Foster, S. J., and McCartney, H. a. 2002. Detection of airborne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape crops by polymerase chain reaction (PCR) assays. Plant Pathology 51:303-310 doi:10.1046/j.1365-3059.2002.00721.x.
23. Calonnec, A., Cartolaro, P., and Chadoeuf, J. 2009. Highlighting features of spatiotemporal spread of powdery mildew epidemics in the vineyard using statistical modeling on field experimental data. Phytopathology 99:493-508.
24. Calonnec, A., Cartolaro, P., Poupot, C., Dubourdieu, D., and Darriet, P. 2004. Effects of *Uncinula necator* on the yield and quality of grapes (*Vitis vinifera*) and wine. Plant Pathology 53:434-445.
25. Calonnec, A., Cartolaro, P., Naulin, J. M., Bailey, D., and Langlais, M. 2008. A host-pathogen simulation model: powdery mildew of grapevine. Plant Pathology 57:493-508.
26. Campbell, C. L., and Madden, L. V. 1990. Introduction to Plant Disease Epidemiology. Wiley-Interscience, NY.
27. Cardoso, T. C., Ferrari, H. F., Bregano, L. C., Silva-Frade, C., Rosa, A. C. G., and Andrade, A. L. 2010. Visual detection of turkey coronavirus RNA in tissues and feces by reverse-transcription loop-mediated isothermal amplification (RT-LAMP) with hydroxynaphthol blue dye. Molecular and cellular probes 24:415-417.
28. Carisse, O., Bacon, R., and Lefebvre, A. 2009. Grape powdery mildew (*Erysiphe necator*) risk assessment based on airborne conidium concentration. Crop Protection 28:1036-1044.
29. Carisse, O., Bacon, R., Lefebvre, A., and Lessard, K. 2009. A degree-day model to initiate fungicide spray programs for management of grape powdery mildew (*Erysiphe necator*). Canadian Journal of Plant Pathology 31:186-194.

30. Carisse, O., Tremblay, D. M., Lévesque, C. a., Gindro, K., Ward, P., and Houde, a. 2009. Development of a TaqMan real-time PCR assay for quantification of airborne conidia of *Botrytis squamosa* and management of botrytis leaf blight of onion. Phytopathology 99:1273-80.
31. Carmi, A. 1995. Growth, water transport and transpiration in root-restricted plants of bean, and their relation to abscisic acid accumulation. Plant Science 107:69-76.
32. Chakraborty, S., and Newton, A. C. 2011. Climate change, plant diseases and food security: an overview. Plant Pathology 60:2-14.
33. Chakraborty, S., Tiedemann, a. V., and Teng, P. S. 2000. Climate change: potential impact on plant diseases. Environmental Pollution 108:317-26.
34. Chakraborty, S., Luck, J., Hollaway, G., Freeman, A., Norton, R., Garrett, K. A., Percy, K., Hopkins, A., Davis, C., and Karnosky, D. F. 2008. Impacts of Global Change on Diseases of Agricultural Crops and Forest Trees. CAB Reviews 3:1-5.
35. Chellemi, D. O., and Marois, J. J. 1991. Development of a demographic growth model for *Uncinula necator* by using a microcomputer spreadsheet program. Phytopathology 81:250-254.
36. Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Kaczmarski, E. B., and Fox, A. J. 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. Journal of clinical microbiology 38:1747-1752.
37. Cortesi, P., Bisiach, M., Ricciolini, M., and Gadoury, D. M. 1997. Cleistothecia of *Uncinula necator*-an additional source of inoculum in Italian vineyards. Plant Disease 81:922-926.
38. Cortesi, P., Bisiach, M., Ricciolini, M., and Gadoury, D. M. 1997. Cleistothecia of *Uncinula necator*--an additional source of inoculum in Italian vineyards. Plant Disease 81:922-926.
39. Davies, C., Boss, P. K., and Robinson, S. P. 1997. Treatment of grape berries, a nonclimacteric fruit with a synthetic auxin, retards ripening and alters the expression of developmentally regulated genes. Plant Physiology 115:1155-1161.
40. Delp, J. 1954. Effect of temperature and humidity on the grape powdery mildew fungus. Phytopathology 44:615-626.
41. Doster, M. A., and Schnathorst, W. C. 1985. Comparative Susceptibility of Various Grapevine Cultivars to the Powdery Mildew Fungus *Uncinula necator*. American Journal of Enology and Viticulture 36:101-104.
