**Interim Progress Report for CDFA Agreement Number 18-0339-000-SA**

**Project title:** Investigation of the impact of grapevine red blotch virus (GRBV) on grape ripening and metabolism.

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Role: Support in coordination and execution of experimental plan and dissemination.

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Role: Support in metabolomics analyses.

Report coverage: July 1st, 2018- July 15th, 2019

**Research Area:** Pest/Pathogen Interaction; Pathogen and Disease Management

**Keywords:** red blotch disease, aroma and flavor, grape ripening, metabolic pathways, primary and secondary metabolites

## Introduction:

Grapevine red blotch virus (GRBV), a causative agent for red blotch disease (RB), is a recently discovered virus that has been identified in vineyards in 14 states across the US as well as in Canada. Symptoms of GRBV include red blotches on leaves as well as reddening of primary and secondary veins for red varieties and chlorotic regions within leaf blades and marginal burning similar to potassium deficiency on white varieties (Sudarshana, Perry et al. 2015). Over the past four years, the Oberholster group has researched the impacts of GRBV on grape development and composition and the resulting impact on wine quality across varieties, sites, seasons, and rootstocks. Results indicate mostly a substantial impact on berry ripening in all varieties studied (Oberholster 2015, Oberholster 2016), along with variable impacts on primary and secondary metabolites depending on site and season (Oberholster 2015, Eridon 2016, Oberholster 2016). Through transcriptomics and metabolomics, the present study aims to investigate the impact the virus has on transcriptional factors and regulatory networks. Previous research investigated the impact of GRBV on Zinfandel infected fruit and found that there was an inhibition of the phenylpropanoid metabolic pathway along with other regulatory networks responsible for berry ripening (Blanco-Ulate, Hopfer et al. 2017). This research needs to be expanded across varieties, sites, seasons, and rootstocks to determine any potential varietal, as well as environmental, impact on GRBV and RB disease expression. Only once virus functioning is understood, can tools be developed to mitigate the impact of GRBV other than the removal of infected vines.

## Objectives of Proposed Research and Path to Application

The main objectives of this project are the following:

1) To determine the impact of GRBV on grape metabolism during ripening;

2) To determine the potential impact of variety, rootstock, site, and season on GRBV functioning.

The first step is to understand GRBV and grapevine interaction. How does GRBV infection influence grape metabolism and thus ripening? What potential synergy exist between environmental stresses and red blotch disease expression? Answers to these questions are the first step in developing a red blotch disease management strategy. Outcomes from this study will add much needed information to understand the influence of GRBV on grape metabolism and development. This can be used to develop a measurement tool to determine disease impact as well as vineyard management recommendations to mitigate potential impact on grape quality and guide judicious removal of grapevines.

**Methodology to Accomplish Objectives**

To answer these questions, grape berries sampled during ripening from two different sites (*Vitis vinifera cv*. Cabernet Sauvignon and Merlot) over two seasons will be analyzed as described in objective 1. Grapes from the 2015-2016 and 2016-2017 seasons have already been collected as part of a previous and currently funded AVF proposal (2016-1953 and 2017-1675) and are currently stored at -80°C. Studies have shown that tissue samples that were stored for several years were stable under -80°C (Andreasson, Kiss et al. 2013). The Cabernet Sauvignon grapevines are grafted on to two different rootstocks, 420A and 110R, allowing us to investigate the potential impact of rootstock selection.

Objective 1: To determine the impact of GRBV on grape metabolism during ripening.

