FINAL REPORT

Washington State Grape and Wine Research Program

DUE 5:00 p.m. December 12th, 2016
by email to: ARCGrants@wsu.edu

Wine Research Advisory Committee Research Review – January 18-19, 2017

PROJECT TITLE: Characterization of indigenous yeasts associated with wine grapes and early-stage fermentations in Washington State

Project Duration: 2014-2017
WRAC Project No.: 13C-3061-5313

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Description of participation:		Description of participation:	

BUDGET AND OTHER FUNDING SOURCES

FINAL FINANCIAL REPORTING

BUDGET *(LIST COMPLETED BUDGET NUMBERS)*

	Year 1 FY15	**Year 2 FY16**	**Year 3 FY17**
	Jul 1-Jun 30, 2015	Jul 1-Jun 30, 2016	Jul 1-Jun 30, 2017
Item			
Salaries			
Benefits			
Wages			2,300
Benefits			55
Equipment			
Supplies			13,645
Travel			4,000
Miscellaneous			
Total	17,629	18,155	20,000
Footnotes: Itemized budgets were not available for Years 1 and 2.			

Total Project Funding: 55,784

Project Budget Status*: Include timeline, any changes, developments, problems or delays that may have significant deviations from original budget rate of expenditure.*
The rate of budget expediture is favorable at this time. However, about six more months will be needed to complete the project, because it was in stasis for some months until the Ph.D. student was picked up by a new thesis advisor in Year 2, and studied for preliminary exams in Year 3. The student plans to stay in Pullman until her manuscripts are drafted, which is anticipated for December 2017.

OTHER FUNDING SOURCES/SUPPORT None
(*Please include all other funding sources that have been awarded and/or anticipated.)*

Agency Name:
Amount requested:
Amount awarded:
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Project Summary: Production and consumption of wines made solely with native yeasts and bacteria have risen across Europe, USA and Washington state. Production of alcohol, flavors and aromas during native fermentation is driven by the microbial communities that live on grape berries and in wine production environments (1,2,11,12,17,18,28). However, the lack of predictability of microbial composition in native fermentations poses the risk of vintages with undesirable flavors or aroma. To address a knowledge gap about the native yeasts present on Washington grapes and their persistence during native fermentation, we used advanced DNA sequencing technologies and sequence analysis procedures to determine yeast diversity in Cabernet Sauvignon vineyards and fermentations from two Washington AVAs. The project complements ongoing studies at the Wine Science Center (23; Piao and Henick-Kling, unpublished), and opens doors to more comprehensive diversity testing and comparisons of yeast diversity among Washington AVAs, vineyards and grape cultivars.

A useful characteristic of certain native yeasts is their ability to suppress grape diseases, including Botrytis bunch rot (15,16,26,27,31). Botrytis bunch rot is a serious yield reducer in wet, cool grape production regions. Biological control offers a complementary tool to fungicides, as the bunch rot pathogen can develop resistance to fungicides (7,9,13,20). In another aspect of this project, we quantified the disease suppressive activities of eleven strains of native yeasts against nine strains of the Botrytis bunch rot pathogen on synthetic medium and on individual grape berries in the laboratory. We also evaluated the virulence of the pathogen isolates on inoculated grape berries.

To monitor specific native yeasts of interest in biocontrol and wine quality studies, we are developing molecular (DNA-based) diagnostic assays (22). The assays can rapidly detect and quantify yeasts in berry, fermentation and environmental samples with a high degree of sensitivity.

Project Major Accomplishments: The objectives of the project were to: 1) Quantify the types and amounts of native yeasts on Cabernet Sauvignon berries from two Washington AVAs, and to determine how their populations shift during in-lab fermentations, in the presence and absence of sulfur dioxide; 2) Develop rapid, sensitive and specific molecular diagnostics for yeasts of interest from grape berries and fermentation samples; and 3) Assess the ability of selected native yeasts to suppress the growth of the Botrytis bunch rot pathogen.