42. Dukes, J. P., King, D. P., and Alexandersen, S. 2006. Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Archives of virology 151:1093-1106.
43. Evans, K., Scott, E., and Whisson, D. 1997. Heterothallism among South Australian clonal lines of *Uncinula necator*. Australasian Plant Pathology 26:10-20.
44. Falacy, J. S., Grove, G. G., Mahaffee, W. F., Galloway, H., Glawe, D. a., Larsen, R. C., and Vandemark, G. J. 2007. Detection of *Erysiphe necator* in Air Samples Using the Polymerase Chain Reaction and Species-Specific Primers. Phytopathology 97:1290-1297.

45. Fawcett, T. 2006. An introduction to ROC analysis. Pattern Recognition Letters 27:861-874.
46. Fennell, A., and Hoover, E. 1991. Photoperiod influences growth, bud dormancy, and cold acclimation in *Vitis labruscana* and *V. riparia*. Journal of the American Society for Horticultural Science 116:270-273.
47. Ficke, A., Gadoury, D. M., and Seem, R. C. 2002. Ontogenic resistance and plant disease management: A case study of grape powdery mildew. Phytopathology 92:671-675.
48. Ficke, A., Gadoury, D. M., Seem, R. C., and Dry, I. B. 2003. Effects of Ontogenic Resistance upon Establishment and Growth of *Uncinula necator* on Grape Berries. Phytopathology 93:556-63.
49. Fischbach, J., Xander, N. C., Frohme, M., and Glökler, J. F. 2015. Shining a light on LAMP assays— A comparison of LAMP visualization methods including the novel use of berberine. BioTechniques 58:189-194.
50. Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., and Gurr, S. J. 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186-194.
51. Francois, P., Tangomo, M., Hibbs, J., Bonetti, E.-J., Boehme, C. C., Notomi, T., Perkins, M. D., and Schrenzel, J. 2011. Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. FEMS Immunology & Medical Microbiology 62:41-48.
52. Gadoury, D. M., and Pearson, R. C. 1988. Initiation, development, dispersal and survival of cleistothecia of *Uncinula necator* in New York vineyards. Phytopathology 78:1413-1421.
53. Gadoury, D. M., and Pearson, R. C. 1990. Ascocarp Dehiscence and Ascospore Discharge in *Uncinula necator*. Phytopathology 80:393-401.
54. Gadoury, D. M., and Pearson, R. C. 1990. Germination of Ascospores and Infection of *Vitis* by *Uncinula necator*. Phytopathology 80:1198-1203.
55. Gadoury, D. M., and Pearson, R. C. 1991. Heterothallism and pathogenic specialization in *Uncinula necator*. Phytopathology 81:1287-1293.
56. Gadoury, D. M., Seem, R. C., Ficke, A., and Wilcox, W. F. 2003. Ontogenic resistance to powdery mildew in grape berries. Phytopathology 93:547-55.
57. Gadoury, D. M., Wakefield, L. M., Cadle-Davidson, L., Dry, I. B., and Seem, R. C. 2012. Effects of prior vegetative growth, inoculum density, light, and mating on conidiation of Erysiphe necator. Phytopathology 102:65-72.
58. Gadoury, D. M., Pearson, R. C., Riegel, D. G., Seem, R. C., Becker, C. M., and Pscheidt, J. W. 1994. Reduction of powdery mildew and other diseases by over-the-trellis applications of lime sulfur to dormant grapevines. Plant disease 78:83-87.
59. Gadoury, D. M., Cadle-Davidson, L., Wilcox, W. F., Dry, I. B., Seem, R. C., and Milgroom, M. G. 2012. Grapevine powdery mildew (*Erysiphe necator*): a fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. Molecular Plant Pathology 13:1-16.
60. Gallian, J. J. 1982. Effects of volatile fungicides on *Sphaerotheca pannosa* on rose, Botany and Plant Pathology, Oregon State University, Corvallis, OR.

61. Gee, C. T., Gadoury, D. M., and Cadle-Davidson, L. 2008. Ontogenic Resistance to *Uncinula necator* Varies by Genotype and Tissue Type in a Diverse Collection of *Vitis* spp. Plant Disease 92(7):1067-1073.
62. Gee, L. M., Stummer, B. E., Gadoury, D. M., Biggins, L. T., and Scott, E. S. 2000. Maturation of cleistothecia of *Uncinula necator* (powdery mildew) and release of ascospores in southern Australia. Australian Journal of Grape and Wine Research 6:13-20.