Grapes sampled from two vineyards planted with Cabernet Sauvignon (Oakville Experimental Station, Napa County) and Merlot (Paso Robles) will be utilized for this investigation. Treatment vines designated red blotch positive (RB(+)) and red blotch negative (RB(-)) were identified and marked according to detailed visual mapping of the last few years and confirmed with GRBV and leafroll virus (Grapevine leafroll-associated virus type 1 to 4 and strains of 4) qPCR testing of a subset of vines until 20 data vines for each treatment have been identified. These sites have been utilized previously for red blotch investigations and there is a consistent association of the virus in symptomatic vines in red varieties (Sudarshana, Perry et al. 2015). Only healthy vines (vines that tested negative for viruses and did not show symptoms of viral disease, RB(-)) and vines which only tested positive for GRBV and which are symptomatic (RB(+)) were used as data vines. Data vines were randomly subdivided into five biological replicates of four vines each using a random sequence generator (http://www.random.org.sequences). Five berries were collected from each data vine randomly (top, middle and bottom of grape bunches on the outer and inner side of the canopy) for a total of 20 berries per biological replicate. Grapes were sampled three times during ripening at pre-*véraison*, 50% *véraison* (berry softening and color change) and harvest for 2017, and four times during ripening for 2016 at all the previous points with the addition of post-

*véraison*. The post-*véraison* sampling was missed in 2017 due to a heat spike and unexpected fast increases in sugar content. Previous research utilizing untargeted metabolomics found indications that both primary (organic acids, amino acids, sugars) and secondary (volatile aroma compounds and phenolics) metabolites are affected by red blotch disease (Oberholster 2015, Blanco-Ulate, Hopfer et al. 2017). Thus, sampled grape berries will be analyzed by targeted metabolomic analysis focusing on the primary and secondary metabolites (organic acids, amino acids, sugars, varietal aroma compounds, and phenolics). Solid phase micro-extraction gas chromatography mass spectrometry (SPME-GC-MS, Agilent Technologies) will be used for the analysis of volatile aroma compounds (Hjemeland, King et al. 2013, Hendrickson, Lerno et al. 2016). Whereas, ultra-high-pressure liquid chromatography high resolution time-of-flight mass spectrometry (UHPLC/TOF/MS, Agilent Technologies) analysis will be utilized for non-volatile metabolites (Toffali, Zamboni et al. 2011, Theodoridis, Gika et al. 2012, Blanco-Ulate, Hopfer et al. 2017). Finally, proton nuclear magnetic resonance ($^{1}$H NMR) will be used for analysis of primary metabolites (sugars, amino acids, and organic acids) (Fortes *et al*. 2011). Metabolic profiling data will be combined with transcriptomic approaches using next generation RNA sequencing (RNAseq) which will allow changes in gene expression to be monitored and the impact of GRBV on the metabolic pathways during ripening to be elucidated. Grapes sampled at pre-*véraison*, *véraison*, post-*véraison* and harvest were immediately processed upon arrival at the laboratory and berries were deseeded, frozen in liquid nitrogen and stored at -80°C until further analysis. Data processing and normalization will be performed using Agilent's MassHunter Qualitative Analysis software with Molecular Feature Extraction. Compounds will be identified by authentic standards and/or cross-referenced with metabolite databases (e.g. METLIN Metabolite Database, Tandem Mass Spectrum Database, Human Metabolite Database). Transcriptomic approaches using next generation 3'-Tag RNA sequencing (RNAseq) will allow the monitoring of gene expression changes.

The proposed transcriptomics experiments will require the isolation of total RNA from all GRBV-infected and control samples (five biological replicates for each treatment and site) using the Qiagen RNeasy Plant MiniKit. The purity of the extracted and purified RNA will be analyzed by measuring the absorbance at 260 and 280 with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific) and integrity 2100 Bioanalyzer (Agilent Technologies). Sequencing will be performed at the Expression Analysis Core Facility (UC Davis) using the Illumina HiSeq 3000 platform. These analyses will provide a potential list of genes that have been down or up-regulated and alterations that took place in molecular pathways as a result of red blotch disease. Blanco-Ulate et al. (Blanco-Ulate, Hopfer et al. 2017) found that GRBV infection restricted the biosynthesis and accumulation of phenylpropanoids and derivatives in Zinfandel grape berries. Therefore, further information should be gained to understand the impacts on other varieties. Integration of the transcriptomics and metabolomics data will be carried out using multivariate analyses.

