Monitoring Pathogenesis of Natural *Botrytis cinerea* Infections in Developing Grape Berries

Lance Cadle-Davidson*1

Abstract: Quiescent infections play key roles in *Botrytis cinerea* pathogenesis and in the management of Botrytis bunch rot on *Vitis* spp. An improved understanding of the biology of quiescence and identification of resistant germplasm could result in improved disease management. In 2004 and 2005, two methods were applied to monitor quiescence and activation of natural *B. cinerea* infections on 32 genotypes of *Vitis* spp. and interspecific hybrids. In addition to the standard assay for early detection of *B. cinerea*, based on tissue freezing and incubation, a real-time quantitative PCR (qPCR) assay was developed and tested. Based on Taqman chemistry, this qPCR assay quantified as little as 3.2 pg of *B. cinerea* DNA accurately, with a detection limit of 100 fg, and did not amplify grape DNA. The qPCR and freezing assays detected infection levels of *B. cinerea* in both 2004 and 2005 appropriate to the actual disease severity (22.5% and 1.0% bunch rot, respectively). Grape genotypes varied in their resistance to infection, degree of colonization, and severity of disease. qPCR was not as effective as the freezing assay for detecting infection at early stages of development but was able to quantify fungal colonization, resulting in a new capability for monitoring *B. cinerea* pathogenesis over time. The combined ability of the two assays to detect *B. cinerea* early in berry development and monitor colonization provides a resource for informing disease management decisions and for identifying mechanisms of disease resistance.

Key words: real-time PCR, quiescence, latency, disease resistance

Infections of grape berries by *Botrytis cinerea* Pers.:Fr. [teleomorph = *Botrytis fuckeliana* (de Bary) Whetzel] causes Botrytis bunch rot, affecting fruit quality and yield. The disease can be difficult to manage, partly because of nonsymptomatic, quiescent infection early in berry development, which occurs primarily by deposition of conidia onto inflorescences (Coertze et al. 2001, Keller et al. 2003, McClellan and Hewitt 1973, Viret et al. 2003). Following early season colonization, the fungus enters a quiescent phase (Keller et al. 2003), associated with the production of fungal growth inhibitors by the host (Goetz et al. 1999, Pezet et al. 2003, Sbaghi et al. 1996). During quiescence, further fungal growth and colonization may be halted, and signs of the pathogen are not visible. *Botrytis cinerea* activates from quiescence, further colonizing the berry without visible signs before the initiation of ripening (veraison). The prolonged latent period typically concludes with fungal egress (the first visible sign of infection) around veraison (McClellan and Hewitt 1973). However, there are currently no diagnostic tools available for discriminating between quiescent and actively colonizing *B. cinerea* in developing grapes, hampering studies related to the biology of quiescence and activation. When the fungus finally egresses and sporulates, secondary spread among berries frequently occurs before harvest or during postharvest given conducive environmental conditions (e.g., high humidity) or cultural practices that increase host susceptibility (e.g., nitrogen application) (Zitter 2005).

Management of Botrytis bunch rot most frequently relies on minimizing mechanical damage to grape berries, reducing relative humidity or persistence of fruit wetness, and applying chemical fungicides. Cultural control tactics like pruning, leaf removal, and cluster thinning address these goals by increasing airflow, reducing humidity, improving fungicide coverage, or eliminating physical damage caused by berry contact pressure (Percival et al. 1993). Along these lines, the selection of cultivars or clones with looser grape clusters can provide a quantitative reduction in bunch rot (Marois et al. 1986). Other factors providing partial resistance in some grape genotypes include reduced pore and lenticel number, thicker skin or cuticle to limit pathogen ingress or egress, and production of secondary metabolites that restrict fungal growth (Gabler et al. 2003, Goetz et al. 1999, Percival et al. 1993).

Even with the use of cultural controls and partial disease resistance, in regions where Botrytis bunch rot occurs...
frequently or for the production of table grapes, chemical or biocontrol applications are often deemed necessary. Considerations for chemical or biocontrol include host growth stage, weather conditions, pathogen detection, application timing, and spray coverage as well as day-to-harvest restrictions and post-harvest treatments (Chervin et al. 2005, Deng et al. 2006, Gabler et al. 2005, 2006, Nigro et al. 2006, Romanazzi et al. 2006). In warm, dry regions where bunch rot seldom occurs, growers often choose not to spray botrycides (Nicholas et al. 2003), and the results of an occasional epidemic can be devastating, with no signs of the pathogen until it becomes too late for fungicide or biocontrol application.