To date, specific objectives were met as follows: 1) Methods for extracting total DNA, including yeast DNA, from grape berries and a sequencing and annotation pipeline were developed and used to obtain data on yeast diversity for the 2015 harvest samples. (Tables 1-3). Processing of vineyard and fermentation samples from 2016 is under way. 2) Molecular diagnostics assays have been initiated for eleven native yeast species and completed for four species. 3) All eleven selected native yeast species were found to inhibit at least one of five tested isolates of the Botrytis bunch rot pathogen, *Botrytis cinerea*. The yeast strains rapidly colonized grape berry tissue. In an initial experiment, the eleven yeasts displayed three patterns of metabolite utilization in commercial oxidation-assimilation tests, whereas the pathogen isolates, poor utilizers of assimilates (nutrients), showed a distinct fourth pattern. All but one of the nine pathogen isolates caused significant rot symptoms (were virulent) on Thompson Seedless berries (Fig. 1).

Yeast diversity on Cabernet Sauvignon berries and in fermentation samples (**Obj. 1**) was determined from sequences of the internal transcribed spacer1 (ITS1) and D2 regions of the fungal DNA. Sequences were obtained using the paired-end Illumina MiSeq platform and ITS1 primers selective for fungi, including yeasts (29). We chose these regions because ITS1 is

variable at the species level and is widely used to distinguish fungal species, whereas D2 has been used in yeast taxonomy and phylogeny studies (30). The ITS1 approach was deemed more informative because it yielded more yeast species compared to the D2 sequences (Table 1). Also, the latter contained about 21% plant, algal, pollen and other non-fungal species (data not shown). Using the ITS1 data, we found that the AVAs differed in diversity or richness, as measured by the number of yeast and non-yeast fungal operational taxonomic units, or OTUs (Table 2). There were 116 OTUs of yeast genera or species; 71 OTUs (61.2%) were common to both AVAs, 33 (28.4%) were unique to AVA 1 and 12 (10.3%) were unique to AVA 2.

Table 1. Comparison of yeast diversity based on the number of taxonomic groups (OTUs)[1] derived from ITS1 vs. D2 sequences

	ITS1	D2
Total OTUs	1467	286
Yeast OTUs	145	108
Yeast genera	15	18
Yeast species	134	78

[1] Sequences that vary by 3% or less are grouped into a single OTU

Table 2. Yeast diversity in 2015 samples based on ITS1 taxonomic groups (OTUs)[1]

	No. of OTUs
Total ITS OTUs	1467
Yeast OTUs	145 (9.9%)
Non-yeast OTUs	1322 (90.1%)
Pooled yeast OTUs	116
AVA 1	102 (87.9%)
AVA 2	81 (69.8%)
Total non-yeast OTUs	1322
AVA 1	920 (69.6%)
AVA 2	858 (64.9%)

In AVA 1, ten yeast taxonomic groups comprised of 1,000 or more sequences were identified (Table 3). Among the species were *S. cerevisae*, *Hanseniaspora uvarum*, *H. osmophila*, and *Wickerhamomyces anomalus*. The *Metschnikowia* species were well represented in AVA 1. In AVA 2, five yeasts OTUs of 1,000 or more sequences were found; four were identical to those from AVA 1. Our findings indicate that abundant yeasts were present at both locations but the locations also were distinguished by unique OTUs. This was evident for the less abundant species. Many of the yeasts were of species known for biocontrol activity (15,16,26,27,31). Non-yeast fungal species also were abundant. AVA 1 and AVA 2 harbored twenty and thirteen OTUs, respectively; the top eight OTUs are shown in Table 3. Most of the fungi declined during fermentation, but some species, such as *Aureobasidium pullulans* appeared to persist up to mid-fermentation.

Table 3. The number of yeast and non-yeast fungal ITS1 sequences in taxonomic groups from 2015 Cabernet Sauvignon berries at veraison and harvest (vineyard), and early, mid and late stage fermentations.

