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Research article

Controlled water deficit during ripening affects proanthocyanidin synthesis, concentration and composition in Cabernet Sauvignon grape skins

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ABSTRACT

The influence of controlled water deficit on the phenolic composition and gene expression of *VvLAR2*, *VvMYBPA1*, *VvMYBPA2* and *VvMYB4a* in Cabernet Sauvignon grape skins throughout ripening was investigated. The assay was carried out on own-rooted *Vitis vinifera* plants cv. Cabernet Sauvignon in a commercial vineyard from *veraison* until commercial harvest. Three irrigation regimes were used from *veraison* until harvest with the following treatments: T1: 3.6 mm day^{-1}; T2: 1.8 mm day^{-1} and T3: 0.3 mm day^{-1}. The content of total phenols and total anthocyanins in grape skins increased during ripening, but water deficit did not produce differences among treatments in the total anthocyanin concentration. Proanthocyanidins (PAs) decreased throughout ripening, although approximately 25 days after *veraison* (DAV), their content slightly increased. This effect was more pronounced in the most restrictive treatment (T3). A similar pattern was observed in the transcript abundance of *VvLAR2*, *VvMYBPA1* and *VvMYB4a*. PAs separation revealed differences in concentration but not in the proportion among fractions among the irrigation treatments. Additionally, controlled water deficit increased the mean degree of polymerization and the flavan-3-ol polymeric concentration in grape skins throughout ripening but with no effects on the extent of PAs galloylation. Our results suggest that the water status of Cabernet Sauvignon grapevines affects the gene expression for proteins involved in the synthesis of PAs, increasing their concentration and also their composition, with further evidence for the efficacy of a convenient, controlled water deficit strategy for grapevine cultivation.

1. Introduction

Phenolic compounds are considered the major determinant of the quality of red wines because of their involvement in the main sensory attributes of wine, such as colour, due to anthocyanins, and their body, mouthfeel, bitterness and astringency, which are conferred by proanthocyanidins (PAs). The sensory properties influenced by PAs depend not only on their concentration but also their composition and mean degree of polymerization (Brossaud et al., 2001; Vidal et al., 2003; Chira et al., 2012). Grape skins and seeds contain PAs, but the skins contain procyanidins and prodelphinidins, and are characterized by a lower proportion of galloylation and a higher mean degree of polymerization (mDP) compared with seeds (Downey et al., 2003; González-Manzano et al., 2004; Chira et al., 2015).

In many viticultural regions, regulated-deficit irrigation is a common practice because of its well-known effect on wine (Chaves et al., 2010; Roby et al., 2004; Acevedo-Opazo et al., 2010; Casassa et al., 2015; Zarrouk et al., 2012; Bonada et al., 2015; Kyraleou et al., 2016). In fact, deficit irrigation leads to a reduction in the size of the berries, resulting in a higher skin to pulp ratio, producing an effect of concentration of compounds (Kennedy et al., 2002). More importantly, water deficit stimulate the secondary metabolism in

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berries. Water restrictions at the time of *veraison* induce both a transient advancement of grape berry sugar accumulation, and an increase in the abscisic acid (ABA) concentration in the berries. Both sugars and ABA are signals for gene expression and protein synthesis involved in the phenylpropanoid pathway in berry skins, leading to the accumulation of flavanols, flavonols and anthocyanins (Pastenes et al., 2014; Castellarin et al., 2007; Deluc et al., 2009; Villalobos-González et al., 2016). However, it is still a matter of debate as to what extent the severity of water restriction affects not only the concentration but also, most importantly, the composition of phenolic compounds in grape berries, especially proanthocyanidins.