Given the poor predictability of bunch rot and the costly controls required to manage disease when it does threaten, growers would benefit from the ability to track pathogen growth inside developing grapes infected by B. cinerea. A bioassay involving berry surface sterilization, killing of host tissue by freezing, and subsequent fungal colonization of the dead berry provides a qualitative assay for detection of B. cinerea infection (Holz et al. 2003) but does not quantify the extent of colonization or discern quiescent from active infections. Antibodies have proven reliable for detection and quantification of B. cinerea in juice and wine (Dewey and Meyer 2004, Meyer et al. 2000) but lack the sensitivity to detect small quantities of fungal biomass early in pathogenesis and berry development. PCR has been successfully used to detect low levels of quiescent infection of inoculated grape berries (Gindro et al. 2005) but lacks precise, quantitative capacity for tracking fungal growth. Quantitative PCR (qPCR), on the other hand, has been harnessed in the detection and quantification of B. cinerea in Arabidopsis (Brouwer et al. 2003, Gachon and Saïdrenan 2004), ornamental plants (Suarez et al. 2005), and strawberries (Mehli et al. 2004), ornamental plants (Suarez et al. 2003, Gachon and Saïdrenan 2004). The detection and quantification of other hand, has been harnessed in Quantitative PCR (qPCR), on the early in pathogenesis and berry development. PCR has been used successfully to detect low levels of quiescent from active infections. Antibodies have proven reliable for detection and quantification of B. cinerea in juice and wine (Dewey and Meyer 2004, Meyer et al. 2000) but lack the sensitivity to detect small quantities of fungal biomass early in pathogenesis and berry development. Quantitative PCR (qPCR) has been successfully used to detect low levels of quiescent infection of inoculated grape berries (Gindro et al. 2005) but lacks precise, quantitative capacity for tracking fungal growth. Quantitative PCR (qPCR), on the other hand, has been harnessed in the detection and quantification of B. cinerea in Arabidopsis (Brouwer et al. 2003, Gachon and Saïdrenan 2004), ornamental plants (Suarez et al. 2005), and strawberries (Mehli 2004), ornamental plants (Suarez et al. 2003, Gachon and Saïdrenan 2004). The detection and quantification of other hand, has been harnessed in Quantitative PCR (qPCR), on the early in pathogenesis and berry development. PCR has been used successfully to detect low levels of quiescent from active infections. Antibodies have proven reliable for detection and quantification of B. cinerea in juice and wine (Dewey and Meyer 2004, Meyer et al. 2000) but lack the sensitivity to detect small quantities of fungal biomass early in pathogenesis and berry development. Quantitative PCR (qPCR) has been successfully used to detect low levels of quiescent infection of inoculated grape berries (Gindro et al. 2005) but lacks precise, quantitative capacity for tracking fungal growth. Quantitative PCR (qPCR), on the other hand, has been harnessed in the detection and quantification of B. cinerea in Arabidopsis (Brouwer et al. 2003, Gachon and Saïdrenan 2004), ornamental plants (Suarez et al. 2005), and strawberries (Mehli

Table 1 Grapevine cultivars and genotypes assayed for natural Botrytis infection in 2004 using the freezing assay and qPCR. Cultivars, breeding lines, and species are organized using data provided from GRIN (USDA-ARS 2008) to categorize approximate affinities suggested by parentage; species are listed alphabetically.

