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Utilization of a freeze-thaw treatment to enhance phenolic ripening and tannin oxidation of grape seeds in red (*Vitis vinifera* L.) cultivars

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ABSTRACT

Phenolic ripening represents a major interest for quality wine producers. Nevertheless, climatic or genotypical limitations can often prevent optimal maturation process. During winemaking seeds can be easily separated and technologically processed to improve their quality. Relying on the key role of oxidation for phenolic ripening, a freeze-thaw treatment was proposed to improve the fruit quality for potential use in challenging growing conditions. The experiment was carried on in two distinctive viticultural areas, Michigan and Italy. Five cultivars (Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot noir and Chambourcin) and six cultivars (Cabernet Sauvignon, Sangiovese, Syrah, Croatina, Barbera and Nebbiolo) were used in Michigan and Italy, respectively. Samples were collected at different phenological stages, to describe the natural ripening process and grape seeds were characterized before and after a freeze-thaw treatment. Colorimetric and spectrophotometric data highlighted similarities among natural and artificial seed ripening promising future applications for the wine industries.

1. Introduction

The importance of seed color at harvest time for grape quality evaluation has been understood for millennia – the famous Roman agriculture writer Columella (4-70 A.D.) suggested the process of seed darkening as the best grape ripening index in his book: “De Re Rustica” (Rustioni & Failla, 2016). During ripening, a grape seed starts as a bright green color, and slowly changes to yellow, and eventually dark brown shades (Ferrer-Gallego, García-Marino, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón, 2010; Ristic & Iland, 2005; Rodríguez-Pulido et al., 2012). The seed coat modifications during ripening aims to provide mechanical protection to the embryo and to maintain seed dormancy (Ristic & Iland, 2005; Rolle et al. 2013). Seed browning during berry ripening is considered to be the result of oxidation of flavan-3-ols and tannins (Adams, 2006; Kennedy, Matthews, & Waterhouse, 2000; Ristic & Iland, 2005; Rustioni & Failla, 2016), which are initiated by an oxidative burst at veraison (Pilati et al., 2007). The major role of phenolic oxidation in coat browning has been shown to occur during the development of seeds and fruits in different species including *Arabidopsis thaliana*; *Phaseolus vulgaris*; *Zea mays*; *Litchi chinensis* (Pourcel et al., 2007).

In addition to tannin color, the oxidation of phenolics is expected to affect their gustatory perception, including astringency (McRae & Kennedy, 2011). It is well known that astringency perception is a complex tactile sensation caused by a loss of lubricity in oral saliva (Cheynier et al., 2006; McRae & Kennedy, 2011). The interactions between tannins and saliva proteins that are responsible for this perception involve a number of mechanisms, including hydrophobic interactions (Van der Waals and π-π stacking), hydrogen bonding, self-association (causing cross-links between protein-tannin complexes) and finally, protein aggregation and eventual production of colloidal particles (McRae & Kennedy, 2011). Through the formation of new bonds, and the modification of the molecular structures and interactions (McRae & Kennedy, 2011; Pourcel et al., 2007), intra-molecular bonding is increased, which reduces tannin flexibility; altering linear and extended tannin forms into more condensed structures (McRae & Kennedy, 2011; Poncet-Legrand et al., 2010). Flavanol polymerization reactions, regardless of the polymers formed (proanthocyanidins, oxidation products, or ethylflavanols), generally enhance the astringency (Cheynier et al., 2006). Nevertheless, the availability of binding sites (associated to the structural flexibility) and the steric hindrance of the tannin polymer could prevent the protein access to binding sites, creating a threshold in the correlation among tannin size and protein binding efficacy (McRae & Kennedy, 2011).

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Despite the current knowledge of seed color and tannin oxidation, and their relationship to fruit quality and wine making, environmental conditions pose a challenge to their development in different viticultural areas: ideal growing conditions for *Vitis vinifera* consist of temperate climate regions with warm, dry summers seeing moderate precipitation, and mild winters. The first challenge is the increase in heat accumulation in warm viticultural grape growing regions, which is prominent in many Italian vineyards. Increased summer temperatures are capable of altering the pathways of secondary metabolites during ripening, resulting in poor polyphenolic maturation and low fruit quality at harvest (Frioni et al., 2017). The second challenge is the annual variation in climatic conditions experienced in many cool climate viticultural regions, including Michigan. Each issue can be associated with global climate change, and they are limiting the sustainable production of grapes with a consistent optimal fruit quality at harvest in several viticultural areas of the world (Schultze, Sabbatini, & Luo, 2016).

Technological procedures targeted towards improving phenolic oxidations are widespread among wine producers located in suboptimal environmental climates. The most prominent examples include: must oxygenation during maceration, use of wood barrels and, in general, wine micro-oxygenation (Gómez-Plaza & Cano-López, 2011). The main restriction of these techniques concerns the non-selective nature of tannin oxidation obtained through musts and wines, which involves interactions with other molecules that potentially affect additional organoleptic characteristics (e.g. accumulation of aldehydes). The oxidation effects on different wines has been described by Bueno, Culleré, Cacho, and Ferreira (2010).