Similar approaches to those detailed in Blanco-Ulate et al. (2017) will be followed for both enzymatic activity and phytohormone analyses to verify the impacts found on the phenylpropanoid metabolism. Lower abundances of the hormone abscisic acid, known to be linked to anthocyanin biosynthesis, and increased levels of auxin, known to suppress berry ripening (Blanco-Ulate, Hopfer et al. 2017) have previously been documented. Further investigation into the interactions of transcriptional regulators and hormone networks needs to be performed. Confirming hormonal response to RB infection can lead to the development of hormone treatments that could potentially decrease the negative impacts of the disease. More information regarding grape responses to GRBV infection in other grape varieties and environmental conditions is necessary to ascertain how both grapevine genotype and environment may impact disease outcomes, especially as previous research by the Oberholster group found clear influences of variety, site and season on GRBV expression.

Objective 2: To determine the potential impact of variety, rootstock and season on GRBV functioning.

Grape samples were collected from two different varieties (*Vitis vinifera cv*. Cabernet Sauvignon and Merlot) over two seasons as described above. Targeted metabolomic and transcriptomic analysis (objective 1) of each variety will enable us to determine whether variety has any influence on the functioning of the disease on a molecular level. Additionally, the Cabernet Sauvignon vineyard block contains two rootstocks, 110R and 420A. Initial investigations suggest that grape berries from GRBV symptomatic vines on 110R rootstocks are more impacted by RB disease than those on 420A rootstocks. Additionally, previous research indicated a large environmental (seasonal) impact on both visual expression of RB disease as well as its impact on grape composition. No correlation has been found between RB disease visual expression and grape and wine compositional impact.

A potential pitfall and limitation was RB disease spread to data vines after qPCR testing. Vines were monitored for any visual signs of infections during the season and re-tested if needed. Further limitation is the capabilities of the analytical instrumentation that will be utilized.

**Research Activities:**

Objective 1:

After several months of researching and testing methods to determine the most reliable method with the highest throughput, a protocol for RNA extraction and quality assurance has been established. The RNA from each sample was isolated using a guanidine thiocyanate lysate buffer made in house and the Qiagen RNeasy Plant Mini Kit in conjunction with the Qiagen PowerClean Pro Cleanup kit. DNA was removed prior to the library preparation using the DNaseI RNase free kit from New England Biolabs. RNA integrity and purity was analyzed using a 2100 Bioanalyzer and NanoDrop 2000c spectrophotometer, respectively. Afterwards, the Expression Analysis Core

at the Genomic Center at the University of California, Davis, prepared libraries for each sample and sequenced using 3' Tag RNA sequencing method.

In December, a subset of samples was sent to be sequenced by the Expression Analysis Core. Two samples from each rootstock (1103P, 110R, and 420A) and from each year were analyzed for a total of 12 samples. Overall, 93% of the reads aligned to the grape genome and 65% of the reads aligned uniquely to the genes. Multidimensional Scaling (MDS) was used to determine the similarity between samples based on gene counts, which is shown in Figure 1. In general, the MDS plot separates the samples depending on the scion/rootstock interaction. Moreover, samples were separated within the scion/rootstock combination based on the season. For Merlot on 1103P and Cabernet Sauvignon on 420A, there is a clear separation of samples collected in 2016 than in 2017. This was not observed for Cabernet Sauvignon on 110R, yet, the samples are separated based on the presence or absence of the virus. However, these samples were prepared to determine which sequencing technique to use, and, therefore, were not from the same ripening stage. Once the larger set of samples have been analyzed, further deductions will be made.

Currently, the RNA from the 210 samples has been extracted and isolated. Quality control measures were performed and met qualifications for purity and integrity. The samples were submitted to the Expression Analysis Core on April 16$^{th}$, 2019, and sequencing began June 25$^{th}$, 2019. We are awaiting feedback from the Expression Analysis Core and the Bioinformatics Core to take the next step in data analysis.