Genus/species	OTU[1]	Total seqs[2]	Vineyard	Early[3]	Mid[3]	Late[3]
AVA 1 -Yeasts						
Saccharomyces cerevisiae	6	183,356	82	21,531	67,697	94,046
Hanseniaspora uvarum	12/425	35,315	1573	8715	22,077	2950
Metschnikowia sp.	5	33,143	556	30,836	1606	145
Metschnikowia chrysoperlae	8	15,002	969	12,966	933	134
Metschnikowia sp.	33	14,891	277	13,875	692	47
Hanseniaspora osmophila	49/209	3667	156	794	2272	445
Metschnikowia pulcherrima	18/282/758	2762	507	2007	184	64
Wickerhamomyces anomalus	3/156	1314	663	2801	2456	505

Udeniomyces puniceus	30/97	1153	1142	3	2	6
Candida pimensis	28/750	1001	421	528	38	14
AVA 2 – Yeasts						
Saccharomyces cerevisiae	6	80,199	114	6870	28,382	44,833
Hanseniaspora uvarum	12/425	46,609	95	684	30,585	15,245
Candida quercitrusa	9/1241	14,525	3	3	13,529	990
Meyerozyma guilliermondii	17	9577	431	999	867	7280
Metschnikowia sp.	5	1966	47	1681	204	34
AVA 1 – Non-yeasts						
Mycosphaerella tassiana	1/15	32,558	31,340	543	464	211
Alternaria sp.	4	31,151	27,579	2860	494	218
Ascomycota sp.	7	15,034	14,571	248	117	98
Aureobasidium pullulans	2	13,380	9979	1138	2043	220
Ulocladium charatum	14	12,018	11,686	176	84	72
Aspergillus amstelodami	13	6391	5715	286	129	261
Epicoccum nigrum	11	4674	4461	86	63	64
Aspergillus piperis	22	2504	1001	1339	98	6
AVA 2 – Non-yeasts						
Aureobasidium pullulans	2	93,270	5014	70,454	8007	9795
Mycosphaerella tassiana	1	66,357	58,243	7241	358	515
Ascomycota sp.	7	23,082	23,082	2845	642	335
Alternaria sp.	4	15,694	14,412	1107	62	113
Ulocladium charatum	14	15,656	13,862	1608	78	108
Aspergillus piperis	20	8501	7809	612	34	46
Neosetophoma clematidis	22	3999	3727	234	11	27
Aspergillus amstelodami	13	3231	3062	153	5	11

[1] All yeast taxonomic groups (OTUs) and top eight non-yeast OTUs comprised of >1,000 sequences are shown

[2] Number of sequences from all vineyard and fermentation samples in the OTU

[3] Sampling time points were 3, 7 and 21 days for early, mid and late stage fermentation, respectively

Diagnostics assays (**Obj. 2**) were initiated in 2014 and have been on hold in 2015-2016 while the 2015 yeast diversity data were analyzed. The diversity data indicated that *A. pullulans* is also of interest, as it is abundant in vineyards in both AVAs and persists at mid-fermentation (Table 3). Efficient and sensitive assays are available for *C. californica*, *Mt. pulcherrima* and the *M. carribica/M. guilliermondi* species pair, as reported in last year's project report. A commercial PCR kit is available for *A. pullulans* (genesig.com; Primerdesign Ltd., UK). Assays for seven more yeasts associated with biological control and wine quality (13; Alpin and Edwards, personal communication) will be completed in 2017.

