Viticulture Consortium – East

Progress Report for 2006
As the Final Report, this submission contains results from both years of the project

MOLECULAR EPIDEMIOLOGY AND CHARACTERIZATION OF *BOTRYTIS* QUIESCENCE IN DEVELOPING GRAPE BERRIES

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Summary:

Grape clusters naturally infected by *Botrytis cinerea* were harvested and processed to detect quiescent infection and activation using a standard freezing bioassay and a quantitative PCR assay for two growing seasons. In a year with high incidence of Botrytis bunch rot (2004), the freezing assay was able to detect quiescent infection earlier than qPCR, but the freezing assay had very high variability and little statistical power. The qPCR assay was reproducibly capable of detecting several cells of *B. cinerea* in the background of a grape berry and of predicting with less than 5% error the precise quantity of *B. cinerea* in a biological sample. In a year with low incidence (2005), *B. cinerea* was rarely detected by the freezing assay (0.1% of samples vs. 25.3% of samples in 2004) or by qPCR (1.4% of samples vs. 23.3% in 2004), suggesting that both assays are predictive of vineyard disease. Used in combination, a biological assay (*e.g.* the freezing assay) and the qPCR assay have the power to describe the status of *B. cinerea* infection and colonization and can inform studies of disease resistance and the biology of quiescence.

Experimental design:

In 2004 and 2005, we harvested clusters from thirty-two accessions from the PGRU germplasm collection at Eichhorn-Lorenz stages 31 (pea sized), 33 (bunch closure), 35 (veraison), and 38 (harvest). 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, and two clusters were harvested and tested for *B. cinerea* infection using a standard freezing bioassay (Holz et al. (2003) Plant Disease 87:351-358), as depicted in Fig. 1.

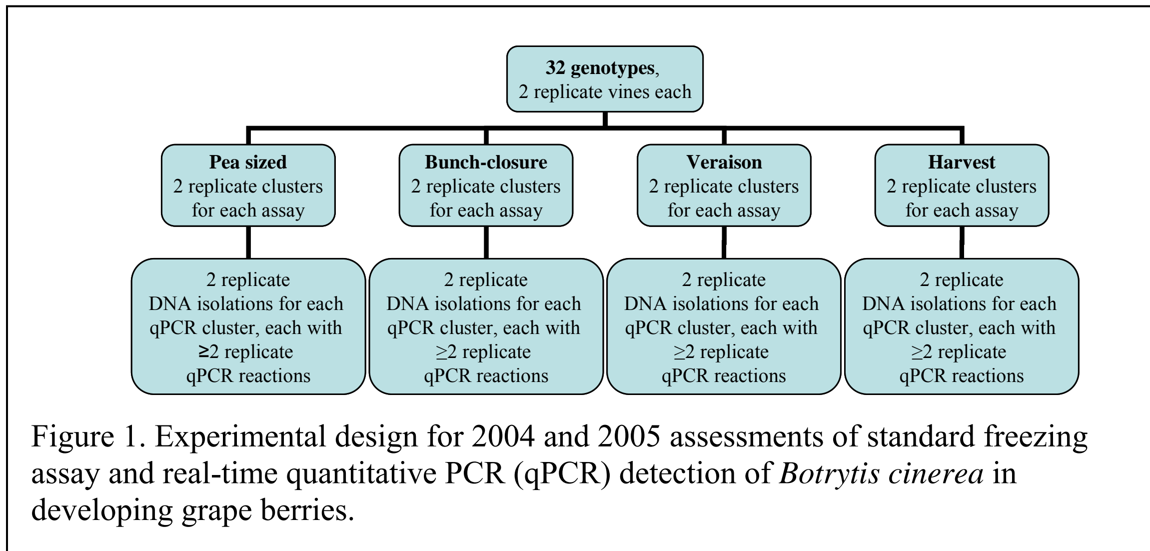

Figure 1. Experimental design for 2004 and 2005 assessments of standard freezing assay and real-time quantitative PCR (qPCR) detection of *Botrytis cinerea* in developing grape berries.

Freezing bioassay:

The weather conditions in 2004 proved to be conducive to significant levels of Botrytis bunch rot. As determined by the standard freezing assay in 2004, the incidence of infected berries averaged across developmental stages ranged from 3.9 to 82.6% for the accessions tested. This sensitive assay was capable of detecting quiescent infection in a large proportion of berry samples. All of the 32 genotypes tested were naturally infected by *B. cinerea*. Whereas previous studies on Merlot and Dauphine have shown a decrease in incidence over the growing season, we saw an increase when averaged across all genotypes (Table 1). However, the assay results in data with high variance and frequently perplexing temporal patterns of colonization. For example, in Eclipse, Bell, and Isabella, berries were infected at pea-size, cured by berry touch, and re-infected by veraison.