<table>
<thead>
<tr>
<th>PI</th>
<th>Name or species</th>
<th>RBT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Brix&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Stage collected</th>
<th>Egress incidence (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>qPCR incidence (%)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>qPCR avg ratio&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>588162 Remaly 63-33A</td>
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<td>26.7</td>
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<td>Closure Veraison Mature</td>
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<td>5.6E-08</td>
<td>1.2E-07</td>
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<tr>
<td>188582 Castel 19-637</td>
<td>2 23.6 5</td>
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<td>64.3</td>
<td>0.0</td>
<td>Closure Veraison Mature</td>
<td>33.3 15.8 91.7</td>
<td>3.1E-07</td>
<td>2.5E-07</td>
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<td>Pea</td>
<td>7.4</td>
<td>0.0</td>
<td>Closure Veraison Mature</td>
<td>12.4 25.0 87.5</td>
<td>4.4E-08</td>
<td>4.9E-08</td>
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<tr>
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<td>Pea</td>
<td>20.3</td>
<td>7.1</td>
<td>Closure Veraison Mature</td>
<td>9.2 31.3 43.8</td>
<td>3.1E-12</td>
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<td>1.9E-06</td>
<td>2.3E-06</td>
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<tr>
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<td>8.3E-11</td>
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<tr>
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<td>Pea</td>
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<td>0.0</td>
<td>Closure Veraison Mature</td>
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<td>3.1E-07</td>
<td>2.5E-05</td>
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<td>2 14.2 3</td>
<td>Pea</td>
<td>15.9</td>
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<td>Closure Veraison Mature</td>
<td>14.0 6.3 7.1</td>
<td>4.7E-12</td>
<td>2.6E-07</td>
</tr>
<tr>
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<td>1.9E-07</td>
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<td>Pea</td>
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<td>0.0</td>
<td>Closure Veraison Mature</td>
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<td>0.0</td>
<td>Closure Veraison Mature</td>
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<td>1.6E-09</td>
<td>5.6E-12</td>
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<td>597128 Bell</td>
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<td>Pea</td>
<td>18.7</td>
<td>0.0</td>
<td>Closure Veraison Mature</td>
<td>0.0 16.7 85.7</td>
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<td>Other interspecific hybrids</td>
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<tr>
<td>279999 Kober SBB</td>
<td>4 20.4 0</td>
<td>Pea</td>
<td>4.2</td>
<td>31.3</td>
<td>Closure Veraison Mature</td>
<td>36.0 12.5 43.8</td>
<td>6.1E-08</td>
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</tr>
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<td>588599 BR 14</td>
<td>2 21.0 3</td>
<td>Pea</td>
<td>15.5</td>
<td>0.0</td>
<td>Closure Veraison Mature</td>
<td>0.0 12.5 87.5</td>
<td>2.6E-06</td>
<td>3.1E-07</td>
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</tbody>
</table>

"...from the Berry samples in the Taqman qPCR assay, calculated as the number of samples that showed evidence of Botrytis DNA amplification.

Incidence of Botrytis infections is calculated as the number of clusters showing symptoms, averaged across 10 clusters: 0 = 0%, 1 = 0.1-4.9%, 2 = 5-9.9%, 3 = 10-24.9%, 4 = 25-59.9%, 5 = 60-100%.

Materials and Methods

Plant material. Thirty-two fruiting Vitis accessions from the USDA-ARS, National Plant Germplasm System (NPGS) collection in Geneva, NY, were chosen from two arbitrarily selected vineyard rows (Table 1). Each accession is represented by two adjacent replicate vines, cane pruned and trained to the Umbrella-Kniffin system. In 2004 and 2005, clusters were harvested from these accessions at Eichhorn-Lorenz stages 31 (pea sized), 33 (bunch closure), 35 (veraison), and 38 (mature). From each of two replicate vines and for each of the four developmental stages, two clusters were harvested, flash-frozen in liquid nitrogen, and stored at -80°C for qPCR analysis. Each of these 16 harvested clusters per vine was the basal cluster of a primary shoot. Collection timepoints (e.g., veraison) were randomly assigned to clusters. Disease severity ratings were taken on 10 of the remaining clusters two weeks following the final sample collection, based on the percentage of the cluster showing Botrytis bunch rot symptoms and categorized in the following scale: 0 = 0%, 1 = 0.1-4.9%, 2 = 5-9.9%, 3 = 10-24.9%, 4 = 25-59.9%, 5 = 60-100%.

Weather data. Daily precipitation data were obtained from a weather monitoring station (NOAA benchmark weather station, 3031840) located 2.5 km from the vineyard at the New York State Agricultural Experiment Station, Geneva. Historical flowering dates are published for each accession at USDA-ARS Germplasm Resources Information Network (GRIN) (USDA-ARS 2008). From the median bloom date to the median maturity date for 2004 and 2005, total precipitation was calculated by summing the daily precipitation totals.

