Early detection of natural *Botrytis* infections

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Introduction. *Botrytis* bunch rot, which affects fruit quality and yield, can be difficult to manage due in part to non-symptomatic, quiescent infection early in berry development. For early infection, individual conidia of *B. cinerea* are deposited onto inflorescences (McClellan and Hewitt 1973; Coertze et al. 2001). Following colonization, the fungus enters a quiescent phase, during which further fungal growth and colonization may be halted without visible signs of the pathogen. *B. cinerea* activates from quiescence prior to the initiation of veraison, further colonizing the berry, and 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 the study of quiescence and activation. When the fungus finally egresses and sporulates, secondary spread between berries will occur under conducive environmental conditions (*e.g.*, high humidity, high nitrogen) before and/or after harvest (Zitter 2005).
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*. Existing technologies for pathogen detection, although useful for some applications of studying pathogen biology and informing grower decisions, have their limitations including insufficient sensitivity or the inability to distinguish quiescent from active infections (Meyer et al. 2000; Holz et al. 2003; Dewey and Meyer 2004; Gindro et al. 2005). The purpose of this study was to develop a quantitative PCR (qPCR) method for detecting and quantifying small quantities of *B. cinerea* DNA in the context of developing grape berries and to identify the strengths and weaknesses of qPCR in comparison with a standard freezing bioassay. The qPCR and freezing assays were applied across a panel of 32 diverse germplasm accessions throughout berry development to test cross-species effects and to identify temporal variation in the activation from quiescence as well as new sources and potential mechanisms of resistance.

Materials and Methods. Thirty-two fruiting *Vitis* accessions from the USDA-ARS, National Plant Germplasm System collection in Geneva, NY were included in the study. Each accession is represented by two adjacent replicate vines, cane pruned and trained to the Umbrella-Kniffin system. In 2004 and 2005, clusters were collected from these accessions at Eichhorn-Lorenz stages 31 (pea sized), 33 (berry touch), 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 -80C for qPCR analysis, and two clusters were harvested and tested for *Botrytis* infection using the freezing assay.
Individual clusters for the freezing assay were processed as described in Holz et al. (2003) with the minor modifications. Data recorded were the total number of berries per cluster and the incidence of egress from discrete berry locations (*i.e.*, bases, cheeks, or stylar ends). The number of discrete egress points was divided by the total number of berries to calculate incidence of egress.
Each qPCR sample was a composite of five arbitrarily processed berries, which were homogenized prior to DNA isolation (Lin and Walker 1997), quantification, and normalization. Negative control (no *B. cinerea* DNA) and standard curve samples for qPCR were obtained by isolating DNA from *Vitis vinifera* cv. 'Pinot Blanc' or 'Pixie' leaf tissue and from *B. cinerea* mycelia. The qPCR assay based on Taqman® chemistry was designed for a previously published intergenic region (Rigotti et al. 2002). The following primers and labeled probe were designed using Primer Express software (Applied Biosystems): BcTaq424f: 5'-GCTTCCCCCGTATCGAAGA-3'; BcTaq491r: 5'-CGAACGGCCAGGTCATCT-3'; and BcFamP: 5'- 6-Fam™-CCCTAGATTTGATTTTACCCTTCGCGTGG- BHQ®-1 -3'. Based on a Blastn query, this 68-nt region should be specific to *Botrytis spp.* and preferential to *B. cinerea.* For qPCR amplification, 15µL reaction mixtures containing 7.5µL iQ Supermix, 2µL normalized DNA, 0.75µL each primer, and 0.50µL probe were assembled in 96-well PCR plates, and reactions were run on a Bio-Rad iQ system. After incubation at 95C for 8min, 60 cycles of two-step amplification were run at: 95C for 15s and 60C for 40s.

Results and Discussion. A qPCR assay was developed based on the species-specific sequence and did not amplify grape DNA within 80 PCR cycles. The assay produced a linear standard curve (R^2=0.95-0.99) ranging from 10ng to 3.2pg of *Botrytis* DNA in a background of grape DNA (20ng total DNA per reaction) in all 30 PCR plates assayed. The limit of detection was 100fg, though standard concentrations below 3.2pg did not have linear responses in threshold value and did not amplify in some replicates. The negative control (reaction mixture with grape DNA only) did not amplify in any of the experiments.
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%. Natural infection by *B. cinerea* was confirmed by the freezing assay for each genotype tested in 2004. In the much drier 2005 season, little *B. cinerea* was detected in the genetic resources 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.
In 2004, some genotypes had high incidence of infection throughout berry development (*e.g.*, Remaily 63-33A and Castel 19-637) while others maintained low incidence of detectable infection throughout their development (*e.g.*, *V. riparia* Rem 65-76, *V. vulpina* Rem 36-77, and Vergennes). However, for the majority of genotypes, incidence of detection was stage-specific. In aggregate, the accessions exhibited generally level incidences over the course of the growing season as detected by the freezing assay, from pea-sized (19.9%) to berry touch (13.9%) to veraison (19.1%) to maturity (16.0%), and increasing incidence according to the qPCR assay, from pea-sized (7.7%) to berry touch (12.4%) to veraison (22.8%) to maturity (46.0%). While both assays are generally predictive of fungal infection, insufficient ability to detect infection in pea-sized berries by qPCR and inability to quantify colonization by the freezing assay suggest a combination of the two assays would provide the most effective insight into *B. cinerea* biology and disease prediction.

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