The phenylpropanoid pathway is tightly controlled by diverse transcription factors that require precise spatiotemporal coordination between plant development and environmental conditions (Cavallini et al., 2015). As for PAs, synthesis requires diverse genes, such as leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) that produce the flavan-3-ol monomers for the formation of proanthocyanidins polymers (Bogs et al., 2005). At the same time, their synthesis is modulated by transcription factors, among which the two well-characterized proteins *MYBPA1* and *MYBPA2* correspond to positive regulators (Bogs et al., 2007), while *MYB4a* and *MYB4b* are also necessary but act as negative regulators of the general phenylpropanoid pathway (Cavallini et al., 2015). Some studies have focused on the general phenylpropanoid pathway in grape berry skins, reporting effects on the concentration of PAs (Castellarin et al., 2007; Deluc et al., 2009; Matus et al., 2009; Genebra et al., 2014). However, few studies have examined the impact of water irrigation levels on the composition of grape berry PAs.

In the present study, we have assessed the effect of controlled water deficit, from weak to moderate water stress and from veraison until harvest, on the synthesis and composition of proanthocyanidins, as well as the expression of secondary metabolism related-genes in Cabernet Sauvignon grape berry skins throughout ripening.

2. Materials and methods

2.1. Chemical reagents and equipment

Methylcellulose (1500 cP, viscosity at 20 g/L) and a standard of (+)-catechin, (−)-epicatechin, (−)-epigallocatechin and (−)-epicatechin-3-*O*-gallate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PVDF membranes of 0.45- and 0.22-μm pore size were acquired from Millipore (Billerica, MA, USA). Anhydrous sodium sulphate, agarose, potassium metabisulfite, vanillin, ethyl acetate, lithium chloride, diethyl ether, sodium hydroxide, hydrochloric acid, sulfuric acid, methanol, ethanol, acetone, ascorbic acid, phloroglucinol and sodium acetate were purchased from Merck (Darmstadt, Germany). Sodium phosphate dibasic anhydrous and potassium phosphate monobasic were acquired from J.T. Baker (Phillipsburg, NJ, USA). All reagents were of analytical grade or higher. Sep-Pak Plus Environmental tC_{18} cartridges (900 mg) and Sep-Pak Plus Short tC_{18} cartridges (400 mg) were obtained from Waters (Milford, MA, USA). DNAse I amplification grade and SuperScript™ III First-Strand synthesis were supplied by Invitrogen™ (USA). LightCycler® FastStart DNA Master SYBR Green I was obtained from Roche (Switzerland). Nitrogen gas was supplied by Indura S.A (Santiago, Chile). Liquid nitrogen was supplied by Linde (Santiago, Chile). Ultrapure water was obtained from a Purelab Ultra MK2 purification system (Elga, St. Albans, UK).

Phloroglucinolysis studies were performed using a 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of a G1315B photodiode array detector (DAD), a G1311A quaternary pump, a G1313A autosampler, a G1322A degasser and a G1316A thermostatted column compartment with a reverse phase LiChro Cart 100 RP-18 column (5 μm, 4.0 mm i.d x 250 mm; Agilent Technologies). RNA measurements were made using an Epoch microplate reader (Biotek, Winooski, VT, USA). Real time PCR analysis was performed using a LightCycler® 96 system (Roche, Switzerland). Absorbance values were measured using a UV-1601 UV−Visible spectrophotometer (Shimadzu, Kyoto, Japan).

2.2. Experimental site, irrigation treatment and berry sampling

The assay was carried out on 12-year-old own-rooted *Vitis vinifera* plants cv. Cabernet Sauvignon, in the organic commercial vineyard Haras de Pirque winery, located in the Maipo Valley in central Chile (33°42′30″S, 70°36′13″W), during the 2014 growing season. The historical average yield for this site was approximately 8 tons ha^{-1}. The trellising system contains vertically trained vines pruned using a double Guyot method, with drip irrigation in north-south oriented rows, planted at 2.5 m between rows and 1.5 m between plants. The site has deep colluvial soil with clay loam texture. The canopy management of the vineyard is typical for vineyards located in this region, and the experimental site experiences a warm semi-arid Mediterranean climate. The 2014 season had typical warm conditions during the period from January to April on the study site, with an average maximum temperature of 28.9 °C and an average minimum temperature of 7.1 °C. No rain occurred throughout the experiment. The average ETp for the period was 3.7 mm day^{-1}.

