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High Temperature and Abscisic Acid Modified the Profile of Anthocyanins in Grape (*Vitis vinifera* L.)

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Abstract: The predicted increase of temperature by effect of climatic change (1.5 °C to 4.5 °C), will affect some berry components. Anthocyanins and flavonols concentration are responsible of wine color. The level of anthocyanin is affected by light intensity, temperature, sugars, growth regulators and vineyard management. Abscisic acid (ABA) increases the synthesis of anthocyanin in grape. The object of study was to evaluate the effect of different temperature in berries *in vitro*, of two cultivars combined with ABA treatments. The treatments were control (C, water) and ABA treatment (1,000 ppm) and temperature: 25, 33 and 40 ºC. In Cabernet Sauvignon, 25 and 33 ºC did not affect anthocyanin total concentration but 40 °C produced a 30% decrease in anthocyanin. ABA treatment increased anthocyanin vs. C at 25 and 33 ºC, mainly due to glucosylated forms. But ABA + 40 ºC showed a 44-60% decrease in all anthocyanins forms compounds. Response of Malbec to the highest temperature (40 ºC), at the end of ripening, was different; higher temperature produced only slight decrease of total anthocyanins concentration (decrease of 7%). Combination of temperature + ABA at 20 °C and 33 ºC increased anthocyanin. But ABA + 40 ºC decreased glucosylated and cumarylated forms of anthocyanin.

Key word: Temperature, ABA, anthocyanins, grape, Malbec, Cabernet Sauvignon.

1. Introduction

Anthocyanins are predominant pigments of grape skins and they are the responsible for the color of the red wine. For winemakers, the anthocyanins and polyphenols are very important, because those are transfer in wine. Their contents in wine determine the organoleptic properties and the health benefit. Anthocyanins accumulation was affected by weather conditions, especially light intensity and temperature [1-6], growth regulators [7-14], water status [15, 16] and vineyard management [17-21].

Temperature is an external factor that affects the coloration of grapes [22]. Authors studied the effect at differences temperature on skin color during the ripening period and they have not had similar results [4, 6, 10, 23]. When Castellarini et al. [24] compared weather condition of two year and anthocyanin concentration, they showed what the years with more days with temperatures over 30 °C have the major anthocyanin content. But, other authors demonstrated what higher temperatures during all days affected the color of grape [25, 26].

Anthocyanin accumulation is enhanced by a plant hormone ABA [14, 27, 28], cold temperature, and high light intensity [23]. Different authors consider that for some varieties the optimal temperatures for anthocyanins accumulation in grape are in 15-25 °C during the day and 10-20 °C at night. When temperature between day/night are greater than 10 °C generally depressed fruit coloration [1]. Experiments with temperature-control were showed that exposing

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vines or clusters to high temperature (30 °C) inhibited anthocyanins accumulation [10, 23].

Anthocyanins are synthesized via the flavonoid pathway in grapevine cultivars that have the wild-type VvmybA1 transcription factor for the expression of UFGT [29]. The encoded enzyme UFGT catalyse glycosylated unstable anthocyanidin aglycones into pigmented anthocyanins (Fig. 1). Two primary anthocyanins (cyanidin and delphinidin) are synthesized in the cytosol of berry epidermal cells. Cyanidin has a B-ring di-hydroxylated at the 3' and 4' positions, whereas delphinidin has a tri-hydroxylated B-ring because of an additional hydroxyl group at the 5' position. Flavonoid precursors are initially recruited from the phenylpropanoid pathway by a small family of chalcone pathway. Parallel pathways downstream of F3'H and F3'5'H [30, 31] produce either cyaniding or delphinidin. The 3' position of cyanidin and delphinidin and sequentially the 5' position of delphinidin can be methoxylated by OMT that generate peonidin, petunidin and malvidin, respectively. Anthocyanins are delivered into the vacuole where they are visible as coloured coalescences (anthocyanic vacuolar inclusions). It still remains unknown whether anthocyanins enter the vacuole as single molecules and thereafter they aggregate or if cytoplasmic vesicles