Phenols are predominantly located in the vacuole, while oxidoreductases are found in the cytoplasm. Thus, their interaction, and subsequent induction of enzymatic browning will not occur unless the cell membranes are damaged (Li, Guo, & Wang, 2008). Freeze-thaw treatments are known to affect the ultrastructure of vacuolated cells, due to the formation of ice crystals during freezing, which affect membranous components of the protoplasm (Mohr & Stein, 1969). This process has previously been observed in studies focused on strawberry shelf-life managements (Holzwarth, Korhummel, Carle, & Kammerer, 2012; Oszmiański, Wojdyło, & Kolniak, 2009). Considering grape seeds, different techniques are already available to separate them from the must during winemaking (Canales, Llaudy, Canals, & Zamora, 2008). Nevertheless, the seed removal (recommended in case of suboptimal phenolic ripening) could also impoverish wines in terms of ‘body’ and ‘structure’, eliminating a tannin source. Thus, in our opinion, seed recycling after freeze-thaw treatment, could be recommended to improve wine quality.

Phenolic heterogeneous pigments obtained by free radical polymerization caused by oxidations (Waterhouse & Laurie, 2006) are difficult to be quantified by traditional chemical assays due to their inhomogeneity and extractability limitations. For example, the oxidation bonds are resistant to acid catalyzed thiolysis (Kennedy et al., 2000) and the tannin oxidation can lead to solubility problems (Poncet-Legrand et al., 2010; Zanchi et al., 2007). Nevertheless, the presence of conjugated double bonds in these phenolic oxidized molecules allows the absorption of light of visible wavelengths (Rustioni, 2017). In fact, the spectrum of oxidized polymeric phenolics was recently characterized in sunburn grape berries, indicating a broad absorption band in the green spectral region, with a maximum around 500 nm (Rustioni, Rocchi, Guffanti, Cola, & Failla 2014). Therefore, the optical properties of seeds measured on-solid could be impacted by the detection limitations of the oxidized polymeric phenolics. However, CIELab color parameters and image analysis have already been proposed as a technique to evaluate seed ripening (Ferrer-Gallego et al., 2010; Obreque-Slier, López-Solís, Castro-Ulloa, Romero-Díaz, & Peña-Neira, 2012; Rodríguez-Pulido et al., 2012).

The present study aims to test a new procedure based on the selective oxidation of grape seeds by a physical treatment (freezing) to improve tannin ripening. Modifications to the seed’s optical properties that occur during natural ripening and freezing treatment will be investigated and described using two different analytical approaches (spectrophotometric and colorimetric), and will utilize grape cultivars grown in two viticultural areas (Italy and Michigan).

2. Materials and methods

2.1. Environmental characteristics

The experiment was carried out during the 2017 growing season in the United States (Michigan) and Italy. Benton Harbor, Michigan (Latitude 42.0841 deg, Longitude – 86.3570 deg, Elevation: 220 m) and Riccagioia, Italy (Latitude 44.98; Longitude 9.08; Elevation 144) are characterized by different climates. Following the Köppen-Geiger Classification (Köppen, 1936), Benton Harbor’s climate is under the Dfa type: continental without dry season with hot summer, while Riccagioia is Cfb: temperate without dry season with warm summer. The following agro-climatological analysis were completed using data collected from weather stations located near the experimental vineyards. The Benton Harbor weather station is a part of Enviro-weather, a weather station network run by Michigan State University, and is located 14 km far from the commercial vineyard in the Lake Michigan Shore AVA (American Viticulture Area) where samples were collected. Torrazza Coste station, 1 km far from the Riccagioia experimental field, belongs to the network of Consorzio Tutela Vini Oltrepò Pavese, the local winegrowers consortium. Data were analyzed for the period 2000, January 1st – 2017, October 31st. In order to provide an evaluation of the availability of thermal resources during the different phases of the growing season two methods of analysis were used:

1. GDD – Winkler Growing Degree Days (Amerine & Winkler, 1944)
2. NHH – Normal Heat Hours (Cola et al., 2014; Parisi et al., 2014)

The advantage of NHH over GDD is the use of a response curve considering optimal temperature for grape growth, taking into account the negative effects of under and over-optimal temperatures on plant growth (Mariani, Parisi, Cola, & Failla, 2012).

Fig. 1 displays these patterns from 2000 to 2017; blue and red lines represent the average value for Benton Harbor (Michigan) and Riccagioia (Italy), respectively, in terms of the seven Winkler classes. The average value for Benton Harbor was 1576 GDD, which follows the Winkler class II: temperate cool, suitable for early ripening grapes for wines to be aged, and medium ripening grapes for white or red wines ready to drink. Riccagoia, with an average value of 2119 GDD, falls into class IV: temperate warm, suitable for late ripening grapes for white or red wines ready to be aged. The difference in thermal resources availability of the two areas is confirmed by the analysis of Fig. 2, where ten-day accumulations of GDD (a1 and a2) and NHH (b1 and b2) are presented. Red lines represent 2017 behavior, and thick, black lines indicate average 2000–2016 values. The dark grey area is bordered by an average ± 1 standard deviation and the light gray area by an average ± 2 standard deviation. The timespan between fruit-set and physiological maturity is represented by the purple area.

2.2. Plant material and experimental design

In Michigan, 5 cultivars were considered: Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot noir (*Vitis vinifera* L.) and the French-American hybrid Chambourcin (Seyval-Villard 12–417 × Chancellor). Samples (3–5 bunches) of each cultivar were collected at the beginning of ripening (T0 – BBCH 85) and harvest time (T2 – BBCH 88) from a commercial grower collection (Meier, 2001). In Italy, vines were used from the ampelographic collection located in Oltrepò Pavese (south Lombardy), described in Rustioni et al. (2013). Six (*Vitis vinifera* L.) cultivars (Cabernet Sauvignon, Sangiovese; Syrah; Croatina; Barbera