Influence of Cluster Thinning on Phenolic Composition, Resveratrol, and Antioxidant Capacity in Chambourcin Wine

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Abstract: The effect of cluster thinning on wine composition, total phenolics, total anthocyanins, antioxidants, and resveratrol content of the winegrape cultivar Chambourcin (*Vitis* spp.) was studied over three years. Titratable acidity and pH of samples were determined using standard methodology; total phenolic, anthocyanin concentration, and antioxidant capacities of wines were measured spectrophotometrically after incubation with specific reagents. Levels of *cis*- and *trans*-resveratrol and their glycosides (piceids) in wine samples were analyzed by direct injection into HPLC coupled with photodiode array detection. Cluster thinning did not affect basic wine composition except pH. Cluster thinning, however, increased linearly the polyphenolic composition of wines as indicated by increases in total anthocyanins, total phenolics, and antioxidant capacity. Cluster thinning also increased linearly the total resveratrol level including free resveratrol (*cis*- and *trans*-) and its glycoside derivatives (*cis*- and *trans*-piceids). Positive correlations were found between total phenolics, total anthocyanins, resveratrol, and antioxidant capacity. It was concluded that cluster thinning of Chambourcin produced wines with increased anthocyanins, total phenolics, and antioxidant capacity and thus wines with increased potential health benefits.

Key words: anthocyanins, crop level, piceid, polyphenols

It has long been known that high-quality wines are usually produced from vineyards having low to moderate yields based on variety and cultural practices (Jackson and Lombard 1993, Kliewer and Dokoozlian 2005, Naor and Gal 2002). Several studies have shown that low crop level can increase anthocyanins and total phenolic compound of fruit and wine (Guidoni et al. 2002, Mazza et al. 1999). Phenolic compounds are known for being responsible for bitterness, astringency, and color intensity of wine, and thus play a major role on wine sensory quality.

In recent years, phenolic compounds in red wines have been linked with health benefits because of their antioxidant properties (Abu-Amsha et al. 1996, Frankel et al. 1995, Lopez-Velez et al. 2003). Among polyphenols, resveratrol (stilbene) has been studied extensively in grapes and wines for its antioxidant properties. Resveratrol has been found to be effective for the prevention of coronary heart disease and has been shown to have activity against certain classes of cancer (Potter et al. 2002, Wang et al. 2002). Recent research has also shown that resveratrol is responsible for enhancing the activity of sirtuin enzymes, which may be responsible for the increased longevity of mice and other unrelated organisms (Baur et al. 2006, Kaeberlein and Rabinovitch 2006). These reports have increased consumer interest in food products that contain high levels of health beneficial compounds also called "functional food." Therefore, the identification of vineyard cultural practices that could influence phenolic compounds, and thus its antioxidant content in winegrapes and wines is desirable.

The benefits of low yield on total phenolic and anthocyanin contents in Nebbiolo (Guidoni et al. 2002), Cabernet franc, Merlot, and Pinot noir (Mazza et al. 1999) wines have been documented. In addition, there are several factors that affect resveratrol accumulation, including the environment, cultivar, pathogens, and cultural practices (Bavaresco 2003). However, to our knowledge, the direct link between the effect of cluster thinning on antioxidant capacity and specific polyphenolics such as resveratrol of grapes and wine has yet to be determined.

The objectives of this study were to evaluate the effect of cluster thinning in Chambourcin, an important red winegrape hybrid in the Eastern and Midwestern regions of the United States, on (1) basic wine composition, (2) total phenolics, total anthocyanins, and antioxidant capacity, and (3) total resveratrol content (*trans*- and *cis*-isomers and their glycosides) in wines from three vintages.

