DEVELOPMENT AND EVALUATION OF
DETECTION-BASED AIR SAMPLING PROGRAMS
FOR GRAPEVINE POWDERY MILDEW

By

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DEVELOPMENT AND EVALUATION OF DETECTION-BASED AIR SAMPLING PROGRAMS FOR GRAPEVINE POWDERY MILDEW

Abstract

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Powdery mildew of wine grape (*Vitis vinifera* L.), caused by *Erysiphe necator*, is one of the most problematic diseases of grapevine worldwide. A real-time PCR assay using species-specific primers was developed for qualitative and quantitative detection of *E necator* in vineyard air samples collected by Rotorod sampling devices. Three methods (FastPrep DNA kit, UltraClean MoBio and FastPrep DNA kit for soil) were used to purify DNA of *E. necator* collected from air samples and evaluated with respect to conidia DNA yields. The DNA yields varied considerably with the extraction procedure used. Results of this study suggest that the less sensitive FastPrep method can at times fail to detect the presence of the pathogen in the air, possibly due to high presence of PCR inhibitors such as dust and soil particles in the Rotorod samples. The false-negative results are of concern when knowledge of the inoculum presence and viability could be useful in determining the initiation of disease management programs. Fungicide treatments may not be initiated or erroneously discontinued if the pathogen is

presumed absent or inactive by a false-negative result. Our results show that DNA quantification was closely correlated with the quantity of spores monitored with Burkard volumetric spore traps, the disease severity assessed in the vineyard and some weather parameters measured within and outside the vineyard. A high degree of correlation was observed between the airborne conidia concentration monitored by the Burkard trap and the DNA concentration extracted from the Rotorod samples, demonstrating the potential of both type of samplers, used in a complementary way, to accurately quantify pathogen occurrence in environmental samples.

The findings of this study describe a rapid, reliable method to assess the presence or absence of the pathogen and the initial step in incorporating an inoculum component in the powdery mildew risk assessment model in widespread use in Eastern Washington. The technique could be used as a means to signal the commencement of fungicide applications. In this regard, the application of control methods would be due to pathogen activity, rather than predicted or assumed activity.

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DEDICATION

This thesis is dedicated to Dr. Robert A. Nilan who transmitted to me his love for science.

INTRODUCTION

Powdery mildew of wine grape (*Vitis vinifera*), caused by *Erysiphe necator*, is one of the most severe diseases of grapevine worldwide (Pearson, and Goheen, 1998). The most deleterious effects of powdery mildew infection are yield losses and reduction in the quality and marketability of the berries (Gubler *et al*. 1996). Severe infections can depress vine vigor because damage to leaves and shoots limits photosynthesis and inhibits the accumulation of sugar in berries. (Pool *et al.*, 1984). Powdery mildew at harvest can cause considerable economic loss because wines made from diseased berries have shown a lower level of quality in their sensory evaluation than wines vinified from uninfected berries. The sensory effects of powdery mildew infections include unpleasant qualities in wine that range from bitterness to off-flavor tastes (Ough and Berg, 1979). According to Stummer *et al*. 2003, even very low levels of infection appear to be detrimental to wine sensory characteristics. Chardonnay wine made from bunches with greater than 30% powdery mildew infection can have lower total soluble solids (TSS), lower wine phenolic concentration and spectral color values compared with wine made from healthy grapes (Stummer *et al.* 2003). There are also data from Ficke *et al*. 2002 indicating the predisposition of infected berries to other fruit rots. Heavy foliar infections can predispose vines to cold damage (Grove, *unpublished*).

Timely fungicide applications are in most instances necessary to manage the disease, which can be expensive because in many instances control requires the

intensive use of Quinone outside Inhibitor (QoI), Sterol Demethylation Inhibitors (DMI), sulfur, and/or oil fungicides. Fuel and labor costs associated with applications continue to escalate (Ball and Folwell, 2003). In addition, resistance of *E. necator* to QoI and DMI fungicides has been documented (Gubler *et al*. 1996; Hollomon and Wheeler, 2002). Therefore, lowering selection pressure is a key component of fungicide resistance management in grapevine IPM programs (Falacy *et al*., 2007).

When the pathogen is not present, inoculum levels are low, or the environmental conditions are not conducive for disease development, the application of protective fungicides may be unnecessary and conflict with the goals of integrated pest management and fungicide resistance management (Falacy *et al*., 2007). Early-season fungicide applications are generally made to control primary infections and therefore delay the onset of epidemics. In Eastern Washington, the immediate bud burst through the first post-bloom sprays are the most critical period because susceptible foliage and fruit can be infected by overwintered inoculum (Grove, 2004). Not all fungicide applications during this time are made at epidemiologically appropriate times or at appropriate intervals. During years when powdery mildew fails to develop or is delayed, early-season fungicide applications may result in both unnecessary expenses and environmental risks (Grove, 2004).

E. necator survives winter in Eastern Washington as cleistothecia in bark crevices and on senesced leaves on the vineyard floor (Grove, 2004). In vineyards where epidemic onset is delayed (despite the early-season occurrence of temperature and moisture conditions favorable for ascospore release) primary inoculum probably

originates from outside sources and is dispersed to these vineyards via air currents. Previous studies in isolated vineyards demonstrated that, due to the absence of overwintered inoculum, the foliar symptoms of the disease have either been absent or developed later in the season, and symptoms on fruit have been rare (Grove, *unpublished*). If the pathogen is not present or environmental conditions are not conducive for primary infection and subsequent disease development, fungicide programs on the industry-standard phenology program (Pest Management Guide for Grape in Washington, 2009) may be unnecessary and undesirable for economic and environmental reasons. Knowledge of the inoculum presence and viability could be useful in determining the appropriate time for the initiation of control measures and improve the precision of forecasting models already widely used in grapevine IPM in the Western United States.

Several models are in widespread use for the prediction and management of the disease. The Kast Model (Kast, 1997) is in widespread use in the Rhineland region in Germany and the Champagne, Bourdeaux, and Burgundy regions of France. In recent years, the use of Gubler-Thomas powdery mildew model has reduced fungicide usage (Gubler *et al*. 1996) in California. Forecasting models allow growers to choose the best fungicide program in terms of application intervals and specific products (Gubler, 1999). The model as applied in Eastern Washington is a modified version that is comprised of three components: a growing degree day algorithm that tracks degradation of the overwintered inoculum supply following bud burst; a primary infection algorithm; and the Gubler-Thomas Risk Index. The first component indicates the presence or absence of an overwintered form of primary

inoculum (ascospores). Cleistothecia persist until about 750 growing degree days (base 10º C) have accumulated after bud burst. Studies in New York and Eastern Washington have demonstrated that cleistothecia require 0.25 cm precipitation or greater at 10º C or greater in order to release ascospores (Gadoury and Pearson, 1990; Grove, 2004; Jailloux *et al*., 1999). The second component of the grapevine mildew model is a temperature/precipitation algorithm that looks for these temperatures and moisture conditions between bud burst and when 750 growing degree-days have accumulated. If the requirements are met within the specified time frame, primary infection occurs and the Gubler-Thomas Risk Index (the third model component) is initiated. The Gubler-Thomas Risk Index ranges between 0-100: Indices of 0-30, 40-50, and 60-100 indicate low, moderate, and high disease pressure, respectively. The index measures how rapidly the fungus is reproducing and is used to provide general guidelines regarding the interval between fungicide applications. Most importantly, the Gubler-Thomas and most other disease forecasting models automatically assume the presence of the pathogen in its assessments of disease risk. In some cases, this assumption could result in the models being "over-predictive" in the absence of the pathogen. A logical model improvement would be the development and integration of an inoculum component that would be required to activate the model or strengthen predictions. An inoculum component would require a means of trapping and accurately identifying propagules of the pathogen.

