Washington State Grape and Wine Research Program
ANNUAL PROGRESS/FINAL REPORT FORMAT
2022-23 Funding Cycle

1. **Summary**:

Cabernet Sauvignon is the major red grape variety planted in Washington State and it is a late budding variety with a long fruit ripening period. Cabernet Sauvignon is typically the last red variety to be harvested due to winemakers desiring both higher alcohol and lower vegetative characteristics. Several reports from wineries have suggested that Cabernet Sauvignon wines made from fruit that was picked after an early frost had occurred, had "rose-like" aromas. We have investigated these claims by studying the impacts of frost on leaves chilled in controlled chambers and by making wines with chilled and dead leaves added prior to fermentation. After conducting two experiments comparing chilled leaves and frozen dead leaves, we concluded that frozen dead leaves were necessary for the off character to be detected in wine. Results from the sensory study show that additions of 2 g/per kg of must or greater were necessary to have a perceivable increase in floral attributes. A model solution experiments of Chardonnay and Cabernet Sauvignon grape leaves showed that marker compounds identified in frost tainted wine were extracted in both aqueous and model wine frozen leaf extracts in short period of time (24-hours). This suggests that all that is necessary for wines to be frost tainted is to have frozen leaves present in the fermentation and that alcohol is not required for this to occur. Pre-cursors (carotenoid breakdown products) to terpenoid-like aromas (2,3-dihydro-4-methyl-furan, (Z)-3,7-dimethyl-1,3,6-octatriene, geranyl acetate) were also found in the aqueous and ethanol extracts of frozen leaves suggesting that yeast metabolism or reactions occurring during wine aging may contribute to the formation of additional frost taint aroma markers.

2. **Final Report**

3. **Project Title**: Evaluation of Freeze Taint in Cabernet Sauvignon.

4. **Principal Investigator/Cooperator(s)**: James Harbertson, Washington State University, 2710 Crimson Way, Richland WA, 509-372-7506 and jfharbertson@wsu.edu; Tom Collins, Washington State University, 2710 Crimson Way, Richland WA, 509-372-7515; Markus Keller, WSU IAREC, Prosser IAREC, Prosser, WA, 509-786-9263

5. **Objective(s) and Experiments Conducted to Meet Stated Objective(s)**:

Objective 1. Identify causal agents of freeze taint aromas.
Sensory and chemical analysis of the 2019 dead leaf addition trial were conducted pre-lock down in late 2019/early 2020. A descriptive analysis panel was carried out. We utilized a consensus built descriptive analysis with clearly defined reference standards. Both aroma and mouthfeel attributes were investigated. We were prevented from doing any rejection/recognition threshold sensory analysis due to the pandemic and the loss of Dr. Frost to another post-doctoral position elsewhere. The panel was trained over 6 sessions and 15 aroma and flavor attributes (red fruit, dark fruit, dried fruit, stone fruit, artificial fruit, tropical fruit, citrus, floral, herbaceous, baking spice, fresh green vegetables, black pepper, sulfurous, cooked vegetables, oxidized) that were rated as well as taste (sweet, sour, bitter) and mouthfeel (hot, astringency). Most of the attributes that were used to describe the wines were typical of Cabernet Sauvignon in Washington and generally around the world. What's unique about the descriptors of these Cabernet wines is the inclusion of the floral, tropical, and stone fruit attributes. An ANOVA found significant differences in astringency, black pepper, citrus, cooked veg, dark fruit, floral, floral aftertaste, fresh green vegetable, stone fruit and sulfurous (Figure 1).

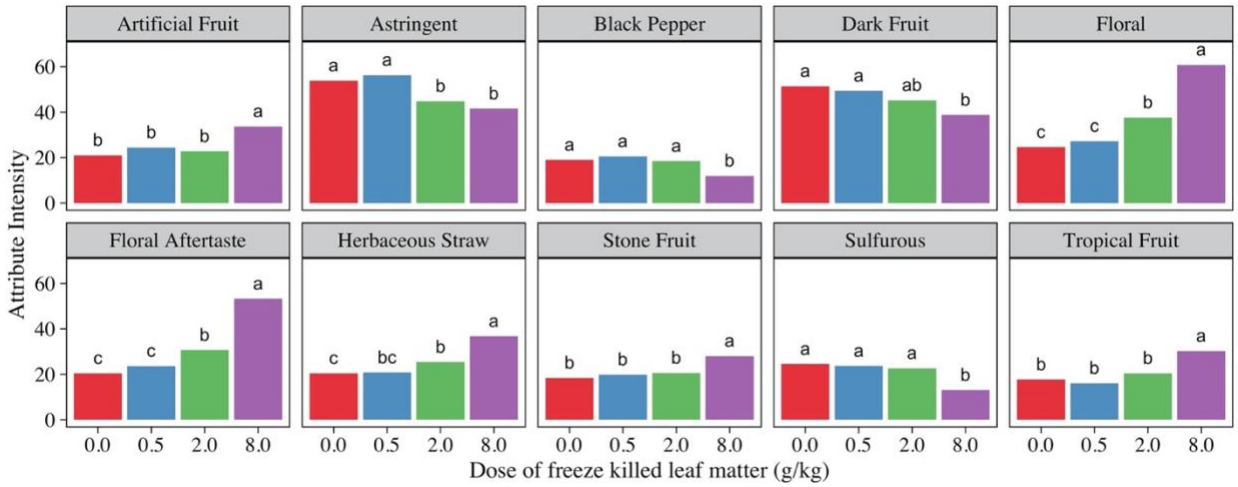

Figure 1 Impact of dead leaf addition on Cabernet Sauvignon sensory attributes.