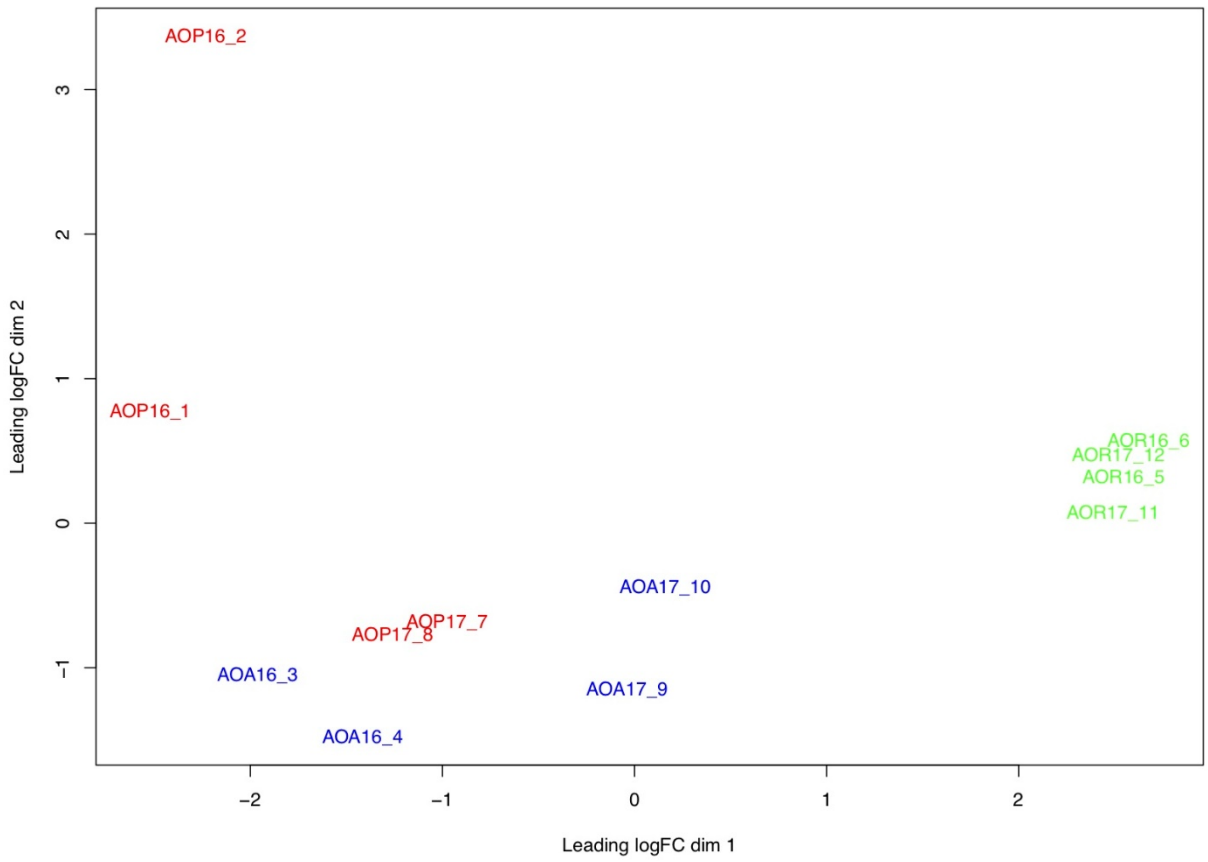


Figure 1. Multidimensional Scaling Plot of gene counts for the subset of 12 samples. AO=Anita Oberholster, P=1103P, A=420A, R=110R, 16=2016, 17=2017, _even= GRBV negative, _odd number=GRBV positive.

In addition, sample preparation techniques for metabolomics analysis were investigated and tested to finalize the methodology that will be used. Non-volatile metabolites will be analyzed using $^{1}$H NMR and UPLC/QTOF/MS. Extractions will be done using 1g of homogenized grape tissue and 4 ml of 60:20:20 methanol:water:cholorform that is acidified with 1% formic acid. Decyl β-D-glucopyranoside will be used as an internal standard. The methodologies for both UPLC/QTOF/MS and NMR have been optimized for this study. Volatile metabolites were analyzed using HS-SPME-GC-MS. In a 20 mL amber vial, 3 g of NaCl, 500 mg homogenized grape tissue, 1 mL of 1M sodium citrate buffer, 25 µl of 0.2 g/L of ascorbic acid solution, and 25 µl of 0.5mg/L 2-undecanone was added. The data collection for volatile secondary metabolites has been completed, and data analysis is underway.