Materials and Methods

Experimental design and treatments. Grafted Chambourcin (Seyve-Villard 12-417 x Seibel 7053) grapevines on

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rootstock Couderc 3309 (*Vitis riparia* x *Vitis rupestris*) were planted in 1996 at a spacing of 1.25 x 3 m (or 2722 vines/ha) at the Horticultural Research Unit 2, Ohio Agricultural Research and Development Center in Wooster (lat.: 40°47'N; long,: 81°55'W, elevation: 311 m asl, Wooster silt-loam soil). Vines were trained to a unilateral cordon at a height of 1.8 m and were pruned to 20 nodes on 5-node spurs per vine. Beginning in 2000, all treatments were thinned to 20 shoots per vine at 24 to 30 cm shoot-length stage. Shoot positioning and cluster thinning, but not leaf pulling, were conducted at pea-size stage to retain 10 (10CL treatment), 20 (20CL treatment), and 30 (30CL treatment) clusters per vine (or 8, 16, and 24 clusters/m of row). Cluster thinning consisted of removing clusters adjacent to the most basal clusters on the shoot (Dami et al. 2006). The time of harvest was determined based on optimum juice composition of the following parameters: soluble solids (SS) (20 to 22 Brix), pH (3.3 to 3.4), and titratable acidity (TA) (9 to 11 g/L). All treatments were harvested at the same time, which was when the optimum conditions for the 10CL treatment were met. Wines were made from grapes harvested from the three cluster-thinning treatments for three seasons (2000, 2001, and 2002).

Winemaking procedures. Approximately 120 kg of fruit per treatment was pooled from vineyard replications and retained for winemaking in 2000, 2001, and 2002. Grapes were stored overnight at 0.5°C, destemmed, crushed, and treated with 50 mg/L SO_2 in the form of potassium metabisulfite ($K_2S_2O_5$). Musts were chaptalized up to the Brix of the 10CL treatment, which produced the highest SS levels. Twelve hours later, Chambourcin musts of each crop level were inoculated with *Saccharomyces cerevisiae* (Lalvin ICV-D254; Lallemand, Montreal, Canada) which had been hydrated and added at a rate of 0.26 g yeast/L must. Fermentation was conducted at a maximum temperature of 32°C in 114-L nalgene polypropylene tanks (Nalge Nunc International, Rochester, NY). Caps were punched down manually twice daily. Each crop level treatment was fermented in triplicate lots and pressed separately at dryness in a small Willmes bladder press (Josef Willmes, Bensheim Hessen, Germany). At the completion of fermentation, wines were racked, sulfited, and cold-stabilized for 60 days at 0.5°C, and stored at the same temperature until bottling. Bottled wines were stored at 15.5°C until analysis. Wines were analyzed for basic and phenolic composition, antioxidant capacity, and resveratrol content in 2005.

Basic and phenolic wine composition. Wine pH was measured using a temperature-compensated AR50 dual-channel pH/ion/conductivity meter (Accumet Research; Fisher Scientific, Pittsburgh, PA) calibrated between pH 4 and 7. Titratable acidity (AOAC method) and free sulfur dioxide (Ripper method) were measured by an established procedure (Zoecklein et al. 1999). Total anthocyanins were measured spectrophometrically at 520 nm for absorbance of 1:100 dilution of wine in 0.1 N HCl, and the anthocyanin concentrations in Chambourcin wines were calculated based on the molar absorbance values of malvidin-3,5-diglucoside (ε = 37,700) (Niketic-Alecksic and Hrazdina 1972). Total phenolics expressed as gallic acid equivalents were quantified by measuring absorbance of the resulting reaction of 1:10 dilution of wine samples with Folin-Ciocalteu reagent in excess base condition at 765 nm (Singleton and Rossi 1965).

Antioxidant capacity. Antioxidant capacity was measured with the modified FRAP (ferric reducing ability of plasma) assay (Benzie and Strain 1996) and the modified ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assay (Ozgen et al. 2006). For the FRAP assay, 20 µL of 1:10 diluted wine samples were mixed with solution of TPTZ [2,4,6,-tris(2-pyridyl)-*s*-triazine] and $FeCl_3$ at pH 3.6. For the ABTS assay, the same quantity of wine was allowed to react with solution of ABTS and potassium peroxodisulfate (pH 4.5). After 10 min of incubation, sample absorbance was measured using a Beckman DU-640 (Beckman Coulter, Fullerton, CA) at 593 nm and 734 nm for FRAP and ABTS assays, respectively. Sample absorbance was plotted against a standard curve of 10 to 50 nmol trolox to determine sample antioxidant capacity. Antioxidant capacities were reported as mmol trolox equivalent per L of wine.