For astringency there was a linear reduction in the amount of astringency detected by the panelists as the dose of dead leaves increased (consistent with the reduction in tannin concentration as measured by protein precipitation, Figure 2). A significant increase in citrus, stone fruit, floral and floral aftertaste was also found as the dose of dead leaves increased. We also observed a significant decline in black pepper, sulfurous, dark fruit, and the vegetal characters, fresh and cooked veg as the dose of dead leaves increased. The data shows that the panelists generally saw a decline in astringency and increase floral attributes in the wine at about 2 g per kg of must. In the same fashion at around 2 g per kg of must there is also lower amounts of the spice and vegetal attributes of the wines. Not all the attributes behave the same but in general it seems like 2 g of dead leaves per kg of must is where the panelists see the change in the wines. A canonical variate analysis was done with the significant sensory attributes to allow an easier visualization of the data (Figure 2). This is a similar method to principal component analysis to generate a map from the sensory profiling data except that the ellipses are confidence ellipses of statistical significance.

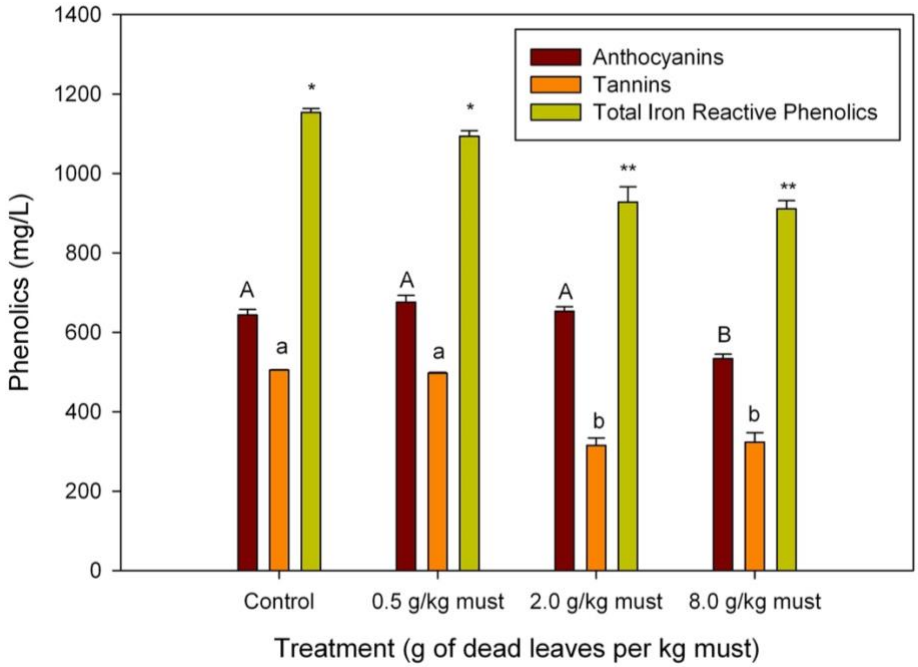

*Figure 2 Impact of freeze-killed leaves on wine phenolics. A 1-way ANOVA followed by a post-hoc comparison of means (Fischer LSD) was done to determine if there were significant differences between treatments. Significance (within a column) is indicated for anthocyanins, tannins, and total iron reactive phenolics by different capital letters, different lowercase letters, or different number of *, respectively.*