63. Gent, D. H., Nelson, M. E., Farnsworth, J. L., and Grove, G. G. 2009. PCR detection of *Pseudoperonospora humuli* in air samples from hop yards. Plant Pathology 58:1081-1091.
64. Gubler, W. D., Rademacher, M. R., Vasquez, S. J., and Thomas, C. S. 1999. Control of powdery mildew using the UC Davis powdery mildew risk index. APSnet Features.
65. Gupta, A. K., and Kaur, N. 2005. Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. Journal of Biosciences 30:761-776.
66. Hall, T. 2000. Epidemiology of grape powdery mildew, *Uncinula necator,* in the Willamette Valley, Botany and Plant Pathology, Oregon State University, Corvallis, OR.
67. Harper, S. J., Ward, L. I., and Clover, G. R. 2010. Development of LAMP and real-time PCR methods for the rapid detection of Xylella fastidiosa for quarantine and field applications. Phytopathology 100:1282-1288.
68. Henckel, P. 1964. Physiology of plants under drought. Annual Review of Plant Physiology 15:363-386.
69. Heyden, H. V. D., Lefebvre, M., Roberge, L., Brodeur, L., Recherche, C. D., Saint-Patrice, R., Carisse, O., Canada, A.-F., and Boulevard, G. 2014. Spatial pattern of strawberry powdery mildew (*Podosphaera aphanis*) and airborne inoculum. Plant Disease 98:43-54.
70. Hoffmann, P., Füzi, I., and Virányi, F. 2012. Indirect Effect of Fungicide Treatments on Chasmothecia of *Erysiphe necator* Schwein Overwintering on Grapevine Bark. Plant Protection Science 48:21-30.
71. Homma, Y. 1937. Erysiphaceae of Japan. Journal of the Faculty of Agriculture, Hokkaido University 38(3):183-461.
72. Hui, S. L., and Walter, S. D. 1980. Estimating the error rates of diagnostic tests. Biometrics 36:167-171.
73. Iland, P., Dry, P., Proffitt, T., and Tyerman, S. 2011. The grapevine. in: The Grapevine: from the science to the practice of growing vines for wine. Patrick Iland Wine Promotions Pty Ltd, Adelaide, South Australia.
74. Isard, S. A., and Gage, S. H. 2001. Flow of life in the atmosphere. MIchigan State University Press, East Lansing, MA.
75. Jailloux, F., Thind, T., and Clerjeau, M. 1998. Release, germination, and pathogenicity of ascospores of *Uncinula necator* under controlled conditions. Canadian Journal of Botany 76:777-781.
76. Jailloux, F., Willocquet, L., Chapuis, L., and Froidefond, G. 1999. Effect of weather factors on the release of ascospores of *Uncinula necator*, the cause of

grape powdery mildew, in the Bordeaux region. Canadian Journal of Botany 77:1044-1051.

77. Jarvis, W. R., Gubler, W. D., and Grove, G. G. 2002. Epidemiology of Powdery Mildews in Agricultural Pathosystems. in: The Powdery Mildews A Comprehensive Treatise. B. R.R. Belanger, W. R., Dik, A. J. Carver, T. L. W., ed. The American Phytopathological Society, St. Paul, Minnesota.
78. Jenkins, D. M., Kubota, R., Dong, J., Li, Y., and Higashiguchi, D. 2011. Handheld Device for Real-Time, Quantitative, LAMP-Based Detection of *Salmonella enterica* Using Assimilating Probes. Biosensors and Bioelectronics 30:255-260.
79. Kottek, M., Grieser, J., Beck, C., Rudolf, B., and Rubel, F. 2006. World map of the Köppen-Geiger climate classification updated. Meteorologische Zeitschrift 15:259-263.
80. Kremheller, H. T., and Diercks, R. 1983. Epidemiology and forecasting of hop downy mildew (*Pseudoperonospora humuli*). Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 90:599-616.
81. Krizek, D. T., Carmi, A., Mirecki, R. M., Snyder, F. W., and Bunce, J. A. 1985. Comparative effects of soil moisture stress and restricted root zone volume on morphogenetic and physiological responses of soybean [*Glycine max* (L.) Merr.]. Journal of experimental botany 36:25-38.
82. Kubota, R., Vine, B. G., Alvarez, a. M., and Jenkins, D. M. 2008. Detection of *Ralstonia solanacearum* by loop-mediated isothermal amplification. Phytopathology 98:1045-51.