The environment in 2005 was not conducive for Botrytis bunch rot in the PGRU experimental vineyard where the research was conducted. Only 0.1% of berries tested positive for *B. cinerea* infection, averaged across all developmental stages.

Spatial assay of infection was not revealing. The total number of berries per cluster and the number of *B. cinerea*-infected pedicels, bases, cheeks, stylar scars, and discrete rachis locations were recorded. In 2004, some berries were preferentially infected at the stylar end and some at the base; few genotypes allowed fungal emergence from the cheeks. However, whether the outgrowth of the fungus at these growth stages says anything about its ingress or colonization is dubious. In 2005, significant patterns could not be discerned due to low incidence of infection.

Quantitative PCR (qPCR):

A limitation of the freezing detection assay is that other fungi emerge from berries (Dugan et al. (2002) Journal of Phytopathology 150:375-381). Identifying *B. cinerea* in a background of *Aspergillus*, *Cladosporium*, *Penicillium* and other fungi requires extensive training and patience. Misidentification has the potential to confound results. A species-

specific assay for *B. cinerea* would alleviate these problems. Therefore, a real-time quantitative PCR (qPCR) assay was developed to assess the results of the freezing bioassay.

Table 1. Average *Botrytis* incidence detected by the standard freezing assay and the qPCR assay on clusters of a subset of accessions collected in 2004.

	597130 [a] Eclipse [b]			597128 Bell			588207 Isabella		
	Freezing[c]	Index[d]	Incidence[e]	Freezing	Index	Incidence	Freezing	Index	Incidence
Pea	14.3%	0.0E+00	0/12	44.1%	0.0E+00	0/12	31.4%	0.0E+00	0/4
Touch	3.9%	6.0E-11	1/12	8.5%	6.2E-11	2/12	0.0%	0.0E+00	0/8
Veraison	8.5%	6.2E-08	1/12	17.1%	1.4E-06	4/16	58.8%	8.3E-11	1/8
Harvest	20.7%	2.6E-09	7/14	nd[f]	2.9E-05	12/14	nd	1.3E-06	4/8

	597270 Paul Revere			181639 Seyve-Villard 23.512			588128 Vergennes		
	Freezing	Index	Incidence	Freezing	Index	Incidence	Freezing	Index	Incidence
Pea	16.4%	0.0E+00	0/14	20.6%	3.1E-12	1/14	12.1%	0.0E+00	0/8
Touch	6.3%	6.8E-13	0/12	17.9%	1.2E-07	2/12	19.3%	1.9E-07	1/8
Veraison	12.6%	1.9E-07	2/10	3.2%	7.8E-08	5/16	13.4%	2.6E-14	1/12
Harvest	nd	3.2E-06	7/18	nd	3.4E-07	6/20	1.8%	1.6E-07	4/12

	588141 *Vitis acerifolia*			588635 *Vitis amurensis*			All genotypes	
	Freezing	Index	Incidence	Freezing	Index	Incidence	Freezing	Incidence
Pea	0.0%	4.2E-09	4/15	14.9%	0.0E+00	0/16	19.9%	26/339 (7.7%)
Touch	12.4%	4.7E-08	1/20	28.1%	0.0E+00	0/16	22.5%	46/372 (12.4%)
Veraison	10.3%	3.5E-12	2/12	52.2%	9.8E-10	1/16	26.0%	90/394 (22.8%)
Harvest	35.0%	6.6E-07	9/12	67.8%	2.3E-06	6/10	32.9%	195/424 (46.0%)

[a] National Plant Germplasm PI accession number.
[b] Cultivar or species name, or all genotypes combined.
[c] Average percent *Botrytis cinerea* incidence detected by the standard freezing assay
[d] Average calculated ratio of *B. cinerea* DNA to grape DNA in samples testing positive for *B. cinerea* by the qPCR assay.
[e] Incidence of *B. cinerea* detection by qPCR.
[f] Not determined because a fruit fly infestation destroyed some harvested samples prior to data collection.

In the first year, we adapted primers that specifically amplify *B. cinerea* DNA (Rigotti et al. (2002) FEMS Microbiology Letters 209:169-174) for a SYBR Green-based qPCR assay to quantify *B. cinerea* DNA in the naturally infected berries described above. However, the SYBR Green protocol was not sensitive enough to detect minute quantities of *B. cinerea* in the background of grape DNA. Therefore, we developed a Taqman assay, which incorporates a template-specific fluorescently labeled probe in addition to the two template-specific primers. Using this new protocol, we were able to detect a single cell of *B. cinerea* in the background of 15,000 grape cells.