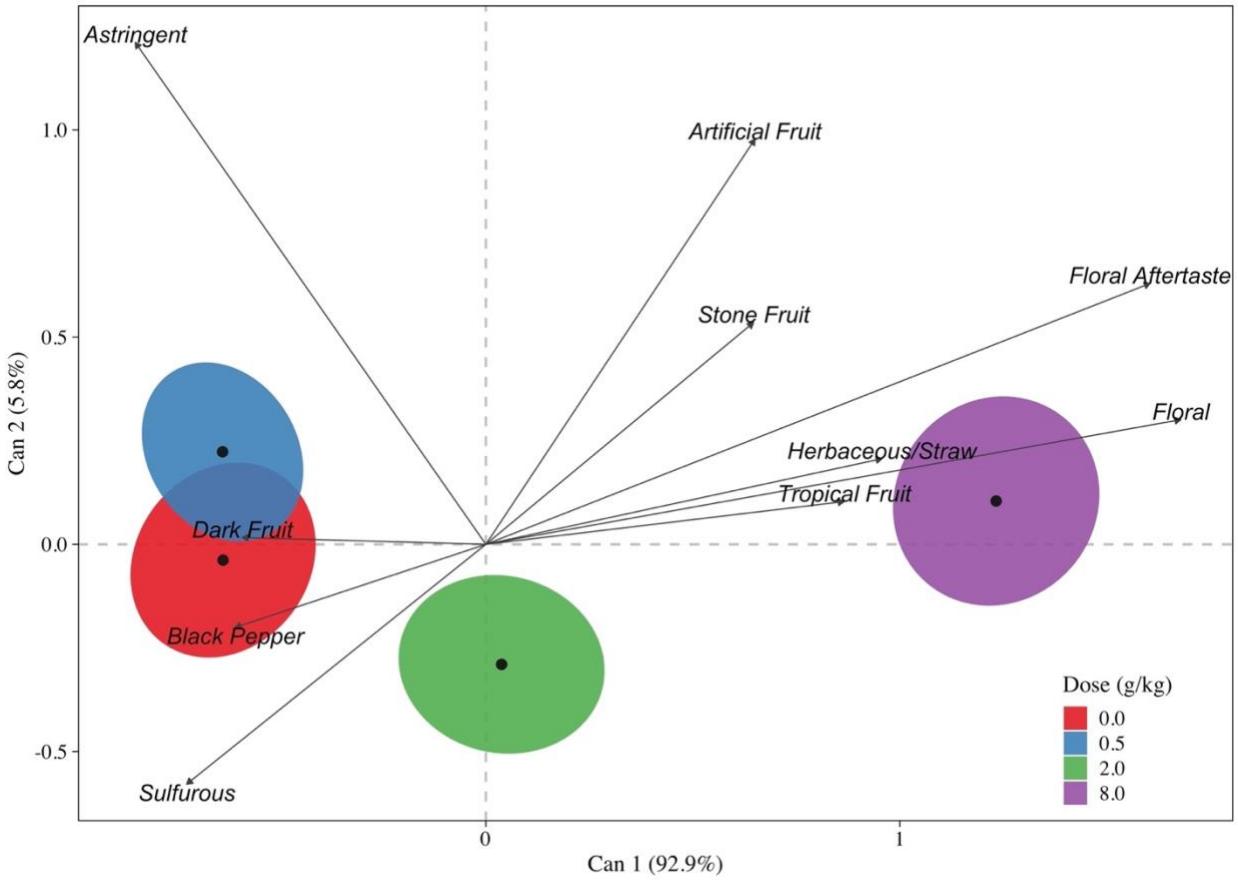

Figure 3 Canonical variate analysis of significant wine attributes.

For our data you can see both the sensory attributes and experimental treatments mapped onto the space. The x-axis predicts 92.9% of the variation in the model and indicates that this is essentially a one-dimensional predictive space along the x-axis. Most of this is being driven by the floral attributes that are on the far-right hand quadrant of the graph. The control and lowest treatment are on the left side of the graph while the 2 g and 8 g per kg of must treatments are moved along the x-axis towards the floral attributes. The control and 0.5 g per kg of must treatment confidence ellipses overlap each other indicating they are not statistically different from each other. Likewise, the 2 g and 8 g/kg of must leaf treatments are separated clearly away from the control and 0.5 g per kg of must treatment indicating they are significantly different. In conclusion the sensory results show a proportional increase in floral attributes with the leaf additions. The floral attributes appear to be suppressing or masking the varietal descriptors for Cabernet Sauvignon like dark fruit and vegetal. This suppression is not limited to the aroma attributes as is also seen in the reduction of astringency. It is unclear why there is a loss of tannins in these wines. Tannins are well understood to bind to cell wall material and proteins, and it is possible that the tannins in the wine are being bound to the leaf material.