The experimental design consisted of completely randomized blocks with five replicates, each of which consisted of seven consecutive plants. Each block corresponded to one row, and blocks were separated by one row. Each treatment was separated by at least five plants in the row. Treatments and replicates were randomly distributed in the experimental area. To reduce the xylem water potential of vines, irrigation was suspended from 25 days before veraison to 10 days before veraison, i.e., the time when irrigation treatment started. The irrigation treatments were applied weekly until five days before last sampling date and were established by means of a combination of drip emitters with different water volumes, resulting in three different treatments, T1: 3.6 mm day^{-1}; T2: 1.8 mm day^{-1} and T3: 0.3 mm day^{-1}. The application of irrigation treatments resulting in the following average values for midday stem water potential throughout the season: T1, $\Psi = -0.8$ MPa; T2, $\Psi = -0.9$ MPa and T3, $\Psi = -1.0$ MPa. As a reference, midday stem water potential values higher than −0.6 MPa are considered no water deficit, while range values of −0.6 to −0.9 and −0.9 to −1.1, are considered weak water deficit and weak to moderate water deficit, respectively (Van Leuween et al., 2009). Plant water status was monitored weekly by measuring midday stem water potential using a pressure chamber. For this process, leaves were enclosed in aluminium plastic bags for 90 min at midday. Berries were sampled for chemical analysis on the following dates: −3, 13, 27, 41 and 60 days after *veraison* (DAV). Samples of 50 berries per replicate were randomly collected from five to seven clusters in each plant throughout the ripening period and immediately weighed, frozen, and stored at −80 °C until processing. The following physical and chemical variables were assessed: weight of 50 berries, skin weight of 50 berries, titratable acidity (g tartaric acid L^{-1}) and total soluble solid (°Brix) in berry juice, by means of a temperature-compensated refractometer (RHB-32ATC) (OIV, 2012). All analyses were performed in quintuplicate. The *veraison* date (8 February 2014) was determined by visual observation and berry firmness. All grapes were harvested at the commercial harvest date, which occurred on the same day for all treatments. This corresponded to the last sampling date (60

DAV), which was determined based on grape chemical parameters and the mouthfeel characteristics of the whole berries.

2.3. Extraction of phenolic compounds from grape berry skins

The grape skins were separated by hand from 50 berries, and subjected to two consecutive extractions under mechanical stirring. The first extraction used a methanol-water solution (80:20 v/v), and the second extraction used an acetone-water solution (80:20 v/v). In each case, 100 mL of solution at 20 °C was used with 60 min of extraction. A sieve was used to separate the solids from the liquid fraction. After each filtration, the two liquid fractions were mixed, centrifuged at 2500 g for 5 min and later evaporated by using a rotary evaporator at 30 °C to remove methanol and acetone. The final solution was adjusted to 100 mL with ultrapure water and was filtered through a 0.45-μm PVDF membrane (Izquierdo-Hernández et al., 2015).

2.4. Spectrophotometric characterization

The total phenol content was determined by UV spectrometry at 280 nm using gallic acid as a standard (Glories, 1984). The total proanthocyanidin content was determined using methylcellulose as the precipitant agent (Sarneckis et al., 2006). The total anthocyanin content was measured using the method described by Ribéreau-Gayon and Stonestreet (1965) and was expressed in malvidin equivalents. All analyses were performed in quintuplicate.

2.5. Fractionation of proanthocyanidins using Sep-Pak Plus tC_{18} cartridges

Skin extracts were subjected to a solid phase extraction for fractionation of proanthocyanidins using Sep-Pak Plus tC_{18} cartridges according to the method described by Sun et al. (1998a). All the steps for fractionation of proanthocyanidins were as in previous work (Cáceres et al., 2012). For each fraction obtained previously, flavanols were quantified using the modified vanillin assay described by Sun et al. (1998b). All analyses were performed in quintuplicate.