For this Taqman qPCR protocol, five berries per sample were arbitrarily selected, and total DNA (host and associated microorganisms) was isolated using the protocol of Lin and Walker (HortScience 32:1264-1266). The resulting DNA concentration was quantified using a PicoGreen assay and normalized to a concentration of 10 ng/uL using a liquid handler robot. Following normalization, the relative DNA content from *B. cinerea* was compared using qPCR, as follows.

Standard curves were developed and included in each 96-well qPCR experiment. *B. cinerea* was grown in culture, dried, weighed, and DNA extracted; DNA from uninfected grape berries was isolated, quantified, and normalized as described above. A five-fold template dilution series of *B. cinerea* DNA to uninfected grape DNA from 1:1 to 1:15,625 was established. To assure that the product quantified was the correct gene and specifically from *B. cinerea*, a confirmational PCR reaction was run in the absence of fluorescent probes for 8 randomly-selected positive samples, and the products were purified and sequenced.

In samples from 2004, the qPCR assay failed to detect any *B. cinerea* infection in pea-sized berries in 23 out of 32 genotypes. Because this could be due to PCR inhibitors present in the isolated DNA, we developed a Taqman assay for grape DNA to be run in parallel as a positive control for qPCR amplification and showed that our DNA isolations were clean. The 2004 results of qPCR, when combined with frequent detection of *B. cinerea* using the standard freezing assay, suggested that our infrequent detection of *B. cinerea* infection in pea-sized berries by qPCR was due to the low levels of fungal colonization at this early timepoint.

In 2005, the low severity allowed us to test whether the qPCR would correctly fail to detect disease in a year with low incidence, and it functioned as hoped, detecting *B. cinerea* in only 1.5% (8/530) of the samples tested (compared with 23.3% in 2004). Fewer than 1% of samples from pea sized through veraison tested positive, with one cultivar frequently testing positive for *B. cinerea* at harvest but not before. Thus, it appears that the qPCR assay, like the freezing bioassay, is predictive of disease.

Because of the low levels of natural infection or activation in 2005, we included positive control samples of Pinot blanc that we had inoculated at 90% bloom in 2005 and collected one week after veraison. These samples had a greater incidence of infection (10 out of 30), and the average colonization index following veraison was similar to other varieties in 2004 (1.4E-08). This data in combination with the freezing assay suggests that natural infections were not able to establish and maintain colonization in 2005, and that the lower incidence of quiescent infections in 2005 was sufficient to explain lower incidence of disease.

Conclusions:

The long-term goals of this project are to develop assays for early detection of *B. cinerea* infection, quiescence, and activation in developing grape berries and to identify novel and effective control tactics to manage disease. We demonstrated that the predominant existing technique for detection of *B. cinerea* in developing grape berries

often produces artifacts leading to incorrect assessment of infection (Table 1). To be able to detect small quantities of the pathogen in the background of the relatively large mass of the berry, we developed a quantitative, real-time PCR technique (qPCR) to amplify DNA specifically from *B. cinerea*. This assay is capable of predicting with less than 5% error the precise quantity of *B. cinerea* in biological samples.

The purpose of these technically demanding approaches is simply to "look" inside a developing grape berry and monitor the growth or quiescence of *B. cinerea*, and each assay has its merits, requiring a different subset of expertise, providing a different type of qualitative or quantitative detection of the pathogen, and having its own artifacts in specific host genotypes under high disease pressure. The standard freezing assay provides a qualitative detection of the presence or absence of *B. cinerea* and other fungi, but requires expertise in fungal identification. The qPCR technique, while requiring molecular biology expertise, allows us not only to look inside and know that we are seeing *B. cinerea*, but also to measure how much fungal biomass there is (based on DNA content) and therefore to know when and where *B. cinerea* has further colonized the berry. The two approaches provide complementary information for the improved understanding of *B. cinerea* biology and epidemiology. The tools developed can be applied in production to scout for presence of inoculum and in research to enhance epidemiological knowledge or to identify specific components of disease resistance.

Appendix:

a. Impact Statement:

A quantitative PCR (qPCR) tool was developed for early detection of *B. cinerea* infection and for quantification of *B. cinerea* colonization. Application of this tool resulted in the characterization of resistant germplasm and in the identification of strengths and weaknesses of a standard detection technique. Because qPCR detection was predictive of disease and provides a quantitative measure of fungal colonization, in addition to current availability for research use, our qPCR technique could prove useful for informing disease management decisions in grapevine production following additional development.

b. Presentations resulting from this Viticulture Consortium-East and NYS Wine and Grape Foundation funded project:

Cadle-Davidson, L. 2005. Detection of *Botrytis* quiescence and activation in developing grape berries using real-time PCR. International Congress on Molecular Plant-Microbe Interactions, Merida, Mexico.

Cadle-Davidson, L. 2005. *Botrytis* quiescence and activation. International Grape Genomics Symposium, St. Louis, MO.