Artificial ripening of grape seed phenolics in Pinot noir and Cabernet Sauvignon

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Abstract. Red Vitis vinifera cultivars are often limited in their performance by climate conditions (e.g., rain, humidity, cold winters, and cool summers). Cool climate viticulture regions are characterized by short growing seasons that reduce fruit quality, limiting technological and phenolic maturity. Management of fruit technological ripening, in vineyard or post-harvest, is pivotal for wine quality. However, the impact of vineyard or cellar practices on seed phenolic fraction remains poorly understood. The aim of our project was to evaluate seed color change, phenolic composition, and their extraction potential after an oxidation induced by a freezing treatment. The freezing treatment was followed by 24 hours of incubation at different temperatures in two Vitis vinifera cultivars: Pinot noir and Cabernet Sauvignon. Results are reporting that the freezing caused the seed color darkening and significant phenolic changes, suggesting similarities with the natural process. The phenolic evolution reported different behaviour between cultivars and compound classes. Most of the changes occurred during the first three hours of incubation, indicating that the oxidation reactions take place at the beginning of the thawing process.

1 Introduction

Vitis vinifera requires dry and warm summers and reduced precipitations to reach an optimal fruit maturity for winemaking. Worldwide, optimal regions for viticulture are limited to Mediterranean climates, even if global warming is causing some shift in viticultural regions. At least 160 up to 225 days of growing season length are necessary for Vitis vinifera to reach ripeness [1]. Thus, choosing the most suitable cultivar in relation to the climate is an important decision. Nevertheless, it is not the only factor to keep in consideration, the wine market is complex and price fluctuations of grape and wine, depending on the costumer’s choices, must be kept in mind.

In cool climate regions, grapevine faces ripening limitations. Cool summers, abundant precipitation post-veraison and short growing seasons are important abiotic issues that prevent the achievement of technological and phenolic fruit maturity. Furthermore, extreme temperatures can alter the physiological pathways of the berry, causing an incomplete phenolic maturation [2]. The insufficient phenolic ripening results in wines with low phenolic structure, flavor defects and unpleasant bouquet with the classical characteristic vegetal and unripe odor.

It is possible to categorize the development of the proanthocyanidin in grapevine seed in four different phases [3]. The first two phases concern the biosynthesis, the first one lasts one month, and it starts at flowering. During this period, an intense synthesis of procyanidin occurs. Immediately after, it starts the second phase with the synthesis of flavan-3-ol monomers that stops at veraison. Once seed tannin content reaches the maximum concentration, at veraison, the last two phases of ripening take place. During the third phase, a programmed oxidation process occurs. Then, the fourth phase starts when the seed desiccation is accomplished with a non-programmed oxidative mechanism.

Recent literature [4] described the browning process occurred after an artificial freeze-thaw treatment. Further studies [5] also investigated the change occurred in seed phenolic fraction after grape seed freezing. The freezing treatment facilitated the oxidation process, breaking vacuoles and causing the release of oxidase enzymes.

The present study aims to characterize color evolution and phenolic changes of grapevine seed after a freeze-thaw treatment as an alternative strategy to improve wine quality in cool climate regions. Techniques to manage grapevine seed ripening are not available yet, both in field and during the winemaking process. This technique, easy to perform by the wine industry, economically viable and potentially efficient, could solve issues related to ripening limitation, allowing a wider number of winemakers to achieve quality.

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2 Materials and methods

The experiment was carried out during the 2019 growing season in the laboratory of the Department of Horticulture, Plant & Soil Sciences Building, Michigan State University, East Lansing, MI, 48824, United States of America. The grape samples were harvested in a commercial vineyard owned by 12 Corners Vineyards and Winery, located in Benton Harbor, MI, United States (42°13’N 86°22’W).

2.1 Plant material

Based on previous works [4, 5], two *Vitis vinifera* cultivars were used for the experiment; Pinot noir (PN) and Cabernet Sauvignon (CS), which have high and low seed tannins content, respectively. For each cultivar, basal clusters were sampled at two phenological stages: veraison (BBCH 83) and harvest time (BBCH 89). At veraison, clusters with 50% of the berries at color change and about 10° Brix, were collected on August 19. At harvest, samples were collected when berries reached about 20° Brix, on September 27. Samples were returned to the viticulture laboratory in a cooler and stored at 4 °C until the seed removal.