Currently method development has begun on the extraction and analysis of phytohormones in grapes samples. Methods outlined in Pan et al (2010) and Muller and Bosch (2011) will be used to the extraction of major hormones in grapes. The hormones of interest due to their importance during grape ripening are abscisic acid, salicylic acid, jasmonic acid, indole-3-acetic acid, trans-

zeatin, and gibberellins. For each sample, 100 mg of grape tissue powder (from liquid nitrogen grinding) will be extracted with 1 mL of 20:79:1 (v/v/v) water:isopropanol:glacial acetic acid. An internal standard mix will be added (50 µL to each sample) which contains all hormones of interest as well as their deuterated forms in a concentration of 50 µg/mL. This solution is sonicated at 4-7°C for 30 minutes and then briefly vortexed. To each sample, 1 mL of dichloromethane is added, and sonicated for 30 minutes at 4-7°C. The samples are vortexed and then centrifuged at 13,000 x g for 15 minutes at 4°C. The solvent is removed from the plant tissue and kept separate. To the remainder of the plant tissue, another 200 µL of extraction solvent and 200 µL of dichloromethane is added, sonicated similarly, vortexed, and centrifuged for 15 minutes at 4°C. The supernatant is again collected and combined with the previous fraction. This process is repeated twice more for a total of four collection fractions. To the supernatant collected, two layers would have formed. The bottom layer is collected (1 mL) and transferred to a pre-weighed vial. The solvent is removed using a constant flow of nitrogen at 4-7°C. Once dried, the vial is weighed again to obtain sample mass. Then sample is redissolved in 0.1 mL of methanol and then analyzed using UPLC/ESI-MS/MS with multiple reaction monitoring (MRM).

Objective 2 (pending):

Objective 2 will commence on completion of objective 1.

**Research relevance statement:**

Prior to our research over the past four years, little was known about the impacts of GRBV on grape composition and the resulting wine quality. Through our research, it was found that there are variable impacts on levels of primary and secondary metabolites, depending on the variety, season, and rootstock. In addition, in research performed by Blanco-Ulate *et al.* in 2017, there were observed changes in transcriptional factors and regulatory networks relating to an inhibition of berry ripening in infected fruit. The current project aims to further this research across varieties, seasons, sites, and rootstocks to understand the potential variable impacts the disease has on berry ripening. By doing so, a deeper knowledge of the virus functioning will be gained, and possible mitigation strategies can be suggested.

**Layperson summary of project accomplishments.**

The first portion of this project was method validation. We have finalized and implemented the methodology needed to obtain pure total RNA extracts of high concentrations to enable successful analysis by the Expression Analysis Core. Additionally, the optimal sample preparation for the analysis of both volatile and non-volatile metabolites were determined and analysis will commence shortly.

**Status of funds:**

Up to date, $33,881.44 has been spend on tuition and fees and student support for the Ph.D. student, Arran Rumbaugh. $1,229.39 was spent to travel to the PD/GWSS symposium and present in San Diego in December 2018. Another $7,250.62 has been spend on supplies to extract and purify RNA from the grape samples for sequencing, while $13,117.42 was spend on sample preparation and analysis by SPME-GC-MS and LC-QTOF-MS. We have an account with the Expression Analysis Core at the Genomic Center at the University of California, Davis and payment for services will proceed upon completion of sequencing and bioinformatics analyses. Estimated cost are $32,000.

**Summary and status of intellectual property associated with the project.**

We do not anticipate that this research will result in materials or procedures subject to intellectual property restrictions. However, if it does, their availability and use will be subject to the policies of the University of California for managing intellectual property (http://www.ucop.edu/ott/pdf/consult.pdf).

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