Conventional methods for identifying and measuring spore concentrations in the air rely on trapping followed by identification using microscopic or cultural

techniques (Williams *et al*. 2001; Freeman *et al*., 2002). These conventional methods are time consuming, labor intensive and subjective because they rely on identification of spores by microscopy or culture-based techniques.

Detection of *E. necator* in the vineyard using the polymerase chain reaction (PCR) has become a new experimental tool (Falacy *et al*., 2007) for studying the epidemiology of grape powdery mildew and is under investigation as a means to signal the initiation of management programs. The PCR assay using species-specific primers was developed to identify propagules of *E. necator* in vineyard air samples collected by Rotorod sampling devices (Falacy *et al.,* 2003). This application of molecular techniques provides a rapid, accurate, and more sensitive means of identifying *E. necator* in air samples when compared to the more conventional tools.

Previous research (Falacy *et al.,* 2007) indicates that volumetric spore traps confirmed the presence of ascospores of *E. necator* in the vineyard during the time period when PCR initially detected the fungus in air samples collected using Rotorod samplers. Both Burkard volumetric and Rotorod air samplers reliably record airborne spores used for monitoring spore dispersal and can be used to assess the temporal concentration of ascospores in the field (Aylor, 1993). The use of a PCR detection technique combined with the Rotorod samplers offers a means to improve the precision of air sampling efforts and represents the initial step towards the incorporation of an inoculum component in the powdery mildew risk assessment model currently in widespread use in Eastern Washington. However, the assay described in 2007 (Falacy *et al.,* 2007) was purely qualitative in nature and could be improved by further development as a quantitative assay.

Quantitative methods using real-time PCR (qPCR) have been used to detect and quantify numerous pathogens from environmental samples (Lievens *et al*., 2006). The level of sensitivity of the qPCR assay makes it suitable for signaling commencement of the fungicide management program based on inoculum presence. The sensitivity of the quantitative PCR assay used in conjunction with the air sampling has been assessed both by directly transferring conidia to PCR mixtures and by placing spores on glass rods coated with silicon grease.

The development and implementation of quantitative molecular techniques to detect *E. necator* spores would greatly expand our understanding of the parameters that influence inoculum availability and concentration. Highly sensitive detection and quantification could also be useful in helping growers to make more accurate management decisions through the establishment of treatment thresholds at key epidemiological times. Accurate determination of inoculum thresholds for primary and secondary infections thresholds identified using the Rotorod sampling/PCR detection method could result in the development of effective novel tools for use in powdery mildew management programs.

The objectives of the present study were: (i) develop a qPCR protocol for detection and quantification of the E. necator in vineyard air samples; (ii) optimize the protocol to assess qPCR sensitivity; (iii) investigate potential relationships between actual aerial spore concentrations, the disease level observed in the field, and qPCR signal strengt.

MATERIALS AND METHODS

Vineyard description and air sampling for qPCR. A vineyard comprised of 2 hectares of 3 to 6-year old own-rooted 'Chardonnay' and 'Riesling' vines located at the Irrigated Agriculture Research and Extension Center about 7 km North of Prosser, WA, was used in 2007 to 2008 field studies. Extensive vineyard studies using Rotorod (Sampling Technologies, inc. Minnetonka, MN) air samplers and qPCR were conducted during 2007 and 2008. The sampling method employed single stationary Rotorod traps located at the leeward edges of the vineyard mounted on a pipe at a canopy height approximately 1.5 m above the ground. The device was operated continuously and the rods collected weekly beginning 1 month prior to bud burst (early March) to harvest. During 2007 Rotorod samples were obtained using two 5 cm by 1 mm glass capillary tubes coated in vacuum grease (Dow Corning, Midland, MI) to evaluate the qPCRassay. The glass rods were collected at weekly intervals for processing and placed directly into micro-centrifuge tubes. These samples were subsequently subjected to DNA extraction. During the second year of the study, the glass capillary tubes used to collect aerial material were replaced with two 5 cm by 1 mm stainless steel rods.

In order to assess the temporal concentration of *E. necator* propagules in the air during the course of Rotorod/PCR studies, a Burkard volumetric spore trap (Burkard Scientific Sales, Ldt., Rickmansworth, Hertfordshire, England) was placed in the vineyard. The trap was operated continuously over the course of the study and

was adjusted to sample approximately 10 liters of air per minute. Trapping tapes were removed at weekly intervals, cut into segments corresponding to 24-h periods, stained with acid fuchsin and mounted on glass slides. These were examined at 200× under a compound microscope. The number of ascospores and conidia exhibiting features of *E. necator* trapped per hour were recorded and totaled over 24-h periods to determine when ascospore release events occurred and thereby help assess the validity of any positive or negative results from the PCR analysis of Rotorod samples. A CR-21X (Campbell Scientific, Logan, UT) datalogger positioned 10 m from the trap provided continuous records of temperature, relative humidity, rainfall, leaf wetness, wind speed, wind direction, and solar radiation. The potential for ascospore release was determined using established rules for ascospore release: average daily temperature above 10 C with 2.5 mm of rainfall.

During January 2009, air samples were also collected at the Lenswood research station situated 30 km east of Adelaide, South Australia. The Rotorod and Burkard volumetric spore traps were positioned at the east edge of a vineyard comprised of 15-year old Chardonnay grape variety, planted 1.5m apart in rows 3m wide and trained to a vertical shoot canopy.

Disease incidence and severity evaluations. Indicator vines in the aforementioned vineyards were not treated with fungicides at any time during the study. Beginning at bud burst, 25 leaves on each of four vines were selected at random and evaluated at weekly intervals through veraison for symptoms and signs of infection by *E.*

necator. During 2008, 25 clusters on each of four vines were also evaluated for symptoms and signs of powdery mildew infection. Disease severity was determined by visual analysis of percent colonization of foliage and clusters (2008 only).

DNA extraction. During 2007, fungal DNA was extracted using a modification of the FastPrep™ system (MP Biomedicals, LLC) described by Falacy *et al*. (2007). Samples were added to 2 ml centrifuge tubes containing garnet matrix and ceramic beads supplied by the manufacturer. The provided CLS-Y buffer (1 ml) was added to each sample and processed twice in the FastPrep 120 homogenizer (Bio 101/savant, Vista, CA) for 30 seconds at speed 4. To prevent any pressure build up in the tubes, samples were cooled on ice after the first and second homogenation processes. Samples were centrifuged for 10 minutes at 10,000 x *g* and 600 µl of the supernatant was transferred to a clean 1.5 ml centrifuge tube. The DNA was bound to 600 µl of the supplied binding matrix and centrifuged for 30 seconds at 10,000 x *g*. The supernatant was discarded and the binding matrix containing the DNA was gently resuspended in 500 µl of SEWS-M solution, transferred to a spin filter inserted into a catch tube, and centrifuged at 10,000 x *g* for 1 minute. The solution in the catch tube was discarded and the spin filter was centrifuged an additional minute to remove any remaining buffer solution. The binding matrix was then resuspended in 100 µl of DES solution. After standing for 5 minutes at room temperature, the samples were centrifuged at 10,000 x *g* for 1 minute a new microfuge tube to collect the DNA. DNA extracts were stored a -20 °C.

