Scientia Horticulturae 209 (2016) 148–155

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Effect of early leaf removal on *Vitis Vinifera* L. *cv*. Tempranillo seeds during ripening based on chemical and image analysis

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ARTICLE INFO

Article history:
Received 10 December 2015
Received in revised form 9 June 2016
Accepted 10 June 2016
Available online 30 June 2016

Keywords:
Early leaf removal
Grape seeds
Image analysis
Phenols

ABSTRACT

Phenolic composition, colour and morphology variables were monitored during ripening in grape seeds of *Vitis vinifera* L. *cv*. Tempranillo in 2010. The aim of the study was to determine the effect of limitation induced by early leaf removal (ELR) *vs.* non-defoliated (ND) control vines. The ultimate goal of this research was to assess whether phenolic composition could be predicted based on variables obtained by image analysis. ELR advanced grape maturity also had lower phenolic concentration and smaller and darker seeds. (+)-Catechin and total cinnamic acid contents as well as L^* and aspect ratio were the most significant parameters for distinguishing treatments. Furthermore, area, length, width, L^*, a^*, b^* and heterogeneity predict the phenolic composition in grape seeds. Although it is not yet a substitute for chemical analysis, it could become a quick way to estimate the phenolic composition of grape seeds during maturation. The methodology proposed in this work could be a powerful tool for winemakers.

1. Introduction

The phenolic compounds located in grape skin and seeds provide different properties to red wine depending on the stage of maturation (Robichaud and Noble, 1990). The qualitative and quantitative phenolic composition of grapes depends on multiple factors, including climate, variety, soil, water availability, and degree of ripeness (Bautista-Ortín et al., 2012). Several studies have shown that phenols in seeds accumulate before the onset of ripening or veraison, reaching a maximum around veraison and decreasing towards harvest (Ferrer-Gallego et al., 2010; Kennedy et al., 2000b). Moreover, it has been reported that poorly ripened berries have lower phenol extractability from skin and higher extractability from seeds (Peryot des Gachons and Kennedy, 2003).

Changes in seed coat colour and morphology have also been related to developmental changes in berry anthocyanins and total skin phenolics, which suggests that external appearance and seed colour may be used as an additional indicator of overall berry ripeness (Ferrer-Gallego et al., 2010; Ristic and Iland, 2005). Usually colour is determined by tristimulus colorimetry and expressed in terms of L^*, a^* and b^* variables, corresponding to the uniform colour space CIELAB (CIE, 2004). Colorimeters, spectrophotometers and spectroradiometers are instruments used to measure colour and require homogenization of the sample in order to obtain a uniform colour, a tedious process when samples are heterogeneous or small, such as grape seeds. For this purpose, digital image analysis is advantageous because it can be used to measure colour and other characteristics such as shape, texture and homogeneity (Zheng and Sun, 2008).

Vineyard management is another aspect that affects phenolic compounds, colour and morphology of grape during seed and grape development (Roby and Matthews, 2004). Early defoliation is a viticultural practice that has proved to be effective for regulating yield and improving grape quality (Tardáguila et al., 2010). It is usually carried out pre-bloom, unlike traditional leaf removal, which is typically done between fruit set and veraison on high density canopies to improve fruit exposure and air circulation (Tardáguila et al., 2008). Crop regulation is achieved in early defoliated vines through reduced fruit set, producing smaller and looser clusters that are less susceptible to Botrytis rot (Poni et al., 2006). In these two studies, grape quality also improved in defoliated vines as soluble solids and anthocyanin concentrations increased.

The relationships between these practices and phenolic composition, colour and morphology of seeds are still unknown. The

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http://dx.doi.org/10.1016/j.scienta.2016.06.013
0304-4238/

aim of this study, therefore, was to establish whether source limitation induced by ELR affects phenolic composition, colour and morphology of grape seeds from Vitis vinifera L. *cv*. Tempranillo *vs.* non-defoliated (ND) control vines, from veraison to postharvest. Finally, correlations between phenolic composition and appearance (colour and morphology) of seeds of different agronomic techniques were determined.

The wine industry requires the use of simple, rapid and reliable analytical procedures, which involve minimal or no sample preparation, to assist in making harvesting decisions or in choosing proper winemaking techniques. The methodologies proposed could allow wine producers to make rapid decisions and may, therefore, be of use in wine production areas.

2. Material and methods

2.1. Plant material and experimental design

Red grape berries from *Vitis vinifera* L. *cv*. Tempranillo grown in Extremadura (Spain) in 2010 were used in this study. The experimental vineyard was in Guadajira (38°N, 6°W, n 198 m a.s.l) and vines were trained to a vertical trellis on a bilateral cordon system oriented in an east-west direction (104° SE–76° NW). The vineyard was planted in 2001 on Richter-110 rootstock at a spacing of 2.5 m by 1.2 m (3333 vines ha^{-1}). Irrigation treatment was done by replacing crop evapotranspiration (ET_c) at a rate of 100% ET_c. Drip irrigation was applied with pressure-compensated emitters of 4 L h^{-1} located in a single row 120 cm apart.

The experimental design was a split-plot with four replicates. The plots had six rows with eighteen vines per row. The main plot consisted of two treatments: early leaf removal (ELR) and control or non-defoliated (ND) treatment. Early leaf removal consisted of manual removal of the first seven basal leaves from the main shoot (seven basal nodes) before flowering.