2.6. Analysis of proanthocyanidins following acid catalysis with phloroglucinol

Acid-catalysed cleavage in the presence of excess phloroglucinol was used to analyse average molecular weight (aMW), percentage of galloylation and its mean degree of polymerization (mDP) as described by Kennedy and Jones (2001). All analyses were performed in quintuplicate. The monomers (+)-catechin, (−)-epicatechin, and (−)-epicatechin 3-*O*-gallate were identified by comparing their retention time with commercial standards. The phloroglucinol adducts of (+)-catechin, (−)-epicatechin, (−)-epigallocatechin and (−)-epicatechin-3-O-gallate were identified by their retention time as described in literature. The number of terminal subunits was considered as the difference between the total monomers without phloroglucinol. The number of extension subunits was considered as the sum of all the phloroglucinol adducts. The mDP was calculated by adding the terminal and extension subunits (in moles) and dividing by the terminal subunits.

2.7. Preparation of RNA, cDNA synthesis and qRT-PCR analysis

Ten grape berries randomly collected from each replicate in the field were immediately frozen in liquid nitrogen and then stored at −80 °C until processed. The berries without seed were ground to a fine powder using liquid nitrogen. Total RNA was extracted from 3 g of fine powder using the perchlorate method (Davies and Robinson, 1996) with some modifications. RNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 vol of ethanol 96% (v/v), which was then sedimented, rinsed and dried. The pellet was resuspended in 0.5 mL of sterile water, mixed with 0.25 vol of 10 M $LiCl_2$ and incubated overnight at 4 °C. The resulting homogenate was centrifuged at 15,000×*g* for 20 min at 4 °C, washed with 1 mL of ethanol 70% (v/v) and then centrifuged at 15,000×*g* for 10 min at 4 °C. The resulting pellet was newly dried and then suspended in 50 μL of sterile water. The quantity and quality of the total RNA were assessed by spectrophotometry (OD 260/280 nm and 260/240 nm) using a microplate reader and by electrophoresis on 1.2% (w/v) formaldehyde-agarose gels. The RNA was treated with DNase I, amplification grade using the manufacturer protocol. For cDNA synthesis, 2 μg of total RNA was reverse transcribed using the SuperScript™ III First-Strand synthesis system PCR and stored at −20 °C until processed.

Gene expression analysis was carried out by real time PCR using a SYBR Green method. The specific primers used are noted in Table 1 and were designed using Primer 3 software (Rozen and Skaletsky, 2000). Each 20 μL PCR contained 500 nM of each primer, 5 μL of 1:100 diluted cDNA, 10 μL of LightCycler® FastStart DNA Master SYBR Green I, and 3 μL of water. The thermal cycling conditions used were pre-incubation at 95 °C for 600 s, 45 cycles of 95 °C for 10 s, 58–62 °C for 10 s, and 72 °C for 10 s, followed by a melt cycle of 5 °C increment per min from 65 °C to 97 °C. The relative gene expression was calculated using a previous standard quantification curve with five serial dilutions of cDNA, which was constructed for each gene to calculate amplification efficiency. Gene expression levels were normalized to the expression of the first sample for the T2 treatment, to obtain a calibrated ΔΔCt for each gene. LightCycler® Instrument software v 1.1 was used to calculate cycle threshold values and observe melt profiles. Ct values for Actin did not vary more than one unit between all samples analysed for each real time treatment throughout the experiment. All primer pairs amplified a single product of the expected size, which was confirmed by melt-curve analysis and agarose gel electrophoresis. All experiments were performed with five biological replicates and three technical replicates. Previous work was consulted for all steps (Pastenes et al., 2014).