During 2008, a modification of the FastPrep kit protocol was necessary due to the use of metal rods. The FastPrep™ 120 homogenizer with its vertical, angular motion, caused some tubes to break due to forces incurred by the metal rods. The FastPrep homogenizer was substituted with the Vortex- Genie 2 (MOBIO Laboratories, INC.) to shake the tubes. The beads were also removed from the tubes prior to processing to allow free movement of the stainless steel sampling rod in the extraction tube during the vortexing process.

Previous experiments conducted by Falacy *et al*. suggested that most samples contained non-target material that may inhibit PCR reactions, thus limiting the sensitivity and/or the detection of the target pathogen DNA. To address this concern, the fungal DNA during the second year of the study was also purified using a modification of the Ultraclean MoBio (MOBIO Laboratories, Carlsbad, CA) protocol for soil DNA isolation to remove such inhibitors. Prior to the addition of the rod samples, 60 µl of the provided S1 solution and 200 µl IRS solution were added to the 2ml MoBio bead beating tubes. Samples were shaken for 10 minutes using the Vortex- Genie 2, and then boiled for two minutes at 100° C for protein denaturation and membrane degradation. The supernatant (400 µl) was transferred to a clean tube, mixed with Solution 2 (250 µl), and incubated at 4°C for 5 minutes. After centrifugation for 1 minute at 10,000 x *g*, 600 µl of the solution was transferred to a 2.0 ml clean tube. Solution 3 (1.3 ml) was added and the tubes were mixed by inversion. The DNA-containing solution (650 µl) was loaded into a spin filter, centrifuged for 1 minute at 10,000 x *g* and the flow through was discarded.

This step was repeated three times. After adding 300 µl of S4 solution to the filter, centrifugation was repeated twice to remove excess liquid from the filter. Fifty µl of S5 solution were added and the samples were centrifuged for 30 s at 10,000 x *g* to collect the DNA and then stored at -20°C.

During 2008, DNA was also purified using the FastDNA SPIN kit for soil (MP Biomedicals, LLC) to remove non-target material that may inhibit PCR reactions. Samples were added to 2 ml centrifuge tubes containing Lysing Matrix E. sodium phosphate buffer (978 µl), and MT Buffer (122 µl) was added to the samples and processed for 10 minutes using the Vortex- Genie 2. Samples were centrifuged for 10 minutes at 10,000 x *g* and 650 µl of the supernatant was transferred to a clean 2.0 ml centrifuge tube. Proteins in the supernatant were precipitated using the supplied protein precipitation solution (650 µl) and centrifuged for 5 minutes at 14,000 x *g*. The supernatant fluid was transferred to a clean 1.5 ml tube and resuspended in binding matrix solution (1.0 ml). To allow binding DNA to the silica matrix, tubes were incubated at room temperature for 3 minutes. Part of the supernatant (500 µl) was removed and discarded being careful to avoid settled binding matrix. The remaining amount of supernatant (approximately 600 µl) was transferred to a spin filter and centrifuged 1 minute at 14,000 x *g* after which the flow through was discarded. The pellet was gently resuspended in 500 µl SEWS-M solution and centrifuged twice at 10,000 x *g* for 1 minute. The spin filter was air dried for 5 minutes at room temperature. The binding matrix was resuspended in 100 µl of DES solution. The samples were centrifuged at 14,000g for 1 minute and the

DNA-containing solution collected. Extracted DNA was stored a -20 °C.

Real-time PCR assay. DNA was analyzed using an ABI 7000 real-time PCR sequence detection system (Applied Biosystems Inc., Foster City, CA) using species-specific Taqman primers and probe (Applied Biosystems Inc.). Primers (Uncin 144 and Uncin 511), designed in a previous study carried out by Falacy *et al.,* 2007, were selected because of their high specificity to *E. necator.* The qPCR specificity and the detection limits have been tested by collecting air-samples from remote vineyard in Washington and Oregon with no occurrence of *E. necator* (Falacy *et al*. 2003).

To quantify unknown concentrations of pathogen DNA, a standard curve was generated by the amplification of a 10-fold serial dilution (10; 10^{-1}; 10^{-2}; 10^{-3}) of *E. necator* template DNA solution that was quantified spectrophotometrically. To minimize variability, each dilution was assayed in triplicate. PCR assays were conducted in 200 µl optical tubes consisting of 45 µl reactions containing the PCR master mix: 5 µl reverse primer (9 pMol), 5 µl forward primer (9 pMol), 5 µl RNAse free water, 5 µl probe (2.5 µMol), 25 µl Taqman Universal PCR master mix (Applied Biosystems); and 5µl target DNA.

The thermalcycling profile consisted of an initial cycle of 2 min at 50°C followed by a single cycle of 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. For all experiments, three reactions were executed for each set of known DNA concentration standards, and two reactions for each sample. Data

acquisition and analysis were conducted using Applied Biosystems software. (Applied Biosystems Inc.)

Sensitivity of the real-time PCR assay. The relationship between concentrations of conidia and the strength of signal obtained using real-time PCR was established using direct amplification of both a single conidium and a conidial suspension. Single conidium were gathered from young colonies of *E. necator* cultured on Chardonnay grape and transferred to leaves using a single eyelash attached to a glass Pasteur pipette while viewing under a dissection microscope. Harvested conidia (1, 5, 10, 30, or 50) were transferred into individual microcentrifuge tubes containing 5 µl RNAse-free water. The tubes containing suspension were centrifuged at 14,000 x *g* for 30 seconds then incubated at -20°C overnight to disrupt the spores. Serial dilutions of a solution of a known concentration of conidia (obtained by washing infected grape leaves) were also analyzed by real-time PCR utilizing the aforementioned thermal cycling parameters.

One, 10, and 50 conidia, respectively, were transferred to metal individual air-sampling rods coated with vacuum grease while viewing under a dissection microscope. DNA from conidia placed on the rods was then purified using the FastPrep™, Ultraclean MoBio, and FastDNA SPIN (soil kit) methods and analyzed as described above. One, 5, 10, 30, and 50 conidia were also transferred directly into extraction tubes. DNA from these conida was purified using the FastPrep™ and Ultraclean MoBio methods and analyzed as described above.

Data Analysis. JMP Statistical Analysis Software (SAS Institute, Cary, NC 27513) was used in all data analyses. Regression analysis was used to describe the relationship between DNA signal strength (Y) and numbers of conidia (X) used in sensitivity studies comparing the Mobio and FastPrep methods as well as direct amplification studies using the former technique. Regression analysis was also used to describe the relationship between DNA signal strength (Y) and airborne conidia concentrations (X_c), foliar disease (X_{fi}) incidence and severity (X_{fs}), and cluster disease severity (X_{sc}; 2008 only) in vineyard studies. The relationship between DNA signal strength, aerial conidia concentrations, and the relationship between disease incidence/severity were conducted on data points taken beginning at the first detection of visible foliar symptoms and signs. Data taken until the first frost were included in the 2007 analyses. Variances in conidia counts and the percent disease incidence or severity were stabilized using natural logarithm and square root transformations, respectively (Neter and Wasserman, 1974). The relationships between the dependent and individual independent variables were evaluated using coefficients of determination (R^2), significance of regression coefficients, F-ratios, and the pattern and distribution of residuals (Neter and Wasserman, 1974).