83. Kubota, R., Alvarez, A. M., Su, W. W., and Jenkins, D. M. 2011. FRET-Based Assimilating Probe for Sequence-Specific Real-Time Monitoring of Loop-Mediated Isothermal Amplification (LAMP). Biological Engineering Transactions 4:81-100.
84. Lanza, S. T., Collins, L. M., Lemmon, D. R., and Schafer, J. L. 2007. PROC LCA: A SAS procedure for latent class analysis. Structural Equation Modeling 14:671-694.
85. Lanza, S. T., Dziak, J. J., Huang, L., Xu, S., and Collins, L. M. 2011. Proc LCA & Proc LTA Users' Guide (Version 1.2.6). The Methodology Center, Pennsylvania State University, University Park, PA. .
86. Legler, S. E., Caffi, T., and Rossi, V. 2014. A model for the development of *Erysiphe necator* chasmothecia in vineyards. Plant Pathology 63:911-921.
87. León, J., Rojo, E., and Sánchez-Serrano, J. J. 2001. Wound signalling in plants. Journal of Experimental Botany 52(354):1-9.
88. Luo, Y., Ma, Z., Reyes, H. C., Morgan, D., and Michailides, T. J. 2007. Quantification of airborne spores of *Monilinia fructicola* in stone fruit orchards of California using real-time PCR. European Journal of Plant Pathology 118:145-154.
89. Lybbert, T. J., and Gubler, W. D. 2008. California Wine Grape Growers' Use of Powdery Mildew Forecasts. in: ARE Update. University of California, Giannini Foundation of Agricultural Economics.
90. Madden, L. V., Hughes, G., and Van den Bosch, F. 2007. The study of plant disease epidemics. American Phytopathological Society St. Paul.

91. Mahaffee, W., Grove, G., and Stoll, R. 2011. Inoculum detection to manage grape powdery mildew. Practical Winery and Vineyard Journal Spring:26-32.
92. Mahaffee, W. F., and Stoll, R. 2016. The ebb and flow of airborne pathogens: monitoring and use in disease management decisions. Phytopathology (In Press).
93. Malvick, D., and Impullitti, A. 2007. Detection and quantification of *Phialophora gregata* in soybean and soil samples with a quantitative, real-time PCR assay. Plant disease 91:736-742.
94. Marx, V. 2015. PCR heads into the field. Nat Meth 12:393-397.
95. McCartney, H. A., Fitt, B. D. L., and West, J. S. 2006. Dispersal of foliar plant pathogens: mechanisms, gradients and spatial patterns. Pages 159-192. in: The Epidemiology of Plant Diseases B. M. Cooke, D. Gareth Jones, and B. Kaye, eds. Springer Netherlands.
96. McGrath, M., Staniszewska, H., Shishkoff, N., and Casella, G. 1996. Distribution of mating types of *Sphaerotheca fuliginea* in the United States. Plant disease 80:1098-1102.
97. Merry, A., Evans, K., Corkrey, R., and Wilson, S. 2013. Coincidence of maximum severity of powdery mildew on grape leaves and the carbohydrate sink-to-source transition. Plant Pathology 62:842-850.
98. Miller, N., Gould, A., Stoll, R., Mahaffee, W., and Pardyjak, E. R. 2012. An Experimental Study of Momentum and Heavy Particle Transport in a Row-Oriented Agricultural Canopy. Pages 2.6. in: 30th Conference on Agricultural and Forest Meteorology/ 1st Conference on Atmospheric Biogeosciences. Boston, MA.
99. Miller, N. E., Stoll, R., Mahaffee, W. F., Neill, T. M., and Pardyjak, E. R. 2015. An experimental study of momentum and heavy particle transport in a trellised agricultural canopy. Agricultural and Forest Meteorology 211:100-114.
100. Mori, Y., and Notomi, T. 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. Journal of Infection and Chemotherapy 15:62-9.
101. Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochemical and biophysical research communications 289:150-154.
102. Mori, Y., Kitao, M., Tomita, N., and Notomi, T. 2004. Real-time turbidimetry of LAMP reaction for quantifying template DNA. J. Biochem. Biophys. Methods 59:145 - 157.
103. Moyer, M. M., Gadoury, D. M., Wilcox, W. F., and Seem, R. C. 2014. Release of *Erysiphe necator* ascospores and impact of early season disease pressure on *Vitis vinifera* fruit infection. American Journal of Enology and Viticulture 65:315-324.