The focus of the past year has been to compare the inhibitory activities of eleven native Washington yeasts (5) against Washington isolates of *Botrytis cinerea* (8), and to compare the virulence of the pathogen isolates on grape berries (**Obj 3.**). The yeast-like fungus *A. pullulans*, active against *B. cinerea* on apples (32,33), inhibited five grape *Botrytis* isolates on synthetic medium and four isolates on grape berries. On synthetic medium, the best yeast inhibitors were *Curvibasidium pallidicorallinum*, *Metschnikowia pulcherrima*, *Meyerozyma guilliermondii*, *S. cerevisiae* and *W. anomalus*. The latter four yeasts also reduced disease severity on the berry. However, *C. pallidicorallinum* was not active against the isolates 207cb and 407cb on grape berries. In contrast, the *Mt. chrysoperlae* strains reduced disease severity of 207cb and 407cb on

the berry but did not inhibit the pathogens on synthetic medium. Other yeast strains showed activity only on the berry. Our findings showed that the biocontrol mechanism(s) occurring in the absence of the grape was not necessarily operational on the grape, and suggested that niche competition could be a major factor in vivo. These experiments also showed that the *B. cinerea* isolates varied in sensitivity to the yeasts.

The yeasts grew to significant population densities in wounds on the grape berry. Populations reached approximately 1 million cells after 2 days from a starting inoculum of 200 cells, and often exceeded 10 million cells at 10 days, when Botrytis rot disease severity was evaluated in our experiments. The rapid population increase indicated that the yeast strains were aggressive colonizers of grape berry tissue and potentially successful niche competitors.

In an initial experiment, the yeast strains fell into three metabolic categories when tested for the ability to oxidize or utilize a set of yeast-specific compounds in commercial Biolog plates. *Aureobasidium pullulans*, *Candida saitoana* and *M. guilliermondii* tested positive in the highest number (87-89%) of oxidation and assimilation (nutrient utilization) tests. The *Mt. chrysoperlae* and *Mt. pulcherrima* isolates and *W. anomalus* were moderate at 63-78%, and *C. pallidicorallinum* and *S. cerevisiae* were the lowest at 21-38%. Five isolates of *B. cinerea* (101V3Dd, 111bb, 207a, 207cb and 407cb) also were tested using the Biolog plates. Growth was monitored after 6 days at A630 nm. All five isolates displayed similar oxidation-assimilation profiles (39-48% positives), although they differed from that of the yeasts. The findings in this initial experiment predict that common nutrient utilization profiles among the yeasts and *Botrytis* can account for some of the disease suppression.

Eight of the nine *B. cinerea* isolates caused substantial rot on Thompson Seedless berries over a 10-day interval. An example of disease progression in one of three experiments is shown in Fig. 1. Relative virulence was also determined by comparing the area under the curve for each pathogen isolate (data not shown). Isolates 207a, 407cb and 407da showed a slight but significantly ($P<0.05$) higher virulence than the other isolates, whereas 111b and 207db were slightly less virulent. Our findings indicate that pathogen isolates are not identical in virulence. Together with the differential sensitivity to various biocontrol yeasts, the data indicate phenotypic variations among the pathogen isolates.

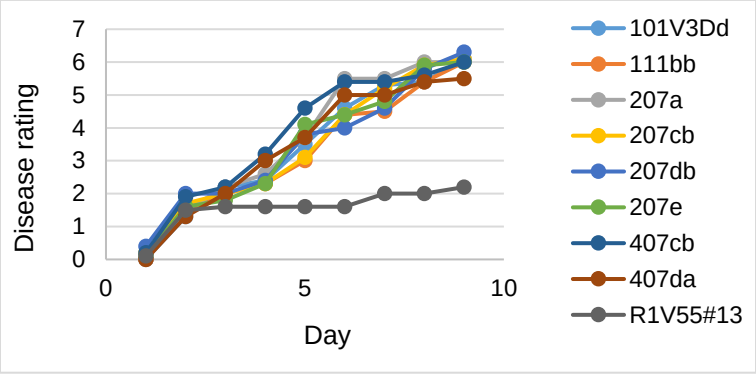

Fig. 1. Botrytis bunch rot disease severity on Thompson Seedless berries. Ten spores of each *B. cinerea* isolate were introduced in wounds (1 wound per berry, n = 12) and maintained at 23°C. Disease severity was rated daily for 10 days using a visual scale of 0 (healthy) to 7 (grape covered with sporulating fungus, berry shrunken).