2.2 Experimental design

Fifteen clusters for each cultivar were collected within a single vineyard row from the east side of the canopy. At veraison and harvest, 2,400 berries (1,200 each variety) were removed from clusters, and 100 berries were equally distributed between treatments and replications based on total berry weight and peel color. Samples were then put in separate Ziploc® bags, crushed, and 2,800 seeds (1,400 for each cultivar) were separated from the pulp, rinsed with distilled water, blotted dry with a KimWipe®, and prepared for the color, chemistry, and microscopic analysis. Each treatment used a total of 360 seeds (120 each replication). The juice was stored for basic fruit quality analysis (Table 1). The seeds to be frozen were put into tubes and then frozen at -20 °C for at least 24 hours prior to analysis. Each group of 100 berries was assigned to one of four treatments. Each treatment and its relative time analysis had three replications. In total four different treatments were tested: T20, T20I20, T20I40, and T20I120. The T20I20, considered as control, had no freezing and thawing at 20 °C; T20I40, no freezing and thawing at 40 °C; T20I20, freezing at -20 °C and thawing at 20 °C; T20I120, freezing and thawing at 40 °C. The T means freezing temperature and the I, incubation temperature. For freezing treatments, analysis began following the removal of seeds from freezing conditions, whereas for non frozen treatments, measurements started after seed preparation. To begin, seeds were placed in a 6 cm plastic weighing boat and, according to the treatment, were kept in one of two different environments: at room temperature (20 °C) or at 40 °C (laboratory convection oven). The total incubation time was 24 hours for all treatments. Samples were evaluated for color and chemical analysis after 5 minutes, 1 hour, 3 hours, 6 hours, and 24 hours of incubation.

2.3 Color analysis

Color measures occurred 5 minutes, 1 hour, 3 hours, 6 hours, and 24 hours after the start of incubation. Each evaluation was repeated 10 times using a Konica Minolta Chroma Meter CR-400 (Konica Minolta, Osaka, Japan). After every evaluation, seeds were mixed and re-placed into the plastic weighing boat. Seed color was characterized using three color dimensions of the Munsell system [6]: lightness (L), chroma (C) and hue (h).

The lightness spectrum goes from 0 (0% light) to 100 (100% light) [7]. The chroma increases with color fullness, representing the purity of the color. Finally, the hue represents the tone of the color and is measured in angles degrees (0° = red; 90° = yellow; 180° = green; 270° = blue). The different dimensions are then combined to obtain the color in consideration by a LCh gradient picker.

2.4 Chemistry analysis

After each color measurement analysis, 20 seeds were separated from the plastic tray and prepared for analysis, weighted, and inserted into a 50 mL tube containing 20 mL of extraction solution. The extraction solution contained 80% of methanol, 19% of distilled water and 1% of formic acid. The seeds were kept in contact with the extraction solution for 24 hours on a rotating plate. After 24 hours, the extract solution was poured into another tube and stored in freezer and 15 mL of new extraction solution were added into the tube with the seeds and located again on the rotating plate for one hour. Later, the 15 mL extract solution was poured into the tube previously placed in freezer. Thus, the 35 mL extraction solution was analyzed by LC-MS using a Waters Acquity UHPLC interfaced to a Waters Xevo G2-XS Q-TOF mass spectrometer. Sample (10 μL) was injected onto a Waters HSS-T3 UHPLC column (2,1 × 100 mm, 1.7 μm particle size) held at 40 °C using a binary gradient of water with 0,1% formic acid (solvent A) and acetonitrile (solvent B). The solvent flow rate was 0.3 mL/min; it was started at time 0 with 100% A, held for 0.5 min at 100% A, ramped to 50% B at 6 min, ramped to 99% B at 6.5 min, held at 99% B until 8.5 min, reverted back to the starting condition of 100% A at 8.51 min, and held until 10 min.

Compounds were ionized by electrospray ionization in positive-ion mode with a capillary voltage of 3.0 kV, a
The color index, combining the color parameters, gives a schematic pattern of the color evolution (Fig. 1). The lowest is the color index value, the darker is the color. At veraison, for both PN and CS is possible to distinguish 4 pattern pairs between the treatments: the first and the second pairs are formed by the freezing treatments (T-20°C, T-30°C) and the not frozen treatments (T-20°C, T-30°C), respectively, which have similar evolution intensity. The third and fourth pairs are the incubation at room temperature (T-20°C, T-30°C) and the incubation at 40 °C (T-20°C, T-30°C), respectively, which have similar kinetics. At harvest, the starting values were lower than the veraison ones, because of the higher level of maturity. The CI values during the incubation period increased for the treatments at 40 °C (T-20°C, T-30°C) after 6 hours at veraison and after 3 hours at harvest. The T-20°C at harvest (CS) increased after 6 hours. These increases in value are probably due to the brightening occurred after seed desiccation. In general, the color index decreased in all treatments with different intensity and the freezing caused the highest reduction.

Fig. 1. Color Index graph for PN (A, C) and CS (B, D) at veraison (A, B) and harvest (C, D). The black symbols correspond to the not frozen treatments, the white to the freezing treatments. The circle corresponds to the incubation at 20 °C and the triangle at 40 °C. Different letters identify significant different means and *, ** and *** mean significant difference per P<0.05, P<0.01 and P<0.001, respectively.

3.2. Phenolic results

The chemical analysis showed the evolution of the quality and quantity of phenolic compounds. The metabolomics profile of phenolic acids, flavonols, stilbenes, catechins, and procyanidins has been utilized to build a heatmap. The T-20°C, considered as control, was compared with the other treatments (Fig. 2). The range of changes is between ± 6%, expressed as percentage. The compound concentration showed significant changes between the freezing and not frozen treatments. In both cultivars, an increase in total phenolic acid was observed due to freezing treatments, among them, gallic acid, 2/3/4 trihydroxybenzoic acid and procatechuic acid. The incubation temperature boosted this evolution. Phenolic acid and flavonol changed significantly compared with catechins and procyanidins. The single compounds showed variations between the freezing treatments (T-20°C, T-30°C), as gallic acid and 2/3/4 trihydroxybenzoic acid increase was higher among the incubation at 40 °C. In CS the total catechins and total phenols had a decrease after the freezing treatments, especially with incubation at 40 °C compared with PN. The compounds evolution had a specific pattern for the cultivar, the freezing treatments, and the different compound classes.