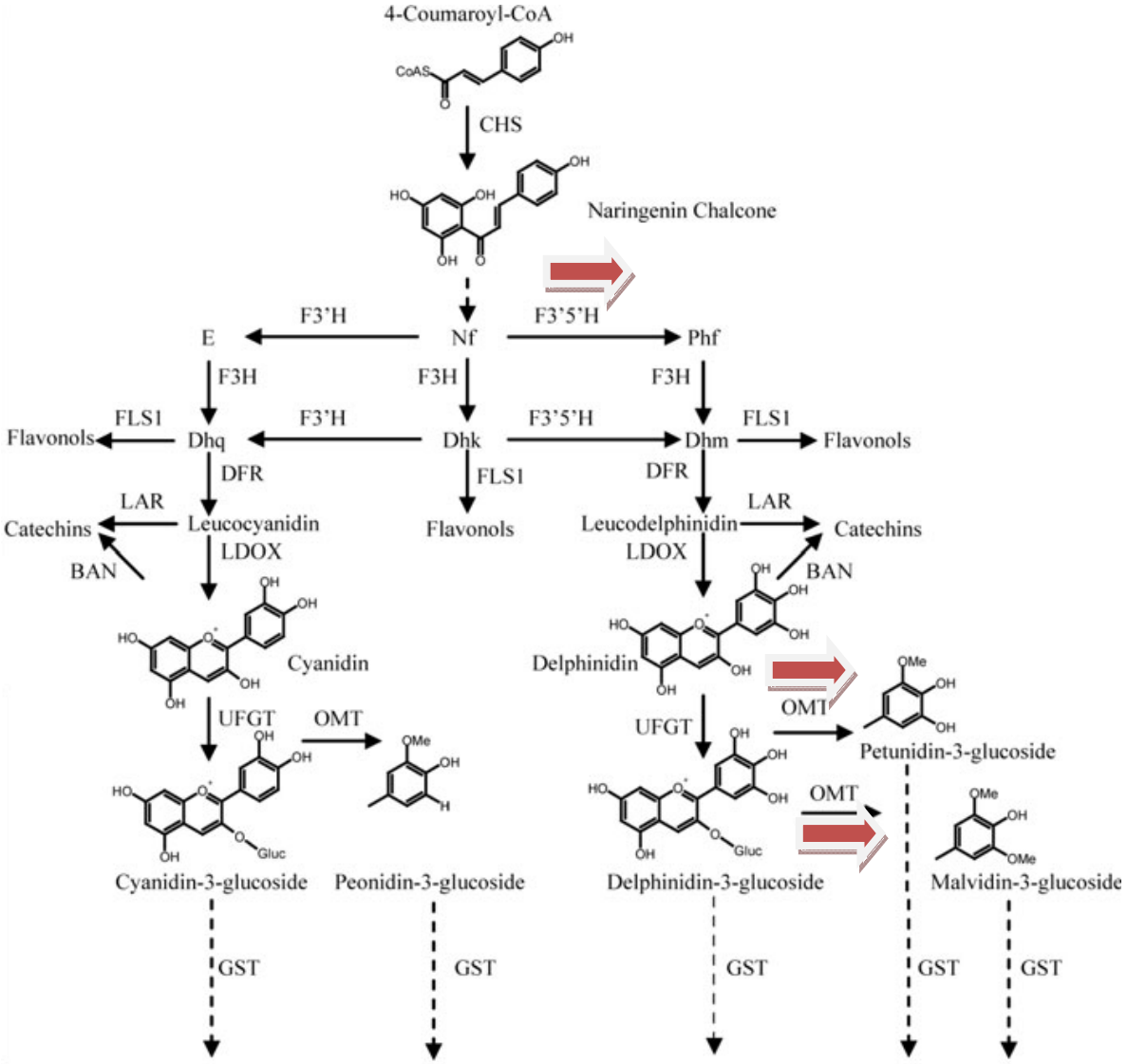

Fig. 1 Key steps of the flavonoid pathway leading to anthocyanin biosynthesis. (Extracted to 24).

containing coalesced anthocyanins interact with the tonoplast [32]. Whatever the mechanism, some members of the GST protein family are believed to participate in vacuolar trafficking and sequestration of anthocyanins [33, 34].

Total amount of anthocyanins and the relative abundance of single anthocyanins are extremely variable among red- to blue-skinned cultivars. Both traits are under genetic control and are developmentally regulated. Physiological studies show that incident radiation on bunches [35, 36], plant water status [37] and exogenous hormones [38, 39] modify anthocyanin content, expanding the range of metabolite variation beyond that due to the genetic synthases (CHS1, CHS2, CHS3) and enter the flavonoid background of a given cultivar. In particular, little is known about whether or not, and if so, to what extent the expression of anthocyanin biosynthetic genes is affected by seasonal water availability throughout the progress of ripening.