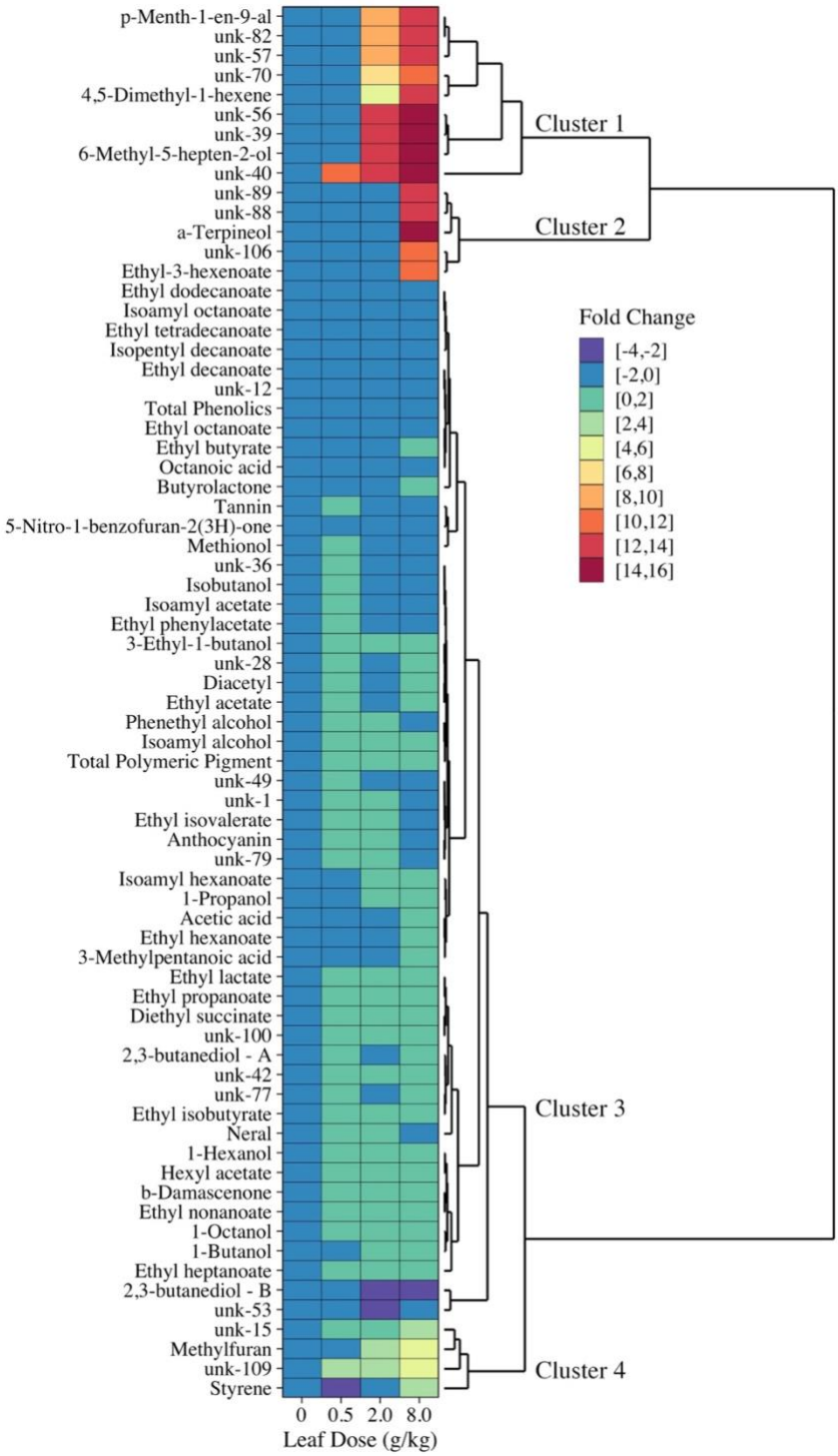

Figure 4 Dendrogram of Euclidean distance matrix between compounds and dose response.

Figure 3 shows a dendrogram of changes in volatile compounds compared to the addition rate of the dead leaves. The mathematics to create such a graph are somewhat complex. A Euclidean distance matrix (EDM) was created between the log2 of the raw gas chromatography data and the dosage rate of the dead leaves. Each trend was evaluated with the EDM, and the amount of change was plotted in color in the graph. It shows that there are four clusters or trend types (behaviors). Cluster 1 is a group of compounds that increase exponentially despite the linear nature of the treatment addition rate. The second cluster of aroma compounds show no change in concentration until the highest dosage rate is used. The remaining two clusters change little or have inconsistent changes with treatment dosage (increasing and/or decreasing). Of note phenethyl alcohol a higher alcohol formed by yeast through the deamination of the amino acid phenyl alanine was deemed unimportant

based on this analysis. Phenethyl alcohol was initially suspected due to its known rose-like aroma, however due to the lack of change from the treatments we can rule it out as a causal agent. The compounds in cluster 1 and cluster 2 are probably the compounds responsible for the floral aroma in the wine though the compounds in cluster 2 may be less important given they are only present in the highest treatment. There are fourteen compounds that we have measured but unfortunately, we can only identify five of the compounds. Of the five three have known aroma characteristics ranging from fruity (pineapple, ethyl-3-hexanoate, citrus (α-terpineol, monoterpene, citrus/pineapple, p-menth-1-en-9-al monoterpene, likely citrus), to green coriander aroma (6-methyl-5-hepten-2-ol). It is unclear if 4,5-diemethyl-1-hexene has any aroma.