2.2. Sampling

Vitis vinifera L. *cv*. Tempranillo grape samples were collected in 2010 from each experimental plot at six different developmental stages: from veraison to over-ripeness. The first sampling was performed when the ELR reached approximately 20° Brix. Four replicates were made in each experimental plot, except at some stages of the ND treatment when samples were not considered optimal for performing the analysis (stage I n = 3, stage V and VI n = 2). Sampling was carried out as follows: 100 berries were collected from both sides of the vines in a row within the vineyard. Edge rows and the first two vines in each row were avoided. The samples (whole grapes) were immediately frozen and stored at −80 °C until analyses were performed.

2.3. Must analysis

Total soluble solids (TSS) (°Brix) by refractometry, total acidity by titration (expressed as mg tartaric acid L^{-1} of must) and pH by a pH-meter, were analysed at different maturation stages in the must samples. Total phenolic compounds (TPC, expressed as gallic acid, mg g^{-1} of berry fresh weight) and anthocyanins (expressed as malvidin glucoside, mg g^{-1} of berry fresh weight) were extracted and determined using methods proposed by the Australian Wine Research Institute (Iland et al., 2005). Berry tannin concentration (expressed as catechin, mg g^{-1} of berry fresh weight) was determined according to Sarneckis et al. (2006). All analyses were made in triplicate.

2.4. Phenolic extraction and analysis

Phenolic extraction was carried out as described in Nawaz et al. (2006) with some modifications. Grape seeds were manually separated, freeze-dried and ground to obtain a homogeneous powder. Sample (2 g) was homogenized in 10 mL of 75% ethanol, agitated for 1 h in a shaking apparatus (VWR Incubating minishaker, Pennsylvania), and further centrifuged at 4190*g* for 5 min. The supernatant was collected and the residue was submitted to a second extraction using 7.5 mL of 95% ethanol as solvent. The extracts (2 mL) were combined and concentrated (Eppendorf® Concentrator plus/Vacufuge® plus, Germany) to dryness and further re-dissolved in 1 mL of water-methanol-acetic acid (88:10:2, v/v/v). The extracts were injected directly into the chromatographic system after filtration through a 0.45 μm nylon syringe filter (Merck-Millipore, Germany). All analyses were performed in triplicate.

Analysis of the individual phenolic compounds was performed according to the methodology described by Hernanz et al. (2007) with some minor modifications. High Performance Liquid Chromatography (HPLC) analyses were carried out in an Agilent 1100 series HPLC system (Agilent Technologies, California) equipped with a diode-array detector, which was set to scan from 200 to 770 nm, and a C18 Zorbax ODS column (5 μm, 4.6 × 250 mm) (Agilent Technologies, California), using an injection volume of 10 μL.

The solvents were water-methanol-acetic acid (88:10:2, v/v/v, solvent A) and methanol-water-acetic acid (70:28:2, v/v/v, solvent B) at the following gradient: 0–60 min, 100% B linear; 60–70 min, 50% A and 50% B linear; 70–75 min, 100% A linear; 75–80 min, 100% B linear; 80–90 min, washing and re-equilibration of the column. The flow was 1.0 mL min^{-1}, and the temperature of the column was set at 20 °C.

Identification of phenolic compounds was achieved by comparing their retention times and spectra with those of appropriate standards. Quantification was carried out by external calibration from the areas of the chromatographic peaks obtained by UV detection at the following wavelengths: 280 nm for benzoic acids and flavanols, 320 nm for cinnamic acid derivatives and 370 nm for flavonols. The corresponding calibration curves were made up for the following phenolic compounds: (−)-epicatechin ($r^2 < 0.9998$), gallic acid ($r^2 < 0.9999$) and caffeic acid ($r^2 < 0.9999$). The range of the linear calibration curves for (−)-epicatechin was 10–500 mg L^{-1}, with limit to detection (LOD) of 1.703 mg L^{-1} and limit to quantification (LOQ) of 5.677 mg L^{-1}. The range of the linear calibration curves for gallic acid was 1–25 mg L^{-1}, with LOD of 0.069 mg L^{-1} and LOQ of 0.231 mg L^{-1}. The range of the linear calibration curves for caffeic acid was 0.5–45 mg L^{-1}, with LOD of 0.144 mg L^{-1} and LOQ of 0.481 mg L^{-1}. The different phenolic compounds analysed were tentatively identified according to their order of elution, retention times of pure compounds. Quantification of other compounds was made using the calibration curves belonging to the most similar compound. (−)-epicatechin, (+)-catechin, and procyanidins B1 and B2 were quantified with the calibration curve of epicatechin. Gallic acid, ethyl gallate, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid and syringic acid were quantified as gallic acid; and caffeic acid, *p*-coumaric acid, and *m*-coumaric acid as caffeic acid. Standards, epicatechin, gallic acid and caffeic acid, were acquired from Sigma Aldrich®, Analytical Carlo Elba® and Fluka®, respectively.

2.5. Image acquisition

The DigiEye® imaging system based on the calibrated digital camera was used (Luo et al., 2001). This device consists of a closed illumination box, specially designed by Veri Vide Ltd. (Leicester, UK), and a digital camera (10.2 megapixel Nikon® D80 with Nikkor® 35 mm f/2D objective) connected to a computer (Pentium