The objective of this study was to determine the combined effect of ABA and high temperatures on anthocyanin profile in two varieties of grape *in vitro* cultivation.

2. Materials and Methods

Berries of *Vitis vinifera* cv. Cabernet Sauvignon and Malbec were sampled randomly from four plants at maturation and post-harvest. The berries were washed with deionized water, sterilized in 15% sodium hypochlorite for 30 min and rinsed with sterilized water. Following procedures were carried out in a clean bench and all instruments used were sterilized. Each berry was cut longitudinally into halves with a razor blade. The ten half-cut berries were placed on a filter paper in a sterilize Petri dish and 5 mL of test solution was added before filter this by a membrane (0.45 μm). The Petri dish was covered with Parafilm "M" (Laboratory Film, Pechiney, Chicago, EEUU). Then the ABA added and the Petri dish was maintained under the photoperiod light-darkness 16/8 hours.

The + ABA treatments was obtained added 10 mL of ABA solution 1 $g \cdot L^{-1}$ for Petri dish (+ A) and the – ABA treatments was obtained added 10 mL of water deionized and sterilized for Petri dish (– A). These treatments were combined with different temperatures: 25, 33 and 40 °C, being: 25 °C plus ABA (25 + A), 25 °C without ABA (25 – A), 33 °C plus ABA (33 + A), 33 °C without ABA (33 – A), 40 °C plus ABA (40 + A), 40 °C without ABA (40 – A).

2.1 Grape Extraction for UV-Vis Analysis and HPLC

For determining anthocyanin concentration, the skin was removed from the grape and placed in a methanol-HCl (99:1) solution for 48 h at –20 °C for extraction. The liquid fraction was filtered through 0.4 mL cellulose acetate filter. Anthocyanin pigments were quantified according to Maza et al. [40]. The determination of total polyphenols (TP) was made from the same extract but absorbance was measured at 280 nm.

UV-Vis analysis: A Varian Cary WinUV spectrophotometer with 10 and 1 mm—optical path cells was used to perform the absorptiometric measurements. For anthocyanin content, the solution was diluted 1:50 (v/v) with acidified distilled water (1% v/v HCl) and absorbance was measured at 520 nm against a blank of reagents.

For polyphenol content, the solution was diluted 1:100 (v/v) with distilled water and absorbance was measured at 280 nm against a blank of reagents.

HPLC: The measurements were made with HPLC LKB 2152, Wavelength monitor 2141 and Integrator HP 3395.

For HPLC analysis, these samples were filtered using a hydrophilic PTFE membrane filter (0.45 mm) before injection. Reversed phase HPLC separations were conducted using the Lichrosorb RP-18 5 μm 250 mm × 4.6 mm Cat.N°54949, Suppelco Inc. columns. The flow rate was 0.8 mL/min, and the sample volume was 20 μL. Anthocyanins were detected using the

SPD-10A UV-Vis detector (Shimadzu, Japan) with absorbance at 518 nm. For linear gradient elution, the method using 10% formic acid, 3% acetonitrile and 87% water bidistille as solvent A and 10% formic acid, 50% acetonitrile and 40% bidistilled water as solvent B. the gradient was follows: 0 min, 6% B; 15 min, 30% B; 30 min, 50% B; 35 min, 60% B; 41 min, 6% B; 45 min, 6% B.

The individual anthocyanins were identified by comparing their retention time with those of standards and published data.

2.2 Statistical Analysis

One way analyses of variance (ANOVA) and the Fisher's multiple comparison of means in order to discriminate between the averages by the minimum difference, with a significance level of $P < 0.05$ were applied.

3. Result and Discution

Fig. 2 shown temperature effects on total anthocyanins content (TA) in grape. In two moments, high temperatures (40 °C) decreased TA. Analysis to profile evidenced decrease tri-hydroxylated and di-hydroxylated anthocyanins content when temperature increases (Table 1). This ratio was higher at 33 °C because of the content of di-hydroxylated compounds was 33% minor and the tri-hydroxylated was only 3%. Finally, the color of these berries was darker because it increases the ratio blue/red. For the winemaking this is favorable due to tri-hydroxylated anthocyanins are most stable during the elaboration and conservation of wine.