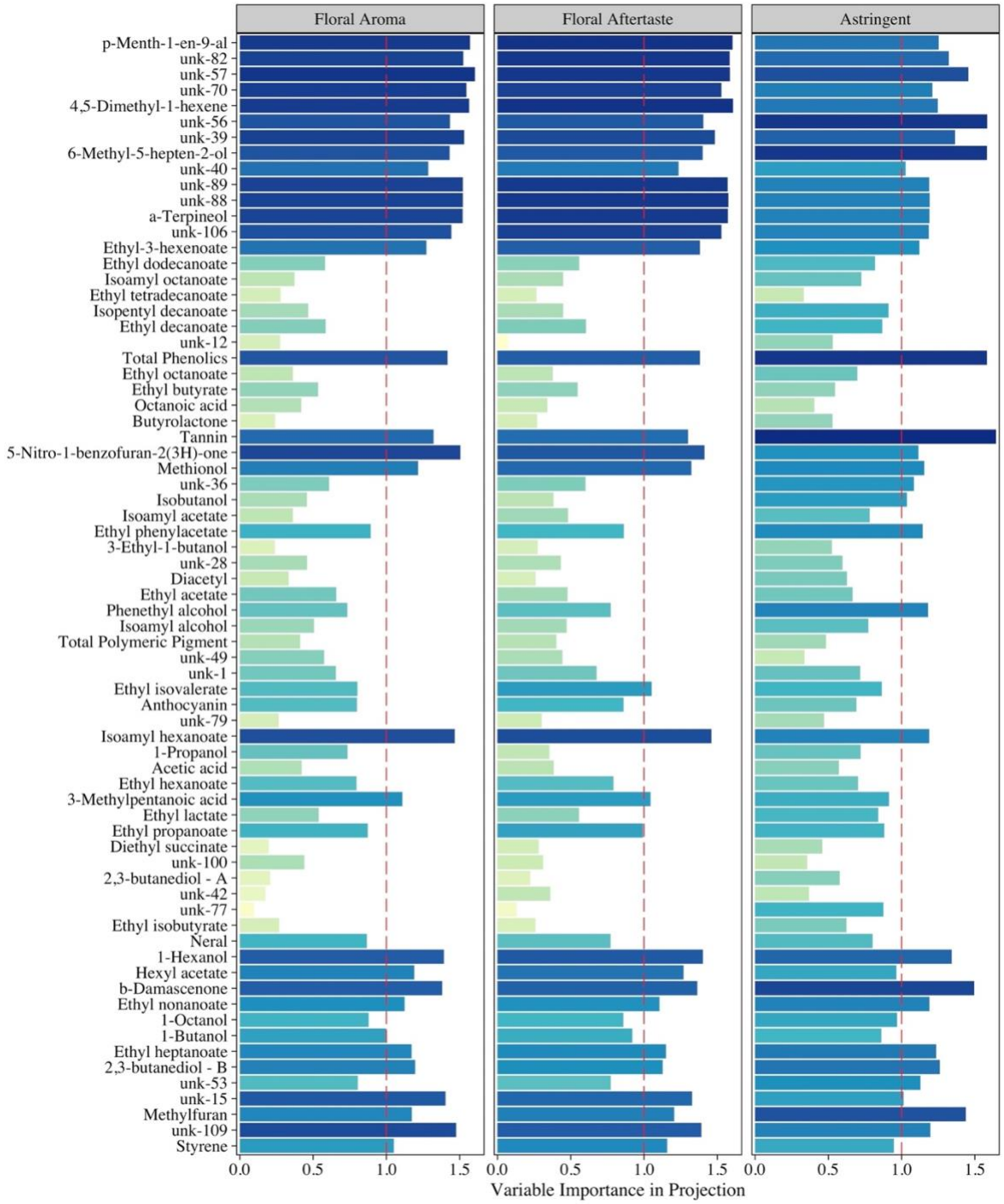

Figure 5 Variable Importance in projection plot

A partial least square regression model of the chemical data and sensory data was created to determine which chemical parameters best correlated with the sensory attributes. A Variable Importance in Projection (VIP) that

estimates the importance of each variable was created and we have shown floral aroma, floral aftertaste, and astringency (Figure 4). The compounds identified by the EDM in cluster 1 and 2 are all found to be in important in explaining the floral and floral aftertaste perception by the panelists. Interestingly several other compounds also are found to be important including those that are not volatile such as tannins and total iron reactive phenolics (TIRP). Tannins and TIRP are important for explaining astringency and as we showed earlier that astringency significantly declined with the larger doses of dead leaves, so it is unsurprising that they are also important in predicting the floral characters. Several compounds identified by the EDM as cluster 3 or 4 were also identified as being important to explaining the floral attributes. This underscores that although the compounds found in cluster 1 and 2 by the EDM may be important in explaining the floral attributes that other compounds found in cluster 3 and 4 may be altering their perception. For instance, β-damascenone is known not to have an aroma of its own in wine but it has been found to suppress vegetal aromas while enhancing fruity aromas (Skouroumounis and Sefton 2002) and in this work it appears that it is behaving in a similar way.

Objective 2. Recreate environmental conditions that cause freeze taint.
Utilize existing technology designed to evaluate cold hardiness by Dr. Keller's laboratory to induce freeze taint in leaves, petioles, stems, and fruit.
Due to the post-doc leaving after the first year of the project and the COVID-19 pandemic my graduate students could not travel to Prosser to pursue this work. We instead focused on model extractions of tissues (see objective 3 model solution extractions)