The **key outcomes** realized from the project so far are as follows. **A)** Wine-associated yeasts found in diversity studies throughout the world (e.g., 4,6,10,14,23,25,34) were also found in Washington vineyards. However, Washington vineyards also harbored unique yeasts, especially among the less abundant (data not shown), and some yeasts notably were absent in the sampled vineyards. As expected, diversity differed between the vineyards (AVAs). **B)** Non-yeast fungi were also abundant on grape berries, and some persisted to mid-stage fermentation. The

biocontrol strain *A. pullulans* was especially abundant and persistent. The role of non-yeast fungi in wine quality remains unknown. **C)** Unlike a previous study (3), sulfur dioxide appeared to have no significantly effect on yeast diversity or abundance in the fermentation samples (data not shown). **D)** Selected native yeasts rapidly colonized grape berry tissue and reached high populations densities, making them good niche competitors for biological control. **E)** Common nutrient utilization profiles between the yeasts and *Botrytis* isolates accounted for some of disease suppression, but additional biocontrol mechanisms, including killer toxin production (31), likely come into play on the berry or in fermentation. **F)** Common nutrient utilization profiles among the yeasts might restrict their efficacy in if used in combination.

This research has long-term **benefits to growers and winemakers** in the state of Washington. The ultimate goal of the diversity research is to provide information about beneficial and undesirable Washington yeasts to vineyard managers, winemakers, and researchers interested in flavor/aroma analysis. Yeast diversity in the vineyard is a key element in native fermentations, and as it is largely location-specific (4,6,10,14,23,25,34), should be directly determined for vineyards in Washington AVAs. Native yeasts also affect wine quality in the presence of introduced *S. cerevisiae* (2,11,34). Information will be shared with the Henick-Kling laboratory to provide a more comprehensive picture of diversity to growers and winemakers. The long-term goal of the biocontrol research is to develop yeasts that suppress multiple strains of the *Botrytis* pathogen in the vineyard and during postharvest storage. One infection court for postharvest Botrytis rot in apple appears to be the flower, suggesting a strategy for postharvest control on table grapes. However, biocontrol of Botrytis bunch rot on wine grape berries will require optimization of formulation and application. If such an approach is successful, it will provide a management tool for reducing fungicide application and pathogen resistance. Finally, molecular diagnostics for specific yeasts of interest obviates the need for costly and time-consuming diversity analysis. Samples from throughout the state can be collected at any time and shipped to diagnostic laboratories for testing. The technology is transferrable to commercial services, industry users and researchers, and will facilitate monitoring from vineyard to bottle.

Information Dissemination, Extension, and Outreach Activities:

Wang X, Edwards C, Henick-Kling T, Glawe DA. 2014. Non-*Saccharomyces* yeasts associated with grape must and fermenting juice in Washington State. WAWGG Annual Meeting. Poster, abstract p. 24-25.

Wang X, Edwards C, Glawe DA. 2015. Persistence of indigenous grape yeast species during alcoholic fermentation. WAWGG Annual Meeting. Poster, abstract p. 8-9.

Scott Weybright. 2015. Harnessing wild yeasts to produce refined wines. Article for:
WSU News, June 10, 2015
CAHNRS News, June 10, 2015
Voice of the Vine, June 2015

Wang X, Kramer E, Glawe D, Okubara P. 2016. Grape berry colonization and biological control of *Botrytis cinerea* by indigenous vineyard yeasts. WAWGG Annual Meeting. Oral presentation and poster, abstract p. 16-17.

The above poster was presented at the American Phytopathological Society Pacific Division annual meeting, La Conner, WA. Abstract p. 56.

Okubara P, 2016. The power of native yeasts. Washington Advancements in Viticulture and Enology (WAVE), Richland, WA.

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