Methylation of tri-hydroxylic anthocyanins was favored by high temperature which eventually outweighs the decrease of anthocyanins. However, high temperature did not affect O-methyltransferase of di-hydroxylated anthocyanins. There are specific enzymes for each group (di or tri hydroxylated compounds) [32], and they could be regulated by different factors. 33 °C was the optimum temperature for methylation of di-hydroxiled anthocyanins.

When the grape had 24°Brix, total anthocyanins decreased vs the 22°Brix. Naturally, the anthocyanins content maximum is produced before the optimal °Brix for the harvest. Not only total anthocyanins were minor, but also changed the profile. At 40 °C decreased the tri/di hydroxylated ratio for decrease of tri-hydroxyled and increased of di-hidroxylated compounds, and this variation is detrimental for stability of wine.

High temperature decreased methylation of delfinidin (malvidin) but did not affect the petunidin. The methylation is favorable for winemaking because the compounds methyled are most stability.

The ratio 3',4',5'-hydroxylated/3',4'-hydroxylated anthocyanins was higher at 33 °C. This result can be understood for the activity or transcription of F3'H and F3'5'H regulated with temperature.

Temperature on wine color has two effects: one is directly for diminution of anthocyanin total content and another indirect because nonmethyled compound has less stability in wine. The result showed the temperature effect on the enzymatic activity of OMT.

At 22°Brix, ABA applied increased TA at 25 and 33 °C, but at 40 °C produced decreased of this compound. The main effect was increase to malvidin 3-O-glucoside in 58% at 25 °C and 35% at 33 °C. Also, ABA increased the tri-hydroxyled and in minor intensity the di-hydroxyled compounds. The tri/di-hydroxyled ratio was similar for 25 and 40 °C, but for 33 °C it was higher than them. This effect is positive for the colors of wine.

Additionally, the ABA increased the derivates compound (malvidin acetil, peonidin cumaril, malvidin cumaril) giving stability of colors. In absolute values the largest increase experiment was for malvidin, in relative values, the ABA stimulates the synthesis of petunidin.

At 24°Brix, the anthocyanins content was lower than 22°Brix, but malvidin 3-O-glucoside contents increased