Objective 3. Evaluate role of fermentation in development of taint
Small batch winemaking to evaluate formation and change during aging.
During 2020 a couple of wineries indicated that they had issues with abnormal rose like aromas in their white wines after harvesting their grapes mechanically after the frosts in 2019. We hypothesized that the frozen leaf material would have contacted the juice during mechanical harvesting and transportation to the winery and while the fruit was being pressed. The time that frozen leaves could be in contact with the juice would be limited as compared to production of red wines where the leaves would be in contact throughout maceration. We devised and executed an experiment whereby we took fresh and frozen leaves and soaked them in white grape juice produced by a commercial winery (Riesling due to availability and likely hood that it might get harvested late enough for a frost to occur) for 0, 3, 12 and 24 hours to emulate in a more controlled way the length of time that leaves may come in contact with juice after mechanical harvesting and transport after a frost. The time represents normal and worse case scenarios based on conversations with winemakers.
Riesling leaves were taken from the same vineyard a week prior to fruit harvest (Horse Heaven Hills). Leaves were weighed and placed into mesh grain bags (510 g per bag) normally used to produce small batches of beer so that they could be soaked in the wine and then removed easily without leaving any residual leaves. Half of the bags were frozen in a large chest freezer at -3°C for 5.8 days while the others remained in a 5°C cold room. The dosage rate for leaf addition was close to the minimum amount observed to cause the rose aroma to occur in red wine from our 2019 experiment (~1-2 g /L of juice). As the treatments last year were done on a dry weight (DW) basis we estimated that the water makes up ~70% of the leaf fresh weight (FW). An additional 70% of weight was added for the fresh leaf addition (2 x 1.7 = 3.4 g FW or ~1 g DW/ L of juice). The effects of fresh or frozen leaf addition on the basic fruit and wine chemistry as compared to the control are shown (Table 1). Lactic and acetic acid were also measured and were found to be 9 and 45 mg/L respectively and were not affected by the treatment. An informal sensory appraisal was done a year after the wines were in bottle and we found that the wines were not really very different from each other, so we didn't pursue formal sensory evaluation.

Table 1. Basic Chemistry of Riesling juice and wine dead leaf experiment.

Juice Composition	pH	TA (g/L)	Malic Acid (g/L)		
	3.079	10.06	4.49		
Wine Composition	pH	TA (g/L)	Malic Acid (g/L)	Glucose + Fructose (g/L)	Alcohol % (v/v)
Control	3.14±0.007	9.78±0.29	3.55±0.15	0.15±0.01	12.66±0.01
Fresh 3 hr.	3.10±0.004	9.17±0.09	3.47±0.04	0.14±0.01	12.53±0.09
Fresh 12 hr.	3.11±0.010	9.52±0.13	3.51±0.04	0.18±0.02	12.60±0.04
Fresh 24 hr.	3.15±0.005	9.37±0.29	3.51±0.05	0.17±0.02	12.54±0.11
Frozen 3 hr.	3.11±0.001	9.13±0.11	3.51±0.04	0.14±0.01	12.64±0.32
Frozen 12 hr.	3.12±0.010	9.35±0.10	3.56±0.05	0.17±0.02	12.59±0.02
Frozen 24 hr.	3.12±0.029	9.27±0.22	3.55±0.03	0.24±0.09	12.52±0.01

Model solution extractions of Chardonnay and Cabernet Sauvignon leaves

As a result of not finding any significant sensory impacts for the white wine experiment we decided to pursue model solution experiments where we could vary different aspects of the leaf treatment such as cultivar, fresh or frozen and maceration (ground or unground) in model wine (pH 3.3 KHT 12.5% ABV) to determine if we could mimic the winemaking results in laboratory solutions. Further, we wanted to understand if alcohol was required for the extraction which might have explained the lack of obvious result for the white winemaking experiment where alcohol was not present. Fresh Chardonnay and Cabernet Sauvignon leaves were collected from the Roza vineyard in Prosser. Leaves were frozen at -20 °C for 96-hours. The leaves (13 g) were extracted in 250 mL of water or model wine for in sealed beakers on a shaker table for 24-hours (n=3). The extracts were then decanted and subjected to untargeted HS-SPME-GC-MS analysis (like what was used for the earlier analysis of the wines). Each treatment replicate was analyzed in duplicate with an internal standard (2-undecanone). About 50 compounds were found and each compound's peak area was represented using a calibration curve based on the internal standard. PARADISe was used to provide a tentative compound identification based on comparing the collected data to the NIST database (National Institute of Spectral Technology). A 4-way ANOVA was done using XLSTAT to evaluate the treatments on their impact on extracting the various compounds. It was found that extraction of the compounds varied primarily by the state of the tissues (fresh/frozen) and the solvent used for the extraction (model wine or water). A principal component analysis of the chemical data was performed to visualize the differences between the extraction methods (Figure 6). Variety played a very small role in differentiating the treatments. Primarily the solvent type and whether the tissues had first been frozen separated the treatments. Both Cabernet Sauvignon and Chardonnay frozen leaf model wine extracts were differentiated by 8-carbon compounds (ethyl-octanoate, 1-octanol, octanal) amongst other aldehydes (2-hexenal, 2-methyl-4-pentenal, benzaldehyde). The frozen leaf water extracts were characterized by numerous acetate esters (nonyl-acetate, geranyl acetate, octyl acetate) and complex higher alcohols (6-methyl-5-hepten-2-ol) and ketones (3-pentatnone, 3-methyl-1-phenyl-1-butanone). Of note 6-methyl-5-hepten-2-ol was also identified as a potential marker compound in the initial winemaking trial with freeze-killed leaves added. The fresh leaf extracts were

characterized with by-products of the lipoxygenase pathway (2-hexen-1-ol acetate, 2-hexen-1-ol, 3-hexen-1-ol). The fresh ethanol extracts were characterized by carboxylic acid esters (benzoic acid ethyl ester, butanoic acid hexyl ester).