Resveratrol. Resveratrol analyses were performed using a Waters 2695 HPLC unit connected to a 2996 diode array module (Waters Corporation, Milford, MA). The specification of the column was C18 Gemini (Phenomenex, Torrance, CA) 5 µm, 110Å, 250 x 4.6 mm. Wine samples were injected in duplicate after passing through a 0.2-µm 10-mm diam Anotop disposable syringe filter (Whatman, Florham Park, NJ). Separation and quantitation of resveratrol followed the procedure of Lamuela-Raventos et al. (1995). The *cis*-resveratrol and *cis*-piceid standards were obtained by 30-min exposure of *trans*-resveratrol and *trans*-piceid solution in a quartz cuvette at 365 nm using a handheld UV meter to generate partial conversion (Trela and Waterhouse 1996). A *trans*-resveratrol standard curve was used for the calculation of all isomers assuming equal absorptivities of *trans*- and *cis*-isomers at 306 nm and 285 nm, respectively.

Statistical analysis. ANOVA on cluster thinning was conducted using the GLM procedure (SAS version 8.02; SAS Institute, Cary, NC) and orthogonal contrasts were used to delineate linear or quadratic trends. Pearson correlations were obtained using the CORR procedure in SAS.

Results and Discussion

Yield and juice parameters. Results of yield and juice composition were previously reported (Dami et al. 2006). The average fruit yield per plant for each treatment was 3.3, 4.8, and 5.1 kg/vine for the 10CL, 20CL, and 30CL treatments, respectively.

Basic and phenolic wine composition. Increased severity of cluster-thinning treatments consistently increased pH of Chambourcin wines (Table 1). Titratable acidity (TA)

was not affected by crop level in two of three years, but was reduced by cluster thinning in 2002. In this study, the response of wine pH to cluster thinning followed the same pH trend observed in grape juice, which indicated a viticultural rather than an enological effect (Dami et al. 2006). Other studies reported similar responses of juice (Ferree et al. 2004, Kurtural et al. 2006, Reynolds et al. 1994) and wine (Reynolds et al. 1996) pH to crop level reduction. These responses were generally attributed to advanced fruit ripening of the low-crop treatment (Naor and Gal 2002). Grapes harvested at advanced maturity typically produce wines with high pH and low TA (Iland and Coombe 1988).

Total phenolics and total anthocyanins increased linearly with cluster thinning in all wines made in 2000, 2001, and 2002 (Table 1). The three-year average increase of total phenolics and anthocyanins was 46% and 52%, respectively, in the low- (10CL) as compared with the high- (30CL) crop treatment. Total anthocyanins, which accounted for about half of the total phenolics, were positively correlated to the latter in all three years (Table 2). These findings are in agreement with previous studies where cluster thinning was shown to enhance total anthocyanin concentration of grapes and wines (Guidoni et al. 2002, Mazza et al. 1999, Reynolds et al. 1996). Anthocyanins, which are responsible for the color of red wines, are reported as one of the major contributors to red table wine quality. A positive relationship between grape berry development and anthocyanin concentration has been found (Kennedy et al. 2002). Based on these reports and our findings, earlier-maturing grapes from low-crop vines accumulated more phenolic compounds than those produced from high-crop vines. Cluster thinning may have increased polyphenols accumulation indirectly by advancing fruit maturity or directly by altering the source to sink balance (i.e., leaf area to fruit ratio) and as such may have increased the substrate levels necessary for polyphenol synthesis. This hypothesis is currently under investigation.

Antioxidant capacity. Parallel to the phenolic and anthocyanin content, cluster thinning linearly increased wine antioxidant capacity in each year. Even though FRAP values were consistently lower than ABTS values, the two assays were highly correlated in Chambourcin (Table 2). Cluster thinning increased antioxidant capacity of Chambourcin wines by ~42% in the low treatment (10CL) as compared with the high treatment (30CL). The level of antioxidant increase was similar to the level of increase observed with total phenolics (i.e., 46%) (Table 1). As a result, strong positive correlations were found among total

Table 2 Correlations among phenolic components and antioxidant capacity in Chambourcin wines, 2000–2002.