Freezing bioassay. Clusters for the freezing bioassay were harvested into plastic bags and kept cool over ice until processing. Individual clusters were processed as described elsewhere (Holz et al. 2003) with the following modifications. Clusters were surface sterilized for 2 min in 0.6% sodium hypochlorite with 0.001% Tween-20, followed by 30 sec in 70% ethanol and a sterile water rinse. Clusters were transferred to a sterile mesh screen overlaying sterile paper towels and were air-dried in a laminar flow hood prior to incubation in a plastic flat with transparent lid at -12°C for 2 hr, which was sufficient to kill host tissue and allow growth of the fungus. Flats were then transferred to lighted shelving, paper towels saturated with sterile distilled water to establish high relative humidity, and incubated for 10 days at room temperature with a 12-hr photoperiod. Data recorded were the total number of berries per cluster and the incidence of egress from each of three berry regions (base, cheek, or stylar end) (Holz et al. 2003). The number of discrete egress points (up to 3 per berry) was divided by the total number of berries to calculate incidence of egress as an approximation of incidence of infection.

DNA isolation and normalization. Each qPCR sample was a composite of five arbitrarily processed berries with two samples processed for each of the four replicate clusters. Sampled berries were ground in liquid nitrogen by mortar and pestle, and two 400-μg subsamples per 5-berry sample were transferred into 96-well deepwell masterblocks (Greiner, Monroe, NC). Each well contained two 5-mm stainless-steel beads (OPS Diagnostics, Lebanon, NJ). Tissue was maintained in extraction buffer (Lin and Walker 1997) at -80°C until DNA isolation. Prior to DNA isolation, samples were thawed and further homogenized by shaking in a GenoGrinder 2000 for two 1-min intervals at 1500 rev/min. Total DNA (host and associated microorganisms) was then isolated using a published protocol (Lin and Walker 1997) scaled to 96-well format. DNA concentrations were quantified and normalized to 10 ng/μL on a Tecan Genesis RSP 150 liquid handler using a PicoGreen assay (Invitrogen, Carlsbad, CA) and stored at -20°C for up to one month until qPCR analysis.

Negative control and standard curve samples for qPCR were obtained as follows. Leaf tissue for the negative control (no B. cinerea DNA) was collected from Vitis vinifera cv. Pinot blanc (2004) or Pixie (2005) grown from green cuttings in a growth chamber. Mycelia from B. cinerea isolate Be9 (Robbins Farm, Geneva, NY; 200x) grown on PDA were harvested, and -75 mg of mycelia were ground in liquid nitrogen in a mortar and pestle. DNA from negative controls and from B. cinerea Be9 was isolated using the Lin and Walker (1997) protocol, quantified, and normalized as described above. A standard curve was created by mixing equal volumes of Be9 DNA and negative control DNA, producing two replicate 5-fold dilution series (from 1:1 to 1:15,625) with negative control DNA as the diluent, and combining the replicate tubes. For samples collected in 2005, a 10-fold dilution series over seven orders of magnitude was created in the same manner.

Inoculated control clusters. To test whether lack of detection was due to low natural incidence of quiescent infection, quiescent infections were established in 10 arbitrarily selected clusters of V. vinifera Pinot blanc grown in a commercial vineyard in Lodi, NY in 2005. Conidial suspensions of B. cinerea (105 conidia/mL) were sprayed onto clusters at 90% capfall as described by Zitter (2005). Inoculated clusters were bagged in moisture-resistant #401 pollinating bags (Lawson Bags, Northfield, IL) to protect them from fungicide applications and harvested at veraison on 28 July. Three replicate subsamples per cluster were analyzed by qPCR.

Quantitative PCR assays. Primers for species-specific amplification of B. cinerea DNA were previously reported (Rigotti et al. 2002). These primers (C729+: AGCTCGAGAGATCTCTGTA and C729-: CTGCAATGTTCT-GCGTGGAA) were used directly in a quantitative PCR assay using iQ SYBR Supermix (Bio-Rad, Hercules, CA). For qPCR amplification, 25-μL reaction mixtures containing 12.5 μL iQ SYBR Supermix, 2 μL normalized DNA, and 0.4 μM each primer were assembled in 96-well PCR plates, and reactions were run on a Bio-Rad iQ system. After incubation at 95°C for 2 min, 40 cycles of three-step amplification were run at 95°C for 30 sec, 55°C for 45 sec, and 72°C for 45 sec.