RESULTS

DNA extraction. In this study, three commercial kits were used to extract DNA of *E. necator* from air samples collected by Rotorod sampling devices and evaluated with respect to DNA yield. The results (Figures 1-2; Table 1) indicate that DNA yields obtained by the UltraClean MoBio method were higher than those by both the FastPrep DNA Kit and the FastPrep DNA kit for soil. The UltraClean MoBio method was used beginning on 27 May. The FastPrep DNA kit for soil was tested only on 6 samples, beginning on 15 July. During January 2009, DNA of *E. necator* was also extracted from spore traps located at the Lenswood research station (Adelaide, South Australia). The results (Table 1) indicate that the FastPrep method consistently failed to detect the presence of the pathogen in the air samples.

Two methods (FastPrep DNA kit and UltraClean MoBio) were also used to extract DNA of *E. necator* from conidia transferred from infected leaves directly into the extraction tubes. Both extraction methods yielded DNA from a single conidium. The qPCR reading for samples extracted with the FastPrep DNA kit method gave DNA yields of 0.023 ng, 0.011 ng and 3.23 ng for 1, 5 and 50 conidia, respectively (Table 2). The qPCR reading for the samples extracted with the UltraClean MoBio method gave values of 0.007 ng, 0.008 ng, 0.095 ng of DNA for 1, 5 and 50 conidia, respectively. Regression analysis revealed a significant relationship ($R^2 = 0.78$; $F = 10.4$) between DNA signal strength and numbers of conidia at $P < 0.05$ using the Mobio technique. The relationship between signal strength and conidia numbers

using the FastPrep technique were not significant at $P < 0.05$ ($R^2 = 0.70$; $F = 6.9$; $P = 0.08$).

Real -time PCR Assay. A linear relationship between the TaqMan cycle threshold (Ct) values and the logarithm of the target concentration was observed. For all standard curves obtained, the coefficients of determination were high ($R^2 > 0.98$).

During 2007 the FastPrep method was used to extract DNA from air samples collected by Rotorod sampling devices. The initial detection of the pathogen occurred on 7 April with a mean DNA quantity of 0.001 ng. The qPCR signal strength showed a steady increase from May to early August (Figure 1). The maximum quantity (47.48 ng) of *E. necator* DNA was detected on 9 October. The last detection was on 26 October, which coincided with the first freezing event of the Fall season.

During 2008, *E. necator* DNA, purified with the FastPrep method was first detected by the real-time PCR in the air samples on 13 May with a mean DNA concentration of 0.005 ng (Figure 2). The qPCR signal strength peaked on 26 August (Figure 2) with 5.89 ng of mean DNA detected. The last detection was on 7 October, which also coincided with the first freezing event of Fall.

Beginning on 27 May, 2008, *E. necator* DNA also was extracted and purified from the Rotorod air samples using the UltraClean MoBio method (Figure 2). The first *E. necator* DNA detection was on 24 June with a reading of 0.004 ng of DNA. The maximum DNA value was quantified by qPCR on 22 July with a reading of

13.31 ng. During the second year of the study (2008) beginning on 16 July, *E. necator* DNA also was extracted and purified from the air samples using the FastPrep kit for soil (Data not shown).

Sensitivity of the real-time PCR assay. The real-time PCR detection threshold was evaluated by direct amplification of 1 to 50 conidia of *E. necator* harvested from leaves collected in both the greenhouse and the vineyard. The smallest amount of *E. necator* DNA detected by qPCR was from pure conidial DNA. Based on experimental results with known quantities of spores, a consistent positive relationship between the mean DNA quantity and the PCR signal strength was demonstrated. The results are shown in Table 3. Regression analysis revealed a significant relationship ($R^2 = 0.97$; $F = 112.4$) between DNA signal strength and numbers of conidia at $P < 0.0005$.

The qPCR detection threshold was also tested by amplification of *E. necator* DNA purified from conidia (1, 10, 50) transferred to metal rods coated with vacuum grease. Conidial DNA was purified using the FastPrep™ system, the Ultraclean MoBio, and the FastDNA SPIN for soil kit. The obtained DNA was analyzed by real-time PCR. A single conidium was successfully detected using all three methods. The results are shown in Figure 5.

Air sampling results and disease severity evaluations. During the winter of 2007-2008, a cherry orchard adjacent to the experimental vineyard was removed.

Beginning in September 2008, the old orchard site was prepared for planting to another crop. Because of the proximity to air sampling stations, irrigation and dust from site preparation activities had the potential to compromise air sampling results. Therefore only data taken up to site preparation were included in the analyses. *Erysiphe necator* was not detected in air samples collected during the dormant season during all years of the study. The initial PCR detection in 2007 (Figure 3) occurred at bud burst as a result of an extended 12.7 mm rain event on 11 and 12 April (days of year 102 and 103). Five ascospores were obtained by the Burkard trap during this rain event. PCR detection resumed on rods collected on 23 April (sampling period 16-23 April). Three ascospores were trapped by the Burkard sampler during a 5.5 mm rain event on 20 and 21 April (days of year 110 and 111). PCR detection resumed on rods collected on 28 May (sampling period 21-28 May), about 2 weeks prior to the first observance of mildew signs on foliage. Detection continued through the remainder of the growing season. In 2008 (Figure 3), the initial PCR detection occurred on rods collected on 13 May. Fourteen ascospores were trapped during a rain event on 19 April to 3-4 May (days of year 124 and 125). PCR detection resumed on rods collected on day of year 162 (June 10), 6 days prior to the observance of signs of powdery mildew on foliage in the vineyard. The increase in the disease severity assessed on both leaves and clusters coincided with the increase in spore density in the air (Figure 4), monitored by the volumetric spore trap, during both years of study.

Regression analyses of the 2007 vineyard data revealed significant relationships between DNA signal strength and aerial spore concentrations, foliar disease incidence,

and foliar disease severity during both years of the study. Relationship between variables were best described by the equations

$Y = -39.9 + 7.3\ X_c$ (1)

$Y = -0.9 + 4.13\ X_{fs}$ (2)

$Y = -2.1 + 0.33\ X_{fi}$ (3)

(where Y = nanograms of DNA) with coefficients of determination (R^2) of 0.55, 0.81, and 0.67, respectively. Regression coefficients were significant at $P < 0.005$. Random patterns resulted from plotting residuals versus predicted values. Probability plots revealed a normal distribution of error terms.

Analysis of the 2008 data revealed significant relationships between DNA signal strength using the FastPrep procedure and aerial spore concentrations and foliar disease severity (Figure 2). Relationship between variables were best described by the equations

$Y = -8.8 + 2.5\ Xc$ (4)

$Y = 0.69 + 0.55\ Xfs$ (5)

with R^2 of 0.49 and 0.36, respectively. Regression coefficients were significant at $P \leq 0.04$. Random patterns resulted from plotting residuals versus predicted values. Probability plots revealed a normal distribution of error terms. The relationship between DNA signal strength and foliar disease incidence and cluster incidence and severity were insignificant at $P < 0.05$. Results of the 2008 analysis of the relationship between DNA signal strength using the Mobio procedure revealed significant relationships between Y and aerial conidia concentrations, foliar disease

incidence and severity, and cluster disease severity. Relationships between variables were best described by the equations

$$Y = -25.5 + 7.2\ X_c \qquad (6)$$

$$Y = 0.69 + 46.1\ X_{fs} \qquad (7)$$

$$Y = -0.9 + 0.28\ X_{fi} \qquad (8)$$

$$Y = 2.0 + 0.04\ X_{cs} \qquad (9)$$

with R^2 of 0.69, 0.54, 0.55, and 0.38, respectively. Regression coefficients were significant at $P < 0.004$. Random patterns resulted from plotting residuals versus predicted values. Probability plots revealed a normal distribution of error terms.