	Correlation coefficients (r)[a]		
Variables	**2000**	**2001**	**2002**
Total phenolics vs total anthocyanins	0.80*	0.73*	0.97***
Total phenolics vs total resveratrol	0.87*	ns	0.91***
Total anthocyanins vs total resveratrol	0.95***	0.93***	0.93***
FRAP vs total phenolics	0.92**	0.97***	0.98***
FRAP vs total anthocyanins	0.94**	0.82**	0.99***
FRAP vs total resveratrol	0.97***	0.67*	0.94***
FRAP vs ABTS	0.99***	0.93***	0.99***

[a]Coefficient significance: *, **, ***, and ns indicate significance at $p < 0.5$, 0.01, 0.001, and not significant, respectively.

Table 1 Effect of cluster thinning on basic and phenolic composition and antioxidant capacity in Chambourcin wines, 2000–2002.

	Target clusters	pH	Titratable acidity (g/L)	Anthocyanins[a] (mg/L)	Total phenolics[b] (mg/L)	FRAP[c] (mmol/L)	ABTS[d] (mmol/L)
2000	10	3.50	6.4	428	1310	41.1	56.6
	20	3.30	7.5	311	833	28.3	40.9
	30	3.17	8.0	224	895	24.3	36.9
	Linear[e]	*	ns	**	**	***	***
2001	10	3.53	8.1	543	1462	36.3	49.6
	20	3.42	7.9	569	1254	33.8	48.4
	30	3.37	8.0	444	986	29.0	42.5
	Linear	***	ns	***	***	***	***
2002	10	3.75	7.2	1138	1809	44.9	61.5
	20	3.37	7.9	857	1462	36.6	51.0
	30	3.31	8.2	719	1260	32.5	46.2
	Linear	***	***	***	***	***	***

[a]Absorbance determination at 520 nm. Results expressed as malvidin-3,5-diglucoside equivalents.
[b]Total phenolic levels by Folin-Ciocalteu reagent. Results expressed as gallic acid equivalents.
[c]FRAP (ferric reducing ability of plasma) antioxidant capacity assay. Results expressed as mmol trolox/L of wine.
[d]ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid] antioxidant capacity assay. Results expressed as mmol trolox/L of wine.
[e]Orthogonal contrasts: *, **, ***, and ns indicate significance at $p < 0.5$, 0.01, 0.001, and not significant, respectively.

phenolics, total anthocyanins and antioxidant capacity in Chambourcin wines in all three years (Table 2). Therefore, cluster thinning increased the phenolic content of wine and hence its antioxidant capacity. Similar effects of crop level on grape phenolic content have been reported (Mazza et al. 1999). However, this is the first time, to our knowledge, that a direct response of antioxidant capacity relating to cluster thinning has been reported.

Sulfites have been shown to interfere with assays for the determination of total phenolics (Singleton et al. 1999) and antioxidant capacity (Campanella et al. 2004, Saucier and Waterhouse 1999). In an in vitro study, the addition of 50 to 200 mg/L SO_2 to catechin, one of the major phenolics in wine, synergistically enhanced the antioxidant capacity of the sample mixture (Saucier and Waterhouse 1999). In our work, the free SO_2 concentrations in Chambourcin wines from different crop levels were not significantly different at the time of analysis. Therefore, it is suggested that in this study, antioxidant activity was associated with cluster thinning and subsequent increase in phenolic content and not influenced by SO_2 interference.