A second assay based on Taqman chemistry was designed for the same intergenic region. The following primers and labeled probe were designed using Primer Express software (Applied Biosystems, Foster City, CA): BcTaq424F: 5'-GCTTCCCCGTATCGAAGA-3'; BcTaq49Ir: 5'-CGAACGGCCAGGTCATCTG3'; and BcFamP: 5'-6-Fam-CCCTAGATTTGATTTTACCCTCA: BcTaq424F: 5'-GCTTCCCCGTATCGAAGA-3'. For qPCR amplification, 15-μL reaction mixtures containing 7.5 μL iQ Supermix, 2-μL normalized DNA, 0.5 μM each primer, and 0.33 μM probe were assembled 96-well PCR plates and reactions were run on a Bio-Rad iQ system. After incubation at 95°C for 2 min, 40 cycles of three-step amplification were run at 95°C for 30 sec, 55°C for 45 sec, and 72°C for 45 sec.

Detection of infection and statistical analyses. Replicate qPCR reactions were run for each subsample in 96-well PCR plate format. Each plate contained two replicates of the dilution series and two negative controls of the reaction mix with grape DNA only. A regression analysis was conducted for each plate based on ratios of
B. cinerea DNA to grape DNA in the dilution series, and DNA ratios for all amplified samples within a plate were calculated based on the regression. Standard least squares linear regression analysis was conducted using JMP 7 (SAS Institute, Cary, NC). For analysis of variance, predictors included independent experiments (‘experiment’) and the known concentration of Botrytis DNA (as a log of the picogram value) ([Botrytis DNA]). The response variable was threshold cycle ‘Ct.’

Results

Precipitation and natural incidence and severity. From bloom to maturity, 2004 had 66.9% more precipitation than 2005 (41.5 cm and 24.8 cm, respectively) (Figure 1). To indicate relative disease pressure in each year, the average incidence of detection or severity was calculated across all samples for each assay. In the wet 2004 season, the incidence of B. cinerea infection was high in the germplasm collection: 17.6% of berries were infected as determined by the freezing assay and 23.3% were positive by qPCR. Severity of Botrytis bunch rot at maturity was also high, averaging 22.5%. In the much drier 2005 season, little B. cinerea was detected in the germplasm collection: 0.3% of berries were infected as determined by the freezing assay and 1.5% were positive by qPCR. Disease severity at maturity was less than 1% for the genotypes rated in 2005.

Freezing bioassay. Natural infection by B. cinerea was confirmed by the freezing assay for each genotype tested in 2004 (Table 1). Some genotypes maintained low incidence of egress throughout their development (e.g., V. riparia Rem 65-76, V. vulpina Rem 36-77, and Vergennes), while others had high incidence of egress throughout berry development (e.g., Remaily 63-33A and Castel 19-637), as detected by the freezing assay. However, for the majority of genotypes, incidence of egress was stage-specific. In aggregate, the accessions exhibited generally level incidences over the course of the growing season, from pea sized (19.9%), to bunch closure (13.9%), to veraison (19.1%), to maturity (16.0%), and for interspecific vinifera hybrids, the incidence of egress was flat or declining through the growing season (Figure 2). In 2005, B. cinerea egressed from only 45 discrete locations on the 14,423 berries assayed, including egress from 31 locations for 4293 (0.7%) pea-sized berries and from 14 locations for 3825 (0.4%) berries at bunch closure.

qPCR assays. A SYBR Green qPCR assay was developed using previously published, species-specific PCR primers (Rigotti et al. 2002). The assay produced a linear standard curve (R² > 0.95) ranging from 10 ng to 3.2 pg of Botrytis DNA in a background of grape DNA (20 ng total DNA per reaction). However, low natural levels of infection by Botrytis could not be discriminated reliably from nonspecific amplification of the negative control (grape DNA only) with this assay (data not shown).

A qPCR assay based on Taqman chemistry was developed based on the species-specific sequence and did not amplify grape DNA within 80 PCR cycles. In four independent experiments, the assay reproducibly produced a linear standard curve (R² > 0.95 for each experiment).