DISCUSSION

This study resulted in an improved DNA-based assay to detect and quantify *Erysiphe necator* DNA in field samples and has demonstrated relationships between real-time PCR signal strength, aerial spore concentrations, and disease severity. It differs from the techniques described by Falacy *et al.* in its quantitative, rather than purely qualitative, approach. Using pathogen-specific primers and probe and a Rotorod air sampler, it was possible to use PCR amplification to detect and quantify DNA from airborne propagules of *E. necator* in the vineyard prior to observation of disease symptoms and throughout the growing season after disease onset. The initial detections corresponded to predicted ascospore release resulting from rain events with $\geq$ 2.5 mm in 24 h (Gadoury and Pearson, 1990) that were confirmed by the presence of ascospores on impaction tapes from a Burkard volumetric spore trap. Negative sampling results prior to and subsequent to the positive detections indicated that either the pathogen was not present or that the concentration of propagules of *E. necator* in the vineyard air may have been below the detection thresholds of either the Rotorod air sampler or qPCR technique. The strength of qPCR signals was associated with the aerial concentration of conidia and disease severity in the vineyard.

This study confirmed previous work conducted by Falacy *et al*. 2007 on the specificity of the primers Uncin 144 and Uncin 511 for *E. necator,* but represents the first step in the development of a quantitative, rather than purely qualitative assay.

The TaqMan-based method was reliable for detection and quantification of *E. necator* DNA from field samples.

The use of a standard curve, based on known concentrations of total DNA, made it possible to quantify *E. necator* in air samples. The linearity of the correlation between the qPCR cycle threshold (Ct) and the target DNA concentration demonstrated the feasibility of the technique. As reported in other studies (Williams *et al*. 2001; Freeman *et al*., 2002; Lievens and Thomma, 2005), the real-time PCR method is more sensitive and reliable in identifying and quantifying pathogens than conventional methods that rely on microscopic or cultural techniques. However, qPCR can also amplify DNA from dead or non-active organisms, therefore, detection of non-viable propagules may not be excluded (Lievens and Thomma, 2005). Hence, it should be considered that nonviable spores might be present in dormant season or in the vineyard after periods of extremely hot weather.

Aerial spore concentrations and disease severity levels in the field were correlated with the amount of *E. necator* DNA detected air samples. However, coefficients of determination ≤ 0.81 indicated that independent variables accounted for only a portion of the variability in PCR signal strenght. Multiple regression analysis revealed no consistent interactions between independent variables. One possible source of variation in DNA signal strength may be the presence of inhibitors in vineyard dust that interfere with the PCR reactions. Another source could be the gradual decrease in Rotorod battery voltage over the course of the sampling period resulting in a decrease in rotor rotation speed.

The DNA-based assay has been improved in terms of the sampling techniques and the DNA extraction procedure to increase the sensitivity and the efficiency of the assay. During 2008, the glass sampling rods used in conjunction with the Rotorod devices were replaced by metal rods due to the fragility of the former, which were prone to breakage in the field. The binding of DNA to silica and glass, which can lower the final DNA yield, was also a concern. Extensive modifications of the Fast DNA kit protocol were necessary to ensure successful and consistent amplification of target DNA using the metal rods. The FastPrep homogenizer, with its vertical, angular motion, caused the tube to break under the pressure of the metal rods. The FastPrep homogenizer was substituted by a MoBio vortex attachment to shake the tubes. The beads were also removed from the tubes to allow free movement of the stainless steel sampling rod in the extraction tube during the vortexing process. DNA yields varied considerably with the extraction procedure used. Yields were generally greatest with the MoBio method. Williams *et al.* 2001 reported a large amount of inhibitors (e.g. non-target DNA, non-target biological particles and organic dust present in the DNA samples when processing air-sampled material) may interfere with the PCR process thereby reducing the potential sensitivity of the assay. This problem has been alleviated by the use of extraction kits that were better suited for our sample types. The FastDNA kit is suitable for rapid isolation of genomic DNA from plant and animal tissue, bacteria, yeast, algae and fungi. The UltraClean DNA kit is more efficient for isolating DNA from soil samples. Because of dust and soil particles present in the rods samples, the UltraClean kit for soil was more efficient in obtaining highly purified DNA.

Results of this study suggest that the less sensitive FastPrep method can at times fail to extract enough conidial DNA for qPCR detection. This was probably due to the presence of inhibitors in the Rotorod samples that interfere with amplification with PCR. qPCR assays on DNA extractions using the FastPrep method resulted in more Ct values of undetectable DNA, when compared with the values obtained with the UltraClean MoBio method. The false-negative results are of concern when knowledge of the inoculum presence is used as a basis for initiation of disease management programs. Fungicide treatments may not be initiated, or may be erroneously discontinued if the pathogen is presumed absent or inactive by a false-negative result. False negatives resulting from the use of the FastPrep technique may also result from conidial population levels beneath the detection threshold of the extraction technique or Rotorod sampler. However, the FastPrep method was qualitatively reliable in detecting the pathogen early in the season. Further investigation is necessary in order to compare the FastPrep and the MoBio methods in their qualitative efficiency in detect *E. necator* early in the season.

The qPCR procedure appeared to be highly sensitive. A link between the qPCR threshold levels and pathogen DNA concentration was established. When conidia were transferred directly into qPCR master mixes, one conidum was consistently detected with the assay. The sensitivity of this particular technique was improved through freezing the conidia at -20° C overnight prior to amplification. Work by Williams *et al.* (2001) reported that disrupting spores of *Penicillium roqueforti* to release DNA before it is amplified by PCR increases the sensitivity of one- to two folds. Falacy *et al.* (2003) reported direct amplification of DNA from

conidia by omitting the DNA extraction step. Our results suggest that qPCR successfully detected DNA of *E. necator* purified from a single conidium. Fifty conidia were required for consistent detection when spores were placed directly on metal air sampling rods. This reduction in efficiency may be due to several factors. The silicon grease also may interfere with the DNA extraction procedure, as might the presence of the stainless steel sampling rod in the extraction tube. Our results indicate that both the Burkard and the Rotorod sampler devices were reliable and accurate for monitoring airborne ascospores of *E. necator* in the vineyard air. Dhingra and Sinclair, 1995, have described these spore samplers as highly efficient for collecting air particles. Rotorods are simple, low cost, light weight, can be used for large sampling volume, many samplers can be used simultaneously and can be battery operated. The major disadvantage is that collection efficiency is dependent upon spore density and size. On dry, windy days, the rods collected much debris and dust such that the sampling surface was overloaded.

The Burkard trap is an accurate sampler for recording airborne spores in all types of weather conditions (Aylor, 1993). A disadvantage of the Burkard is that the identification of the collected spores is based on morphological characters. Vineyard air is a mixture of pollen, bacteria, fungal spores, and other particles containing DNA (Falacy *et al.,* 2007). Additionally, other crops such as apples, cherries, hops and numerous weed species that host erysiphaceous fungi surrounded the Washington vineyard where the samples were collected. The erysiphaceous fungi that cause epidemics on plantings adjacent to the vineyard are taxonomically distinct organisms but morphologically similar (Falacy *et al.,* 2007). Therefore, identification of the

fungal pathogen based on microscopic examination of morphological characters could be inaccurate and untimely.