104. Moyer, M. M., Gadoury, D. M., Cadle-Davidson, L., Dry, I. B., Magarey, P. a., Wilcox, W. F., and Seem, R. C. 2010. Effects of acute low-temperature events on development of *Erysiphe necator* and susceptibility of *Vitis vinifera*. Phytopathology 100:1240-1249.

105. Nixon, G., Garson, J. A., Grant, P., Nastouli, E., Foy, C. A., and Huggett, J. F. 2014. Comparative study of sensitivity, linearity, and resistance to inhibition of digital and nondigital polymerase chain reaction and loop mediated isothermal amplification assays for quantification of human cytomegalovirus. Analytical chemistry 86:4387-4394.
106. Northover, J., and Schneider, K. 1996. Physical modes of action of petroleum and plant oils on powdery and downy mildews of grapevines. Plant Disease 80:544-550.
107. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic acids research 28:E63.
108. Ohtsuka, K., Yanagawa, K., Takatori, K., and Hara-kudo, Y. 2005. Detection of *Salmonella enterica* in Naturally Contaminated Liquid Eggs by Loop-Mediated Isothermal Amplification , and Characterization of *Salmonella* Isolates. Society 71:6730-6735.
109. Ough, C., and Berg, H. 1979. Powdery mildew sensory effect on wine. American Journal of Enology and Viticulture 30:321-321.
110. Patakas, A., and Noitsakis, B. 2001. Leaf age effects on solute accumulation in water-stressed grapevines. Journal of Plant Physiology 158:63-69.
111. Pautasso, M., Döring, T. F., Garbelotto, M., Pellis, L., and Jeger, M. J. 2012. Impacts of climate change on plant diseases—opinions and trends. European Journal of Plant Pathology 133:295-313.
112. Pearson, R. C. 1994. Powdery mildew. Pages 9-11. in: Compendium of Grapevine Diseases R. C. a. G. Pearson, A. C., ed. APS Press, St. Paul, MN.
113. Pearson, R. C., and Gadoury, D. M. 1987. Cleistothecia, the source of primary inoculum for grape powdery mildew in New York. Phytopathology 77:1509-1514.
114. Peduto, F., Backup, P., Hand, E. K., Janousek, C. N., and Gubler, W. D. 2013. Effect of high temperature and exposure time on *Erysiphe necator* growth and reproduction: revisions to the UC Davis Powdery Mildew Risk Index. Plant Disease 97:1438-1447.
115. Piepenburg, O., Williams, C. H., Stemple, D. L., and Armes, N. A. 2006. DNA detection using recombination proteins. PLoS Biol 4(7):e204.
116. Poon, L. L. M., Wong, B. W. Y., Ma, E. H. T., Chan, K. H., Chow, L., Abeyewickreme, W., Tangpukdee, N., Yuen, K. Y., Guan, Y., Looareesuwan, S., and others. 2006. Sensitive and inexpensive molecular test for *falciparum* malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clinical chemistry 52:303.
117. Pscheidt, J. W., Wittig, H. P. P., Wallace, L. D., Hall, T., and Mahaffee, W. 2000. Evaluation of three grape powdery mildew forecasting programs in Corvallis, Oregon, USA. in: SARDI Research Report Series S. A. Margarey Thiele, K. L. Tschirpig, R. W. Emmett, K. Clarke, and R. D. Margarey, eds., Adelaide, Australia.
118. Rogers, S. L., Atkins, S. D., and West, J. S. 2009. Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. Plant Pathology 58:324-331.

119. Roitsch, T. 1999. Source-sink regulation by sugar and stress. Current Opinion in Plant Biology 2:198-206.
120. Rossi, V., Caffi, T., and Legler, S. E. 2010. Dynamics of ascospore maturation and discharge in *Erysiphe necator*, the causal agent of grape powdery mildew. Phytopathology 100:1321-1329.
121. Roux, K. H. 2009. Optimization and troubleshooting in PCR. Cold Spring Harbor Protocols 2009(4):pdb. ip66.
122. Royle, D. J. 1973. Quantitative relationships between infection by the hop downy mildew pathogen, *Pseudoperonospora humuli*, and weather and inoculum factors. Annals of Applied Biology 73:19-30.
123. Sall, M., and Wrysinski, J. 1982. Perennation of powdery mildew in buds of grapevines [*Uncinula necator, Vitis vinifera*]. Plant Diseases.
124. Skriver, K., and Mundy, J. 1990. Gene expression in response to abscisic acid and osmotic stress. The Plant Cell 2:503.
125. Smith, C. 1970. Production of powdery mildew cleistocarps in a controlled environment. Transactions of the British Mycological Society 55:355-365.