Resveratrol. There are several forms of resveratrol (listed in order of the amount present in grape berries), including the piceids (3-*O*-β-D-glucoside derivatives), free isomers (*trans*- and *cis*-resveratrol), viniferins (oligomers), and pterostilbene (3,5-dimethoxy-4'hydroxystilbene) (Adrian et al. 2000). The *trans*-isomer of resveratrol is usually considered as the active compound, and most health-related research has been conducted using this form. However, *trans*-piceid was the major resveratrol isomer found in Chambourcin wine and accounted for ~67% of total resveratrol (Table 3). Cluster thinning linearly increased total resveratrol in Chambourcin wines in all three years. Low crop level (10CL) had an average increase of 53% of total resveratrol as compared with that of high crop level (30CL). Interestingly, total resveratrol of the 10CL treatment in 2001 appears to be the lowest of the three years, and considerably less spread was observed between 10CL and 30CL treatments, partly explained by no significant yield separation between treatments in 2001 (Dami et al. 2006).

In earlier studies, a negative relationship was reported between resveratrol content and fruit maturity, that is, resveratrol declined during fruit maturity (Creasy et al. 1988), and *trans*-resveratrol had a negative correlation with both anthocyanin and sugar accumulation of grape skin and grape juice (Jeandet et al. 1991). However, in those studies, researchers measured free *trans*-resveratrol only. Later studies showed that piceid (resveratrol glycoside) can be a major source of resveratrol in grape juice (Romero-Perez et al. 1999) and wine (Goldberg et al. 1996). That is why the four forms of resveratrol were measured in this study, and our results agreed with findings (Goldberg et al. 1996, Lamuela-Raventos et al. 1995) that showed *trans*-isomers of resveratrol in wine to be present in greater amounts than *cis*-isomers, and total resveratrol glycoside contents in wine to be greater than total free isomers. Correlation analyses indicated a positive relationship between total phenolics (except in 2001), total anthocyanins, and total resveratrol in Chambourcin wines (Table 2). In addition, total resveratrol had a positive correlation with FRAP. Total resveratrol seems to be closely correlated with anthocyanin accumulation than with total phenolic content.

Even though resveratrol was found to increase with cluster thinning and thus resulted in an increase of antioxidant activity, resveratrol is only one compound among several other polyphenols in wine that play an antioxidant role, including (but not limited to) gallic acid, catechin, myricetin, quercetin, and caffeic acid. Resveratrol may play only a minor role as an antioxidant (Frankel et al. 1995). Therefore, it is suggested that the total level of phenolic compounds present in wine is a better indicator than resveratrol in determining the antioxidant capacity and health benefits of wines.

Table 3 Effect of cluster thinning on resveratrol content in Chambourcin wines, 2000–2002.

	Target clusters	*trans*-Resveratrol (mg/L)	*cis*-Resveratrol (mg/L)	*trans*-Piceid[a] (mg/L)	Total resveratrol (mg/L)
2000	10	6.4	1.9	19.2	27.5
	20	5.1	1.9	13.3	20.3
	30	4.2	1.8	12.2	18.1
	Linear[b]	*	ns	***	**
2001	10	1.4	0.5	12.1	14.0
	20	2.5	0.7	11.3	14.4
	30	1.8	0.5	9.3	11.6
	Linear	**	ns	***	***
2002	10	1.7	0.6	29.6	31.9
	20	2.0	0.7	13.3	16.0
	30	1.9	0.6	13.2	15.8
	Linear	*	ns	***	***

[a]*cis*-Piceid data is not included because of low detectability.
[b]Orthogonal contrasts: *, **, ***, and ns indicate significance at $p < 0.5$, 0.01, 0.001, and not significant, respectively.

Conclusion

Results indicated that it is possible to manipulate wine polyphenolic composition and its antioxidant capacity by modifying production practices using cluster thinning. Further studies are needed to demonstrate that cluster thinning improved the antioxidant capacity of wines by increasing the polyphenolic content in grape berries. Furthermore, the mechanism of how cluster thinning affected the polyphenolic content in berries and wines remains unclear and further studies are warranted. Nevertheless, the findings of health-related phenolics in Chambourcin, which is a hybrid, could be used to increase its market value. Additionally, this may encourage growers to cluster-thin this cultivar if financial incentives were implemented based on health claims rather than the traditional soluble solid content approach. Finally, we have demonstrated once again that cluster thinning is an essential "ripening" and "quality" tool that must be practiced for

red winegrape cultivars grown in cool-climate and short growing season regions.

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