Correlations were observed between the airborne conidia concentration monitored by the Burkard trap and the DNA concentration extracted from the Rotorod samples, demonstrating the potential of the techniques described herein to develop into a rapid and reliable method to assess the presence of inoculum and the initial step in incorporating a quantitative inoculum component in the powdery mildew risk assessment model in widespread use in Eastern Washington. Figure 1 shows obvious discrepancies (e.g., days 283, 290 and 297), between qPCR signal strength and the conidia concentration assessed by the Burkard method, which may be a result of site preparation activities of the orchard adjacent the vineyard where the samples have been collected. In fact, irrigation and dust had the potential to compromise the quality of the spore tapes so that it is difficult to make accurate counts.

The quantitative aspect makes this technology highly attractive for its use in disease management strategies. This could result in a more reliable and cost-effective control of powdery mildew early in the progress of an epidemic. Several instruments are available to perform rapid-cycle real-time PCR for diagnostics directly in the field. Portable thermocyclers are fast, reliable and relatively inexpensive (Schaad and Frederick, 2002).

With further refinement, the technique could be used as a means to signal the commencement of fungicide applications. In this regard, the application of control

methods would be due to pathogen activity, rather than predicted or assumed activity and has shown promise in preliminary vineyard trials (Grove and Mahaffee, unpublished). With further development of treatment thresholds this system may also be useful in signaling the delay of initial fungicide applications or adjusting the interval between subsequent fungicide applications. Such a system could make disease management less expensive, reduce deleterious effects of pesticide use on the environment, and lower the risk of development of fungicide resistance. The technique also provides potential benefits for producers who because lack of access to weather stations do not use model-based disease management.