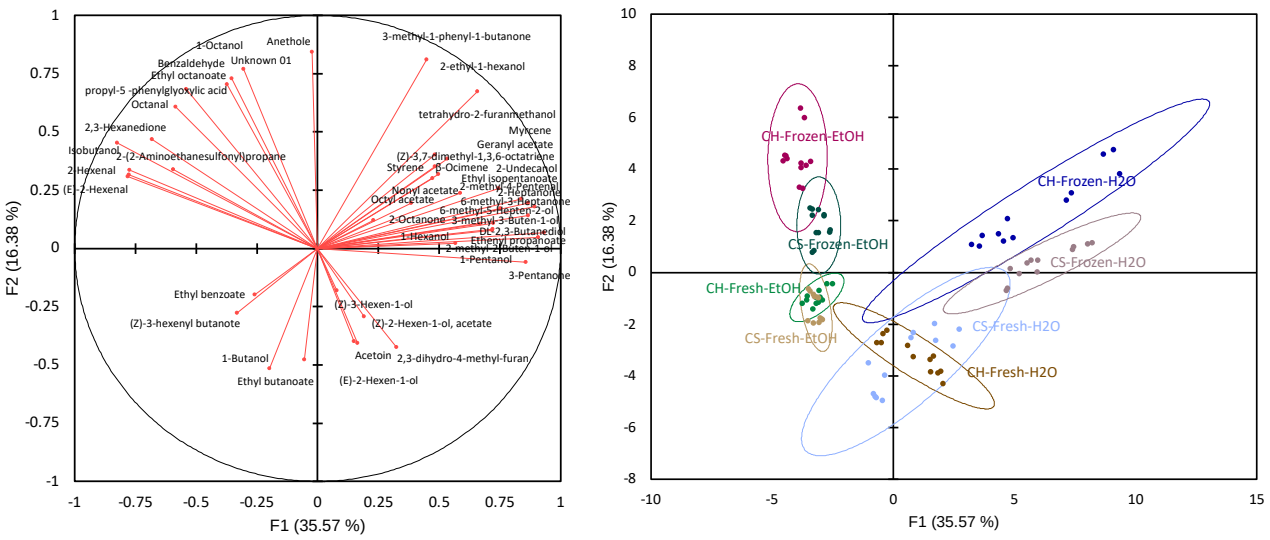

Figure 6. Principal component analysis of the chemical composition of aqueous (H_2O) and ethanol (EtOH) extracts of fresh, frozen, ground, and unground (NG) leaves from Cabernet Sauvignon (CS) and Chardonnay (CH). Confidence ellipses (95%) are drawn around cultivar, fresh/frozen and solvent extracts.

The main conclusions to be drawn from the model experiment is that freezing the leaves alters the composition of the extracts. Secondarily the composition of the extract is also altered by whether ethanol or water is used for the extraction though this appears to be significant but less important. Though the extraction duration in the experiment was short (24-hours) it is evident that it was enough time to alter the composition of the extracts. The number of important compounds associated with the fresh extracts aqueous or otherwise is far lower than the frozen extracts. Disappointingly from the winemaking industry's perspective, the aqueous and ethanol extracts of the frozen tissue though separated mathematically, both contained one of the marker compounds (6-methyl-5-hepten-2-ol) found in the frost tainted wines, but not the extracts made from fresh leaves. This suggests that all that is necessary for wines to be frost tainted is to have frozen leaves present in the fermentation and that alcohol is not required for this to occur. Further, several additional floral compounds were identified in the aqueous frozen leaf extracts (myrcene, β-ocimene, geranyl acetate, isoprenol). Pre-cursors (carotenoid breakdown products) to terpenoid-like aromas (2,3-dihydro-4-methyl-furan, (Z)-3,7-dimethyl-1,3,6-octatriene, geranyl acetate) were also found in the aqueous and ethanol extracts of frozen leaves suggesting that yeast metabolism, or reactions occurring during wine aging may contribute to the formation of additional frost taint aroma markers (Koslitz et al. 2008).

Objective 4. Evaluate Mitigation Strategies from fining agents.
Evaluate and optimize fining agents and filtration technologies to remove freeze taint.

We attempted a battery of various fining agents on 2g/L dead leaf treated Cabernet Sauvignon from 2019 (See Table 2). After an informal bench sensory evaluation with the winemaking team, we decided that the treatments had very little effect. Only activated carbon and milk cream (Half-n-Half) appeared to have any impact at all. As there were also odd milk flavors in the milk cream treated wines it was deemed less useful as a treatment.

Table 1 Fining agents used to ameliorate rose aroma in red wine.

Fining Agent	Rate	Preparation
Carbon	1.2g/L	10x water
Divrrgan (PVPP)	0.5g/L	10x water, let sit 2hr.
Polycacel (PVPP, Cellulose, Casein)	0.7g/L	20x, let sit 2hr.
Freshprotect (PVPP, Bento, Gum, Cellulose)	1.0g/L	10x, let sit 1hr.
Colle Perle (Gelatin)	1.5g/L	Mix 1:1
Inocolle (Gelatin)	1.0g/L	Mix 1:1
Gelocolle (Gelatin, Isinglass)	1.0mL/L	Direct
Cristalline Plus (Isinglass, Citric acid)	0.03g/L	200x water, let sit 3hr.
Bentonite	2.0g/L	10x water
Reduless (Bentonite, Natural copper)	0.15g/L	10x water
Nutrex 370 (Yeast Hulls)	0.3g/L	10x water
Reskue (Inactive yeast)	0.4g/L	10x water
EC1118	0.3g/L	Rehydrate in 10x water
Potassium Caseinate	1.0g/L	10x water, let sit 4hr.
Half-n-half	20mL/L	Direct
Egg White	0.6g/L	Separate, 10x water by weight