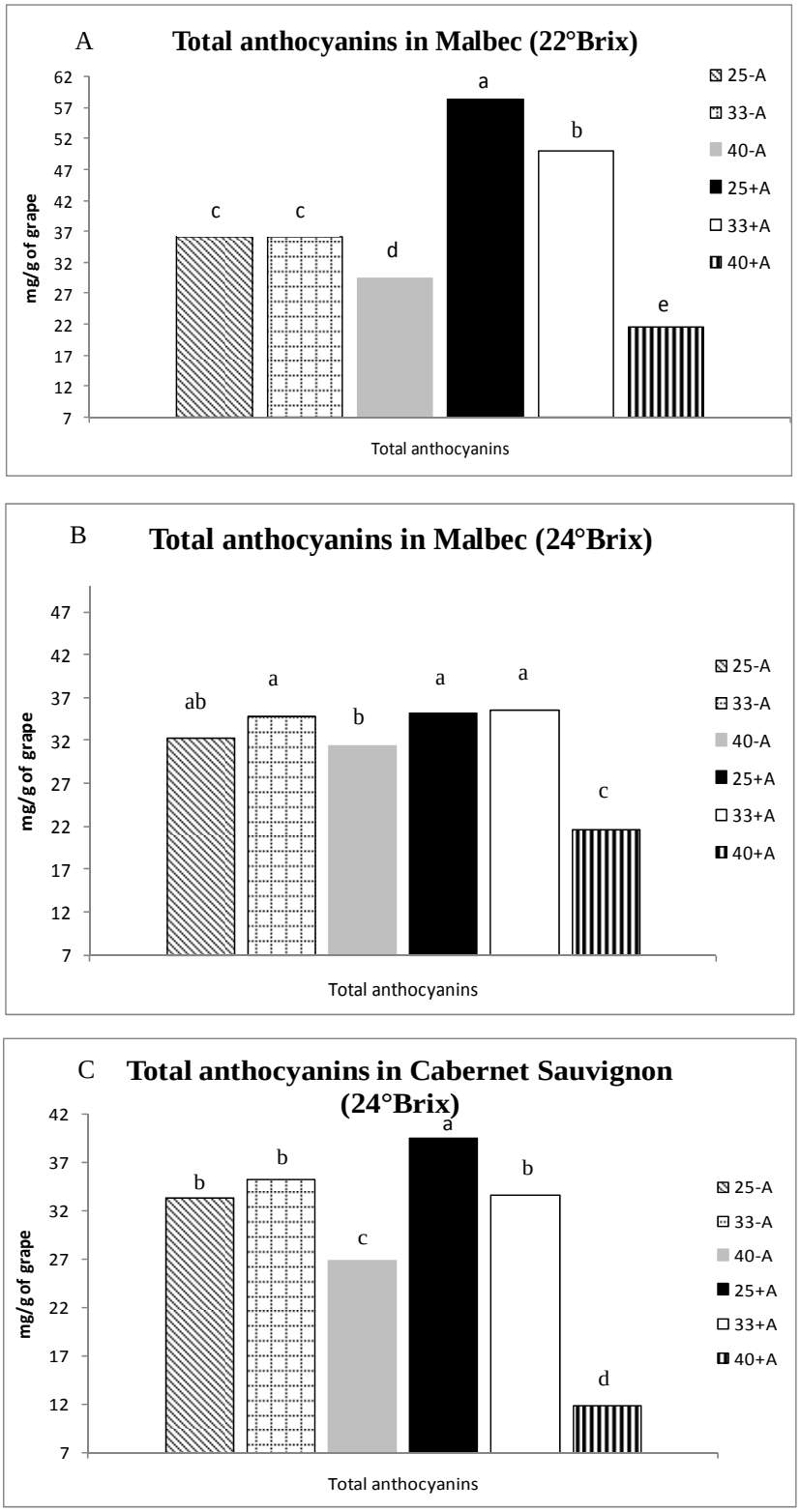

Fig. 2 Total anthocyanins from grapes subjected to different treatments: 25, 33 and 40 °C, being: 25 °C plus ABA (25 + A), 25 °C without ABA (25 - A), 33 °C plus ABA (33 + A), 33 °C without ABA (33 - A), 40 °C plus ABA (40 + A), 40 °C without ABA (40 - A). A: Total anthocyanins in Malbec of 22°Brix; B: Total anthocyanins in Malbec of 24°Brix; C: Total anthocyanins in Cabernet Sauvignon of 24°Brix. Means affected by the same letter are not significantly different at *P* = 0.05.

Table 1 Anthocyanins profile of grape at temperatures different combined with treatment of ABA.

Treatments	Cya3-O-gl	Peo3-O-gl	Del3-O-gl	Pet3-O-gl	Mal3-O-gl	Peo acethyl glu	Mal acethyl gl	Peo cumaril	Mal cumari	Tri/bi-hydroxylated	OMT1	OMT2	OMT3
Malbec 22°Brix													
25-A	0.13	1.21	1.07	1.79	17.62	0.66	1.31	0.66	12.07	15.27	1.66	6.16	9.35
33-A	0.08	0.95	10.9	1.83	16.87	0.40	1.52	0.55	12.94	19.26	1.66	5.77	12.10
40-A	0.83	0.88	0.63	1.28	13.25	0.63	1.19	0.53	11.10	15.77	2.03	6.95	10.70
25+A	0.36	1.82	1.32	2.98	27.45	0.90	2.25	0.90	20.36	14.58	2.26	6.40	5.15
33+A	0.10	1.15	1.3	2.29	22.81	0.89	2.14	0.71	18.69	21.10	1.76	6.38	10.50
40+A	0.06	0.87	0.37	0.97	11.81	0.83	1.79	0.41	9.10	14.13	2.62	8.79	15.00
Malbec 24°Brix													
25-A	0.17	1.07	1.36	1.94	15.04	0.42	1.36	0.49	10.32	15.48	0.78	11.02	9.14
33-A	0.20	1.17	1.33	2.16	17.32	0.26	1.25	0.50	10.59	15.18	0.89	13.07	5.92
40-A	1.14	1.30	1.46	2.13	14.04	0.30	1.33	0.58	10.02	12.19	0.89	9.58	9.12
25+A	0.12	0.97	1.35	2.11	16.86	0.43	1.44	0.49	11.37	18.70	0.72	12.48	8.00
33+A	0.24	1.26	1.30	2.36	19.33	0.49	0.99	0.38	8.98	15.35	0.97	14.84	5.22
40+A	0.06	0.83	0.55	1.14	11.64	0.56	0.67	0.28	5.82	14.87	1.53	21.31	13.57
Cabernet Sauvignon 23°Brix													
25-A	0.54	2.64	2.23	2.36	19.37	0.31	2.96	0.29	2.52	7.53	1.06	8.69	4.89
33-A	0.60	2.57	2.79	2.52	20.73	0.27	2.55	0.30	2.86	8.19	0.90	7.43	4.22
40-A	0.80	2.26	2.06	2.54	14.18	0.35	2.56	0.20	1.75	6.10	1.24	6.89	2.80
25+A	0.96	3.40	2.89	3.40	21.86	0.49	3.62	0.30	2.44	6.46	1.18	7.54	3.55
33+A	0.36	2.33	1.93	2.06	22.38	0.27	0.29	0.29	3.64	9.84	1.07	11.60	6.49
40+A	0.30	1.08	0.69	0.85	6.68	0.22	1.26	0.07	0.59	5.95	1.23	9.73	3.59