FIGURES

Figure 1

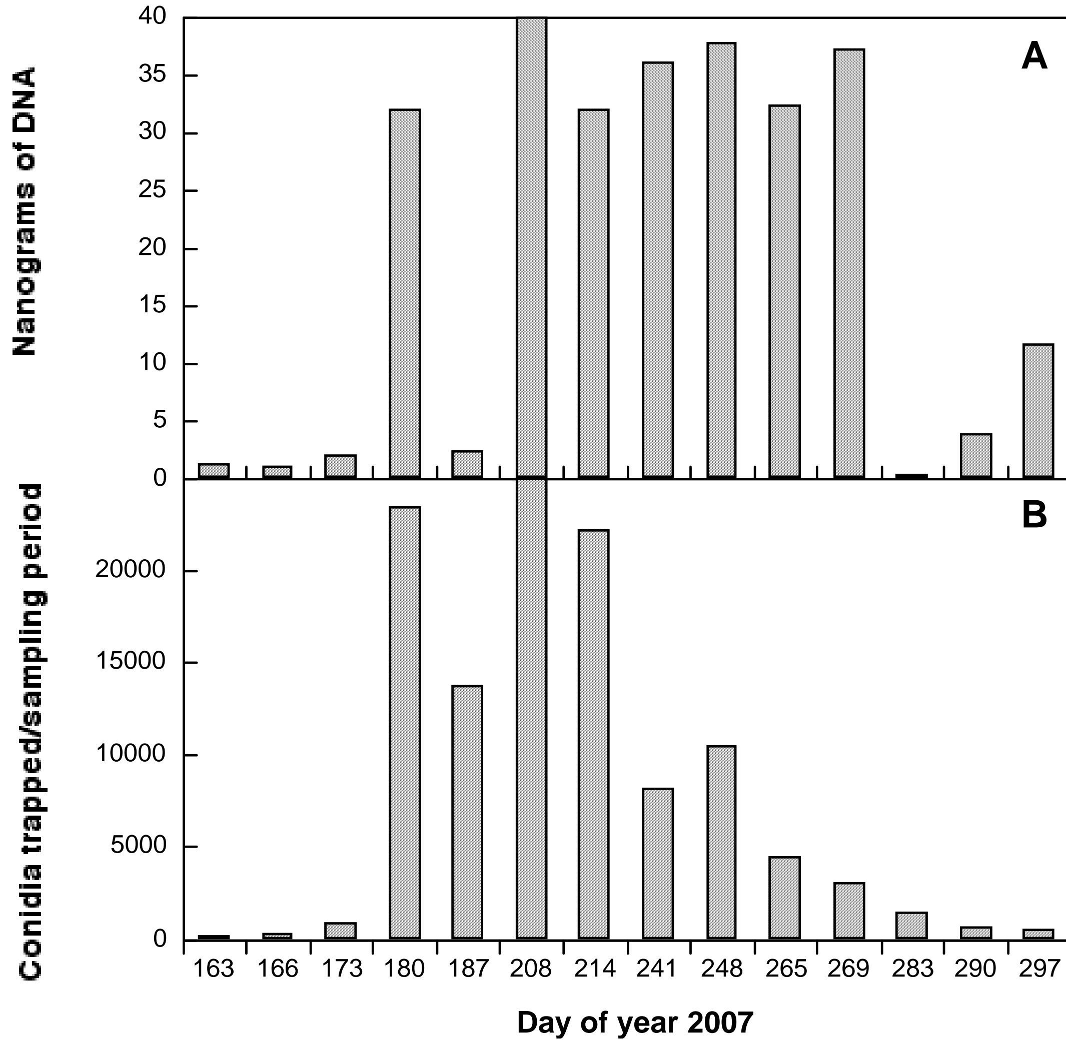


Figure 2

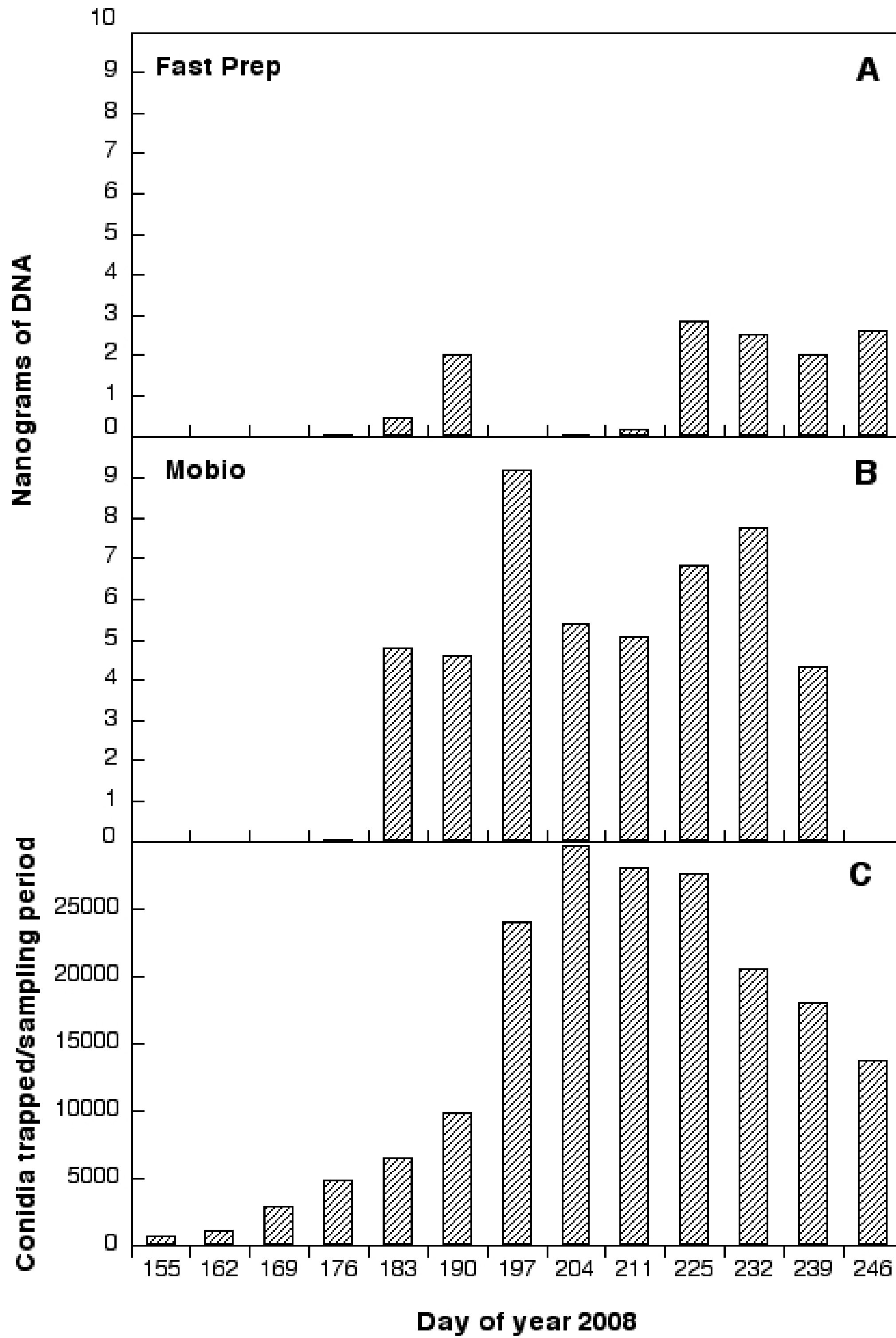


Figure 3

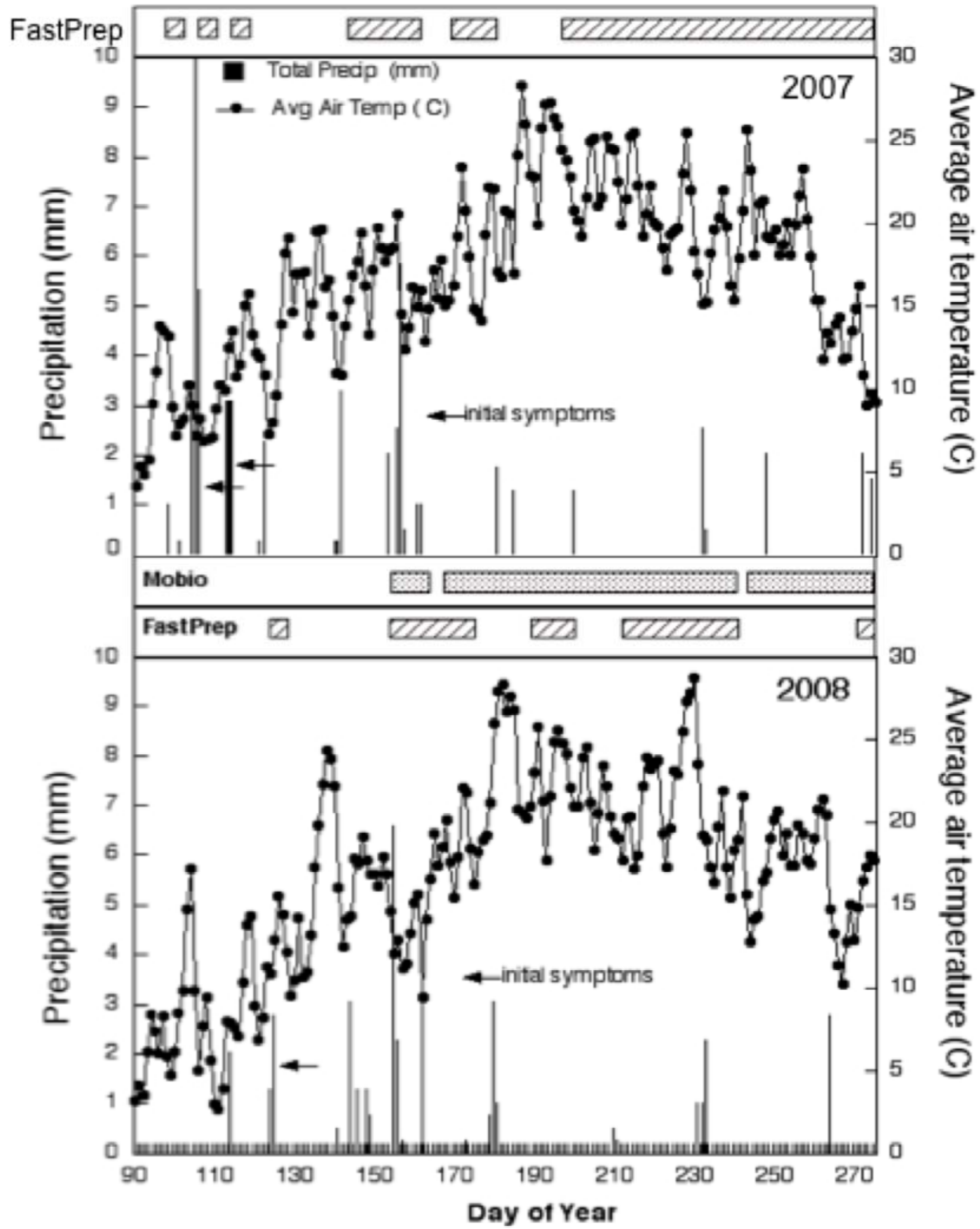


Figure 4

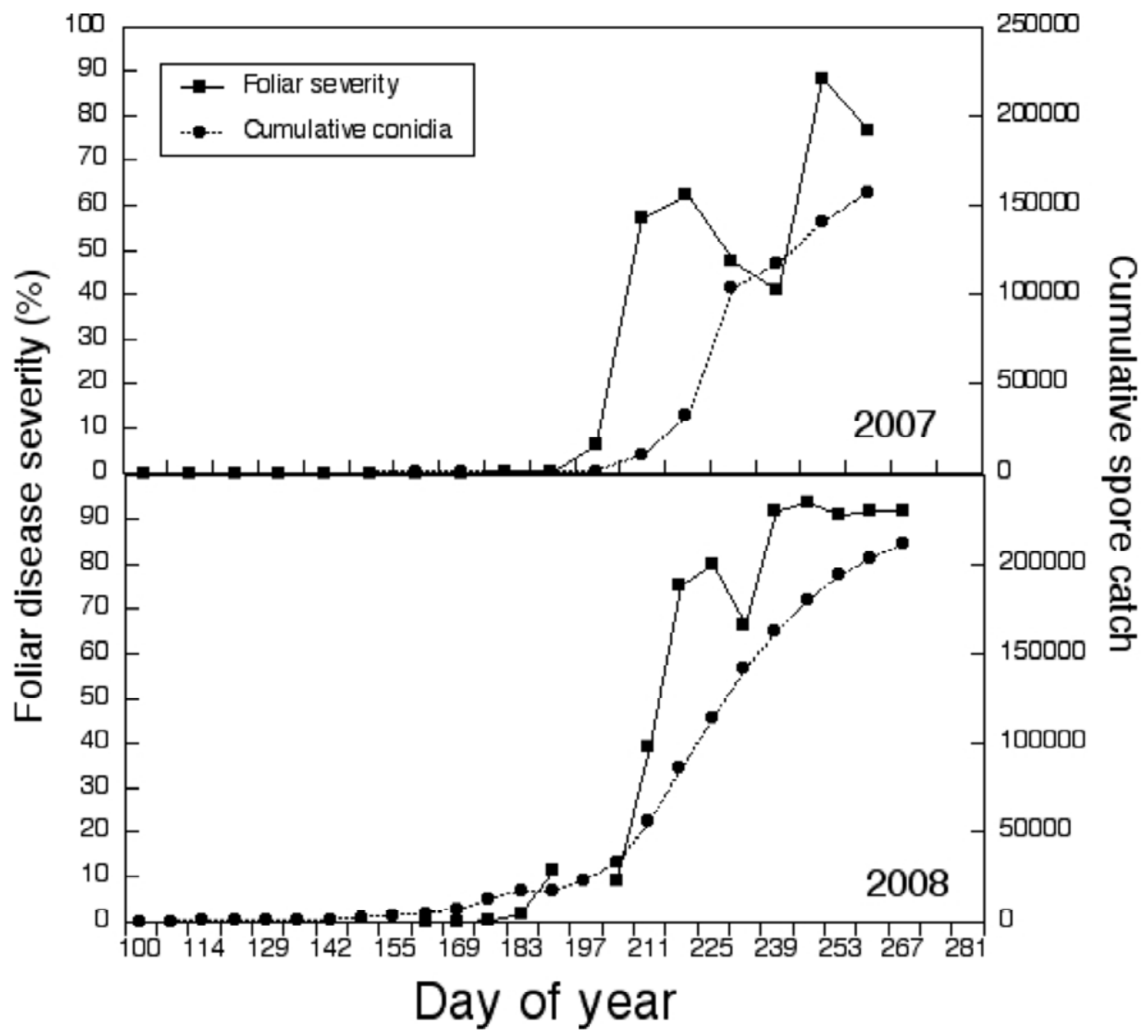
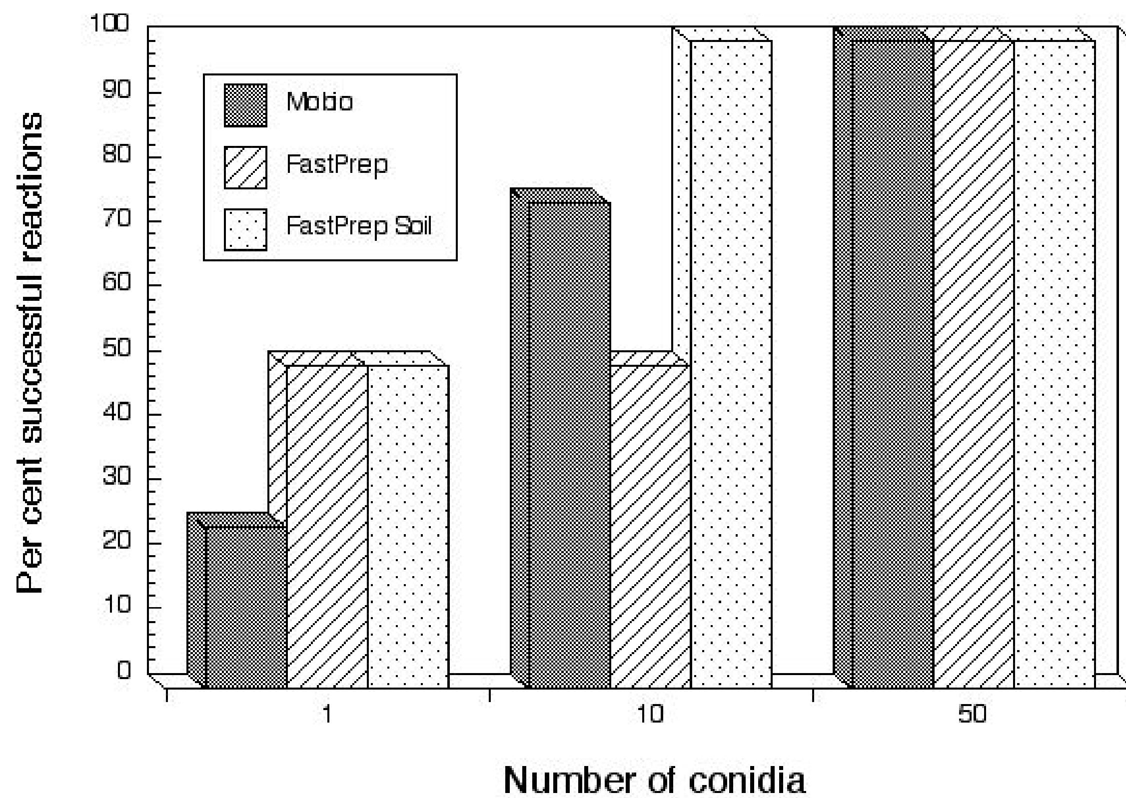


Figure 5



TABLES

Table 1: Mean quantity of DNA in nanograms detected using a Rotorod air sampling device positioned in a cv. 'Chardonnay' vineyard located at the Lenswood Research Station, Adelaide, South Australia. DNA was extracted from sampling medium using the FastPrep, Mobio, and FastPrep DNA for soil kits.

Date	FastPrep Mean DNA Qty	MoBio Mean DNA Qty	Soil FastPrep Mean DNA Qty
1/15/09	undetectable	0.505	1.070
1/22/09	undetectable	1.570	1.080
1/28/09	undetectable	0.534	0.182

Table 2: Mean quantity of DNA in nanograms obtained from extracting DNA from 1, 5, 10, 30, and 50 conidia, respectively, using the FastPrep and Mobio extraction protocols. Conidia were placed directly into extraction tubes using an eyelash attached to a micropipette. Each value represents the average of four experiments at each inoculum level.

Number of conidia	Avg Mean DNA Qty MoBio*	Avg Mean DNA Qty FastPrep*
1 conidia	0.007	0.023
5 conidia	0.008	0.011
10 conidia	0.024	0.005
30 conidia	0.117	0.018
50 conidia	0.095	0.323

* ng of DNA

Table 3: Mean quantity of DNA in nanograms obtained from 1,2,3,6, 10 and 32 conidia, respectively, placed directly into microcentrifuge tube containing 5 microliters of RNAse-free water. PCR reagents were then added to the tubes and real-time PCR was conducted as described in the text. Each value represents the average of four experiments at each inoculum level.

Conidia	Mean Ct[a]	StdDev Ct[b]	Mean Qty[c]	StdDev Qty[d]
1	26.51	0.015	1.16	0.01
2	24.74	0.934	3.53	1842
3	23.97	0.668	5.33	2036
6	22.85	0.001	9.94	0.005
10	21.91	1165	19.17	12152
32	20.54	0.429	38.81	9646

a: Ct (cycle threshold) is the fractional cycle number at which the fluorescence passes the fixed threshold
b: Cycle threshold standard deviation
c: Amount of DNA (ng) target during the amplification cycles of the PCR
d: Mean DNA quantity standard deviation

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