2.8. Statistical analyses

For the chemical parameters and relative gene expression, analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test were used for mean separation with a significance level of 95% ($P < 0.05$). Statistical analyses were performed with R statistical software, version 3.03 (R Foundation for Statistical Computing, Austria).

3. Results

3.1. Water relations

Different combinations of drip emitters per treatment yielded substantial differences in the xylem water potential throughout ripening. The average xylem water potential values were for T1, -0.83 ± 0.03 MPa, for T2, -0.90 ± 0.03 MPa and T3, -1.00 ± 0.02 MPa. These values of stem water potential are common in Mediterranean viticultural sites (Choné et al., 2001) and are considered as weak (T1) to moderate water deficit (T3) (Van Leuween et al., 2009). Significant differences in the potentials were observed among treatments, particularly when irrigation was resumed before veraison (Fig. 1).

Table 1
Accession numbers of sequences and primers used for qPCR.

Gene	NCBI accession number	Primer sequence (5′–>3′)	
		Forward	Reverse
VvLAR2	NM_001281160.1	TTCATTTCCGACCTCCCAGC	TCTTCTTCCACGGTTACACGG
VvMYBPA1	NM_001281231.1	TGGGAAATCGGTGGTCTCTCA	CTTGTGTTCTTCCTCCTCTTGGG
VvMYBPA2	NM_001281024.1	AGCGGAACTTCACACAGTCATCC	GTCGCCAATGTCGAAGCCCAT
VvMYB4a	EF113078.1	GCCGCAGTTAAGGAAGAGGAGA	GGGTTGGTATGGTGGGCTGAT
VvNAD5	GU585873	GATGCTTCTTGGGGCTTCTTGTT	CTCCAGTCACCAACATTGGCATAA
VvUbiquitin	GU585868	TCTGAGGCTTCGTGGTGGTA	AGGCGTGCATAACATTTGCG
VvActin 1	AF369524.1	AGCTGGAAACTGCAAAGAGCAG	ACAACGGAATCTCTCAGCTCCA

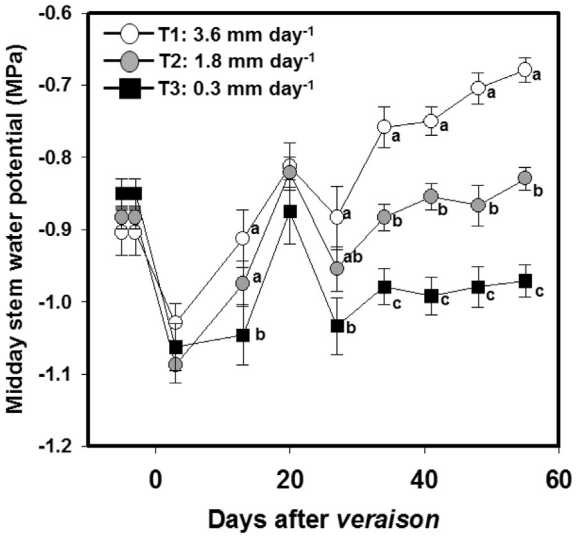

Fig. 1. Midday stem water potential during season. Different letters denote significant differences among treatments ($P < 0.05$, Tukey's HSD test).

3.2. Chemical composition of grape skins

The soluble solids, increased during ripening from 12.9 to 21.6 °Brix on average, with a transient higher content for the more restricted treatments at 45 DAV. The titratable acidity, on the other hand was reduced during ripening, from 15.45 g of tartaric acid L^{-1} to 2.97 g of tartaric acid L^{-1} at veraison and harvest, respectively, with no differences between treatments. Water deficit induced an increase in the ratio of skin weight to total weight throughout ripening, especially in the most restrictive treatment (T3), with significant differences between treatments at 27, 41 and 60 DAV. (Fig. 2).