Cya3-O-gl: cyanindin 3-O-glucoside; Peo3-O-gl: peonidin 3-O-glucoside; Del3-O-gl: delphinidin 3-O-glucoside; Pet3-O-gl: petunidin3-O-glucoside; Mal3-O-gl: malvidin 3-O-glucoside; Peo acethyl glu: peonidin 3-acetylglucoside; Mal acethylgl: malvidin 3-acetylglucoside; Peo cumaril: peonidin 3-p-coumaroylglucoside; Mal cumaril: malvidin 3-p-coumaroylglucoside. OMT1: O-methyltransferase petunidin derivated of delphinidin; OMT2: O-methyltransferase malvidin derivated of delphinidin; OMT3: O-methyltransferase peonidin derivated of cyanindin.

in grape at 25° y 33°C when we supply ABA.

The tri/di-hydroxyled ratio was superior only at 25 °C. At 33 °C was similar a without ABA because increased two derived di-hydroxyled compound (petunidin 3-O-glucoside). Those effects evidenced the stimulation of F3’5’H and O-methyl transferase (derived of cyaniding 3-O-glucoside).

Cabernet Sauvignon was more sensible at temperature change. Grape at 33 ℃ showed a higher content of TA. Tri/di-hydroxyled ratio was incremented at 33 ℃ but decreased 22% at 40 ℃ (more important than Malbec). When the temperature was 25 ℃, tri/di-hycroxylad ratio did not show changes, and this was due to increase of di and tri-hydroxyled anthocyanins. At 33 ℃, the increase was due principally at delphinidin and malvidin 3-O-glucoside. This temperature modified the activity or expression of F3’5’H at increment the delphinidin and derivate compounds contents, favoring the colors blue in wine.

For the winemaking the decreased in TA produced for high temperature (40 ℃) is a negative economical effect and it is of major intensity in Cabernet Sauvignon than Malbec.

ABA supply incremented the TA and malvidin 3-O-glucoside was the principal compound affected. Also, ABA favored the methylation of compounds which is reflected with greater color stability in wine.

4. Conclusion

Global climate is changing and is predicted increases in temperatures. Is necessary to know the response of different cultivars versus temperature increase for to redefine the regions for each cultivar and strategies used to compensate the adverse effects on the color of wines.

With regard to the wine industry, color is crucial to quality for the production of premium red wines. The first sensorial contact to wine is usually made by visual inspection which starts building up the consumer's perceived quality.

We studied the effect of diverse temperatures in two cultivars, and the differences between Malbec and Cabernet Sauvignon is due to genetic differences. Each cultivar responded differentially at temperature increment and the effect prejudicial of 40 °C was major in Cabernet Sauvignon than Malbec. This knowledge allows redefined the region optimal for each cultivar.

The different cultivars responded variably at ABA supply. In general, this hormone incremented TA versus treatments without exogenous ABA. The effect was stimulating two route biosyntheses with different intensity (for di and trihy-droxylated anthocyanin compound). The activity or expression of enzyme F3´5´H was stimulated, as to the O-methyl transferase for the derivate malvidin.

The change of profile of anthocyanins modified the tonality because of the increase of blue/red colors. Also, increment the stability of those compounds. The major stability is favorable for wine as to could be conserved in time.

The supply of this growth regulator will converted an important strategy for the temperature increased effect.

The thresholds of temperature to obtain the major synthesis of antocianos is specific according to variety, should be study each one of them. ABA effect cannot be extrapolated at another cultivar.

Acknowledgments

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