Figure 1 Precipitation and air temperature data for the 2004 and 2005 growing seasons. Bloom timing for bloom categories from Table 1 are estimated based on GRIN data (USDA-ARS 2008) and based on an average in 2005 of five days delayed bloom observed for 79 other accessions (Gee and Cadle-Davidson, unpublished data, 2004-2005). No data exists in GRIN or elsewhere for veraison timing, but a rough estimate of the range of dates for these diverse accessions is provided.
for a 10-fold standard curve ranging from 10 ng to 1 pg of *Botrytis* DNA in a background of grape DNA (Figure 3). Although the concentration of *Botrytis* DNA was highly significant (*p* < 0.0001) and explained 96.3% of the variation in threshold cycle (Ct), experiment-to-experiment variation was also significant (Table 2). Standard curves were assayed for 26 additional, independent PCR plates, and in each case the R² value exceeded 0.95. The limit of detection was 100 fg, although known concentrations below 3.2 pg did not have linear responses in Ct for some dilution series and did not amplify in some replicates (Figure 4). The negative control (reaction mixture with grape DNA only) did not amplify in any of the experiments.

The Taqman assay was used to detect natural infections by *Botrytis*. Average detectable incidence increased over the course of the 2004 growing season, from pea sized (7.7%), to bunch closure (12.4%), to veraison (22.8%), to maturity (46.0%) (Table 1, Figure 2). For most genotypes, *Botrytis* was rarely detected at pea-sized stage or bunch closure but was readily detected at veraison and/or maturity (Table 1). Three genotypes of *V. amurensis* had almost no detectable infection as assayed by qPCR (2/100 samples positive).

![Figure 2](image)

**Figure 2** Incidence of *Botrytis* detection by qPCR and the freezing assay in *Vitis vinifera* interspecific hybrids through the growing season. For each of the seven interspecific *vinifera* hybrids assayed in 2004, the incidence of detection was plotted for each growth stage as determined by qPCR (A) and freezing assay (B). (Regression lines and R² values in Excel.)
Because of the low levels of Botrytis bunch rot in 2005 as detected by the freezing assay, only 530 samples were arbitrarily selected across each developmental stage to be tested using qPCR to detect Botrytis, with only eight (1.5%) testing positive for Botrytis infection. Conversely, 10 of 30 Pinot blanc qPCR samples from clusters inoculated at bloom in 2005 tested positive at veraison for Botrytis infection.

**Discussion**

In 2004 and 2005, the standard freezing assay (Holz et al. 2003) and a qPCR assay were applied in parallel for the early detection of natural *B. cinerea* infections in diverse germplasm. Together, the contrasting data from the two assays provide interesting insight into *B. cinerea* biology in developing berries. The strength of the freezing assay lies in the enumeration of how many berries are infected, while the qPCR assay allows relative quantification of the extent of colonization and thus, when applied over time, detection of active colonization (Table 1, Figure 2).

For example, pea-sized berries of Remaily 63-33A and Castel 19-637 had been infected early by *B. cinerea* (freezing assay) but at levels not detected by qPCR. The fungus began to further colonize berries prior to bunch closure (qPCR). Colonization and incidence levels remained similar from bunch closure until veraison, after which massive colonization of the berry happened in Castel 19-637 (qPCR) and resulted in severe Botrytis bunch rot. Meanwhile, Remaily 63-33A maintained colonization levels similar to those at bunch closure and veraison through maturity (qPCR) and had little bunch rot. Among resistant individuals, these assays identified germplasm with low incidence of infected berries and little colonization (e.g., Vergennes and Eclipse) that could be useful in breeding programs. However, the synthesis of incidence, colonization, and disease was not straightforward for all genotypes, as for instance, high levels of colonization (qPCR) did not always result in disease (e.g., Eden).

In the dry year of 2005, minimal infections (as detected by the freezing bioassay) allowed the opportunity to test for the propensity of qPCR to provide false positive detections. The assay functioned accurately, detecting *B. cinerea* in only 1.5% of the samples tested (compared with 23.3% in 2004). Similarly, with the freezing assay only 0.3% of berries tested positive for *B. cinerea* infection in 2005 (compared with 17.6% in 2004), averaged across all developmental stages. Thus, it appears that the assays are generally good indicators of infection.

However, neither assay was universally effective for detecting infection across all of the genotypes and species tested, particularly in the wild species. For example, infection of all four genotypes of *V. amurensis* was regularly detected at all developmental stages by the freezing assay but rarely detected by qPCR in all *V. amurensis* genotypes except PI 588635. Conversely, infection was detected by qPCR in 10 of 12 samples of *V. riparia* Rem 65-76 at veraison, but in none of the berries examined with the freezing assay.