126. Stummer, B. E., Francis, I. L., Markides, A. J., and Scott, E. S. 2003. The effect of powdery mildew infection of grape berries on juice and wine composition and on sensory properties of Chardonnay wines. Australian Journal of Grape and Wine Research 9:28-39.
127. Tanaka, Y., Sano, T., Tamaoki, M., Nakajima, N., Kondo, N., and Hasezawa, S. 2006. Cytokinin and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in *Arabidopsis*. Journal of Experimental Botany 57:2259-2266.
128. Temple, T. N., and Johnson, K. B. 2011. Evaluation of Loop-Mediated Isothermal Amplification for Rapid Detection of *Erwinia amylovora* on Pear and Apple Fruit Flowers. Plant Disease 95:423-430.
129. Thiessen, L. D., Keune, J. A., Neill, T. M., Turechek, W. W., Grove, G. G., and Mahaffee, W. F. 2016. Development of a grower-conducted inoculum detection assay for management of grape powdery mildew. Plant Pathology 65:238-249.
130. Thomas, C. S., Gubler, W. D., and Leavitt, G. 1994. Field testing of a powdery mildew disease forecast model on grapes in California. Pages 1070 (abstr.). in: Phytopathology.
131. Tomlinson, J. A., Barker, I., and Boonham, N. 2007. Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. Applied and Environmental Microbiology 73:4040-4047.
132. Tomlinson, J. A., Dickinson, M. J., and Boonham, N. 2010. Rapid detection of Phytophthora ramorum and P. kernoviae by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. Phytopathology 100(2):143-9.
133. Tomlinson, J. A., Dickinson, M. J., and Boonham, N. 2010. Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. Letters in applied microbiology 51:650-657.

134. Tsai, S.-M., Chan, K.-W., Hsu, W.-L., Chang, T.-J., Wong, M.-L., and Wang, C.-Y. 2009. Development of a loop-mediated isothermal amplification for rapid detection of orf virus. Journal of virological methods 157:200-204.
135. Turechek, W. W., Webster, C. G., Duan, J., Roberts, P. D., Kousik, C. S., and Adkins, S. 2013. The use of latent class analysis to estimate the sensitivities and specificities of diagnostic tests for Squash vein yellowing virus in cucurbit species when there is no gold standard. Phytopathology 103:1243-1251.
136. Vrana, K. E. 1996. Advancing technologies in gene amplification. Trends in biotechnology 14:413-415.
137. Waggoner, P. 1965. Microclimate and plant disease. Annual Review of Phytopathology 3:103-126.
138. Ward, L. I., and Harper, S. J. 2012. Loop-mediated isothermal amplification for the detection of plant pathogens. Pages 161-170. in: Plant DNA Fingerprinting and Barcoding: Methods and Protocols, Methods in Molecular Biology N. J. Sucher, J. R. Hennell, and M. C. Carles, eds. Humana Press, Totowa, NJ.
139. West, J. S., Atkins, S. D., Emberlin, J., and Fitt, B. D. L. 2008. PCR to predict risk of airborne disease. Trends in Microbiology 16:380-387.
140. Willocquet, L., and Clerjeau, M. 1998. An analysis of the effects of environmental factors on conidial dispersal of *Uncinula necator* (grape powdery mildew) in vineyards. Plant Pathology 47:227-233.
141. Willocquet, L., Berud, F., Raoux, L., and Clerjeau, M. 1998. Effects of wind, relative humidity, leaf movement and colony age on dispersal of conidia of *Uncinula necator*, causal agent of grape powdery mildew. Plant Pathology 47:234-242.
142. Willocquet, L., Colombet, D., Rougier, M., Fargues, J., and Clerjeau, M. 1996. Effects of radiation, especially ultraviolet B, on conidial germination and mycelial growth of grape powdery mildew. European Journal of Plant Pathology 102:441-449.
143. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63:3741.
144. Zheng, Y., Luo, Y., Zhou, Y., Zeng, X., Duan, X., Cao, X., Song, Y., and Wang, B. 2013. Real-time PCR quantification of latent infection of wheat powdery mildew in the field. European Journal of Plant Pathology 136:565-575.
145. Zohary, D. 1995. Domestication of the grapevine *Vitis vinifera* L. in the Near East. Pages 23-30. in: The Origins and Ancient History of Wine Food and Nutrition in History and Antropology S. J. F. P. E. McGovern, and S. H. Katz, ed. Gordon and Breach.