4. Discussion
Regarding the color evolution, the change that occurred during the incubation suggests speculations about the oxidation process. Indeed, the seed color change that occurs naturally is considered crucial for the maturity level of grape seeds [10], and the color modifications are partly related to phenols oxidation [11]. Between the different treatments, the browning process was significant, especially for the freezing treatments (T20°C, T20°C). The freezing process, followed by the thawing, causes the cell and the vacuole membranes disruption (Fig. 3) [12]. Thus, the phenolic compounds, located in the vacuole, are exposed to the oxidative enzymes which are in the cytosol. Evidently, the change occurred at harvest during the incubation was lower respect to veraison. The reason is that at harvest the maturity level was higher, and the color was darker. The freezing treatments (T20°C, T20°C) had similar evolution intensity in the color parameters but different kinetics, resulting in a darker and browner color in all the time points (after 5 m, 1 h, 3 h, 6 h and 24 h of incubation) in comparison to the not frozen seeds. Indeed, the similar change intensity of the color suggests a higher oxidation in comparison to not frozen treatments due to the freeze-thaw process. A slight browning occurred even in not frozen seeds, but it was significantly lower, brighter, and compartmentalized. Thus, a weaker oxidation and chlorophyll degradation might be occurred. The darkest and brownest color was among the freezing treatments confirming the speculation regarding the higher oxidative reactions related to the tissue disruption. At veraison, when the seeds were highly far from the maturity, the browning process during the incubation of the freezing treatments was even higher than the seeds sampled at harvest time before the start of incubation. PN and CS are highly different in total phenolic content and tipology of phenolic compounds. The total phenolic content of CS had nearly halved from veraison to harvest, while in PN slightly decreased. Indeed, PN is a cultivar rich in seed tannins, whilst CS is not. The treatments had different patterns among PN and CS, suggesting a specific effect related to the cultivar. Thus, the seed cultivar diverse response to the freezing treatment was confirmed by the 2020 study on four Vitis vinifera [5]. At harvest, the freezing effect was inferior respect to veraison. The phenolic acids, as gallic acid, and flavonols increased with the freezing treatments compared to the not frozen ones. These classes of compounds are synthetized in the same biosynthetic pathway of flavanols, but further upstream [13]. On the other hand, flavanol compounds recorded a reduction. The cause of this shift from downstream to upstream compounds could be related to the division of polymerized flavanol to single units. The maximum changes occurred after 3 hours of incubation, suggesting that most of the oxidative reactions took place at the beginning of the incubation period. Regarding the incubation temperature, it could affect the metabolic characteristics of enzymes. Between veraison and harvest, the decrease in treatment effect can be associated with the seed ripening phases theorized by Kennedy et al. [3], that reported a non-programmed oxidation occurring after the programmed one. Indeed, the non-programmed reactions have lesser effects on the final composition of seed tannins compared to the programmed one.

Fig. 2. Heatmap displaying the metabolomic profile of phenolic acids, flavonols, stilbenes, catechins, and procyanidins after three hours of incubation. Data was log 10 transformed and treatments were expressed as a percent change from the control (T40°C). Both axes are clustered, and red and blue represent a percentage increase and decrease of compounds, respectively. Data were analyzed by t-test compared to the control, and significantly different means. T40°C, no freezing and thawing at 20°C; T0°C, no freezing and thawing at 40°C; T20°C, freezing and thawing at -20°C and thawing at 20°C; T40°C, freezing and thawing at 40°C; T, freezing temperature; I, incubation temperature. Symbols identify significant difference for *p ≤ 0,05, **p ≤ 0,01 and ***p ≤ 0,001.
Fig. 3. Control seed cells (A) and after freezing (B). The freezing (-20 °C) caused collapsing of cell membranes (B). Vibratome Series 1000® Tissue Sectioning System. Stained with propidium iodide. Pictures taken using a Nikon DS-Fi3 camera attached to an Eclipse Ni-E® upright microscope. Images were processed using NIS-Elements Basic Research Software.

5. Conclusions

The results confirmed that the induction of color darkening through a freeze-thaw treatment is similar to natural seed maturation process. PN and CS reported different compounds evolution, suggesting specific applications of the treatment and further studies should be carried to test cultivars responses. Most of the chemical changes in phenolics occurred before three hours of incubation. The freezing treatments affected the seed tannin content in a significant way compared to the control (T_{20I20}), and further studies focused on wine model solutions are necessary to understand what really happens in musts and wines during the winemaking process. Finally, future studies should focus on the potential impact of this technique on bitterness, astringency, and flavor of the resulting wines.

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