References

Koslitz, S., L. Renaud, M. Kohler, and M. Wust. 2008. Stereoselective formation of the varietal aroma compound rose oxide during alcoholic fermentation. J. Ag. Food Chem. 56:1371-1375. https://pubs.acs.org/doi/pdf/10.1021/jf072518t

Skouroumounis, G.K., and M.A. Sefton 2001 "The formation of β-damacenone in wine" in Winterhalter and Rouseff; Carotenoid-Derived Aroma Compounds ACS Symposium Series; American Chemical Society, Washington DC, 2001

6. **Summary of Major Research Accomplishments and Results by Objective**

- Objective 1. Frozen leaves added prior to fermentation at ≥2 g/kg of must cause reduction in varietal aroma and mouthfeel sensory attributes in Cabernet Sauvignon wines. Several marker compounds were identified.
- Objective 2. Was not carried out due to COVID-19 pandemic and loss of post-doctoral scholar.
- Objective 3. Frozen leaves added prior to fermentation 24-hours (at comparable dosages to those that caused frost taint in red wine) had no discernable impact on white wine sensory attributes. Model solution extracts prepared from fresh and frozen Cabernet Sauvignon and Chardonnay leaves showed more alarming results with all frozen leaf extracts showing rose taint aroma markers and potential pre-cursors (thankfully, fresh leaf extracts did not).
- Objective 4. No commercially available fining agents showed any appreciable reduction in rose taint aromas.

7. **Outreach and Education Efforts - Presentations of Research**:

The outreach and education strategy for this work was to present the work in every available avenue that was possible. We presented in professional meetings (some online due to pandemic), industry meetings and wrote a

scientific publication which is open access and is available freely to anybody who wishes to read it. Our work was also highlighted in an industry-based magazine.

Journal Publication

Frost, S.C., D.J. Fox, M. Keller, T.S. Collins, and J.F. Harbertson. Freeze-Killed Leaf Material Causes Atypical Aromas and Astringency in Cabernet Sauvignon. 2023. Am. J. Enol. Vitic. 74: https://doi.org/10.5344/ajev.2022.22012

Presentations Professional Meetings:

Frost, S., Fox, D., Collins, T. S., Keller, M., and J.F. Harbertson. 2021. Investigation of Potential "Rose Taint" Markers in Cabernet Sauvignon. American Society of Enology and Viticulture 72nd National Conference Virtual (https://www.asev.org/sites/main/files/file-attachments/2021technicalabstracts.pdf?1624910869)

Harbertson, J.F., Frost, S., T.S. Collins, and M. Keller. 2020. Investigation of Atypical Aromas in Cabernet Sauvignon Wines Caused by Vine Frost Exposure. ACS Fall 2020 Virtual Meeting & Expo Agricultural and Food Chemistry

Harbertson. J.F. Investigation of Atypical Aromas in Cabernet Sauvignon Wines Caused by Vine Frost Exposure. University of Hokkaido Wine Academy Online Presentation. August 19, 2020

Industry Based Meetings:
J.F. Harbertson. The Impact of Ripening and Frost Exposure on Washington Wine. Gallo Winemaking Short Course, Lodi California, June 20, 2023

J. F. Harbertson. The Impact of Frost Exposure on Washington Wine. Foley Family Wines Viticulture Educational Series, May 23, 2023

S. Frost. Exploring Rose Taint in Washington State Cabernet Sauvignon. T'aint Just Smoke Taint Winemaking Breakout Session 2023 Unified Wine and Grape Symposium Sacramento, California January 24, 2023

J.F. Harbertson. Impact of frost exposure on Cabernet Sauvignon wines. WAVEx Seminar, Wine Science Center Richland, Washington July 27, 2022

J.F. Harbertson. Investigation of Atypical Aromas in Cabernet Sauvignon Wines Caused by Vine Frost Exposure. WAVE Webinar April 1, 2020

Industry Based Magazine:
B. Avila. Impact of Freeze-killed Leaf Material in Cabernet Sauvignon. Wine Business Monthly February 2023

8. **Research Success Statements**: My research team's work shows the causal agents for frost or rose tainted wines and identified potential marker compounds found both in the wines themselves and frozen leaf extracts from Chardonnay and Cabernet Sauvignon leaves. This work also carefully documents the sensory attributes impacted by frost taint including both aroma and mouthfeel of the wine. This work will benefit the wine industry as they can use this information to help them avoid this problem.

9. **Funds Status**: All funds were expended on post-doctoral scholar salary, graduate student salary, chemicals for analysis, instrument maintenance (and parts), and funds to pay for sensory panelists. A small amount of money was spent on travel (once it was allowable to do so).

Final reports are due to mhansen@washingtonwine.org and arcgrants@wsu.edu by **June 30** of the last year of funding.