The inability to detect *B. cinerea* infections by the freezing assay when they are detectable by qPCR suggests that in some genotypes, fungal growth inhibitors may remain at inhibitory levels even after death of the berry tissues precludes their further synthesis. Previous studies have suggested that growth inhibitors could explain the prolonged quiescent period in grape berries and have shown that the presence of some candidate compounds for growth inhibition follow berry development-related expression patterns (Goetz et al. 1999, Kulakioti et al. 2004, Perret et al. 2003, Pezet et al. 2003, Sbaghi et al. 1996). If these or other inhibitory compounds remain active after tissue freezing, their presence could result in experimental artifacts including the lack of *B. cinerea* detection by the freezing assay.

As a result of the complexity of experiments in the current study, in which the diverse germplasm studied has divergent phenology and uncharacterized timing of ontogenic resistance to infection, weather events could be expected to affect susceptibility and pathogenesis differently for each accession. Because only two replicate vines are maintained in the ARS germplasm repository, small samples sizes limited the ability to fully address biological variation of natural infections. Therefore, while this study provides insight into tools available for studying quiescence and highlights accessions of particular interest, additional studies with more detailed focus and increased biological replication should be pursued to better characterize resistance and the biology of quiescence.

Quantitative PCR is becoming a common tool for detection and quantification of plant pathogens and is particularly useful for pathogens with long latent phases or quiescent interactions. In the current study, two qPCR assays were developed for the detection of *B. cinerea* DNA in the background of grape DNA. While the SYBR Green assay was able to detect and quantify *B. cinerea* with linear response to a dilution series, the assay was not specific enough to prevent signal amplification in the grape DNA-only negative control. The qPCR assay based on Taqman chemistry overcame this limitation and allowed linear detection down to 3.2 pg *B. cinerea* DNA, with a detection limit of 100 fg. The known amount of *B. cinerea* DNA explained 96.3% of the variation in the C<sub>T</sub>. However, experiment-to-experiment variation was also statistically significant, necessitating normalization of within-experiment data before comparisons between experiments.

One fault of the Taqman-based qPCR assay developed in this study is that it often failed to detect very low concentrations of quiescent Botrytis infection, particularly in younger berries. This was notable in 2004 with genotypes like Castel 19-637, for which the freezing assay detected 64.3% incidence of infected pea-sized berries but the qPCR assay failed to detect any *B. cinerea* DNA. Additional colonization presumably resulted in detectable quantities of *B. cinerea* in Castel 19-637 by bunch closure.
as detected by both qPCR and the freezing assay. With a Taqman assay similar to that used in the current study, it was shown that very dilute samples not only have later amplification (higher C\textsubscript{T} values) but also have lower incidence of successful amplification (Hayden et al. 2006), and the standard curve results presented here reflect this finding (Figure 4). Similarly, the incidence of Botrytis detection by qPCR increased continuously through the growing season in naturally infected \textit{V. vinifera} interspecific hybrids (Figure 2). This suggests nondetectable levels of colonization in pea-sized berries but that fungal colonization outpaced berry cell division throughout berry development, even during quiescence. Additional studies are needed to confirm that colonization occurs continuously throughout quiescence.

Monitoring of \textit{B. cinerea} infection and colonization by the qPCR and freezing assays could be used not only to study fungal pathogenesis and identify novel sources and mechanisms of disease resistance but also for application in commercial vineyards as IPM scouting tool for informing management decisions. The freezing assay was better than qPCR at detecting \textit{B. cinerea} infection in pea-sized berries. However, the incidence of detection by the freezing assay remained level or declined through the growing season, suggesting that most of the natural infections were quiescent infections early in berry development. Thus, growers or consultants could use the freezing assay to determine whether quiescent infections are at sufficiently high incidence for an epidemic, and, when they are, a quantitative assay like qPCR could be used for monitoring colonization and informing management decisions.

**Conclusion**

This study was designed to monitor quiescence and activation of natural \textit{B. cinerea} infections across a panel of 32 diverse germplasm accessions throughout berry development. A novel qPCR assay was developed and applied in coordination with the standard freezing assay, providing direction for in-depth studies of the biology of quiescence as well as potential sources of bunch rot resistance.

**Literature Cited**