As shown in Fig. 3A, total phenols increased in concentration, roughly from 14.02 mg GAE g^{-1} skins to 21.09 mg GAE g^{-1} skins on average during ripening, with T2 and T3 reaching significantly higher concentrations than control at 13 and 41 DAV, respectively. As for total PAs, there was a decrease in their concentration from *veraison* until approximately 25 DAV, increasing again afterwards, and reaching higher values for the more stressed treatment (Fig. 3B). Additionally, there was an increase in the total anthocyanins concentration throughout ripening, with no differences among treatments (Fig. 3C).

The monomeric, oligomeric and polymeric flavan-3-ol grape skin proportions are shown in Fig. 4. The less abundant monomeric fraction was reduced during ripening in all the treatments (Fig. 4A). Irrigation treatments affected the concentration but with minor differences among treatments that were restricted to 10 DAV and 45 DAV. The oligomeric fraction on the other hand, more abundant

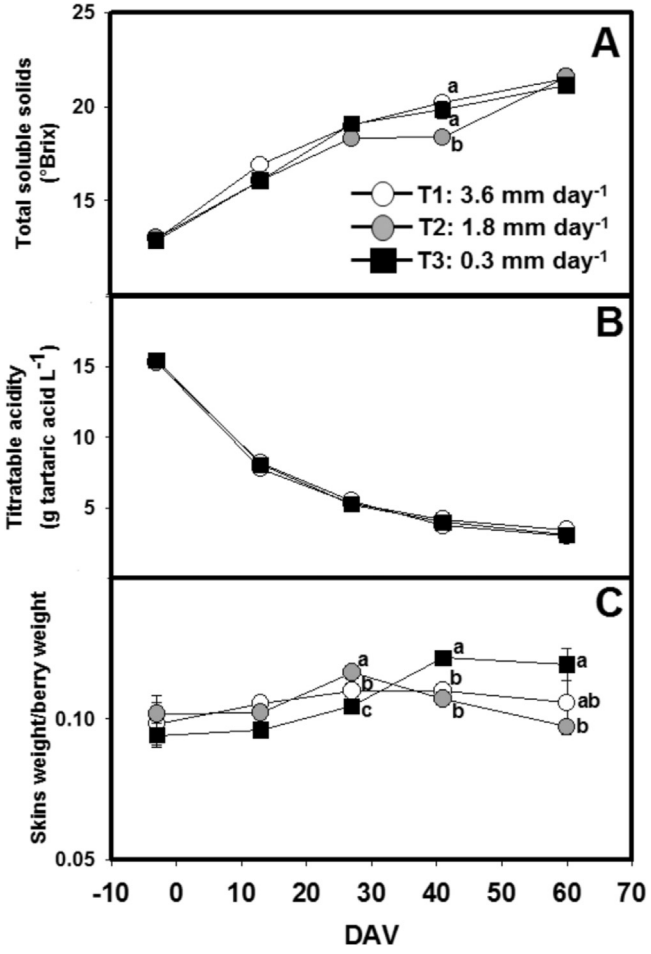

Fig. 2. Progress of berry development: Soluble solids (A), titratable acidity (B) and skin weight/total weight relationship (C) in Cabernet Sauvignon grape berries. Vertical bars indicate standard error of five biological replicates. Different letters denote significant differences among treatments ($P < 0.05$, Tukey's HSD test). DAV: days after *veraison*.

than the monomeric, was reduced from veraison until 30 DAV, increasing again afterwards. No clear trend associated with the water regime was observed and, at harvest, the intermediate stressing treatment (T2) was higher in concentration than the other two more extreme treatments (Fig. 4B). As for the polymeric fraction, this was the most abundant (Fig. 4C), followed a pattern resembling that of the total PAs (Fig. 3B), with the lowest concentration for all the treatments at nearly 30 DAV, and then increasing again until harvest. At the end of the season, the more restrictive treatment accounted for the more abundant polymeric fraction, nearly 20% higher in concentration than T2 and T1 on average (Fig. 4C).

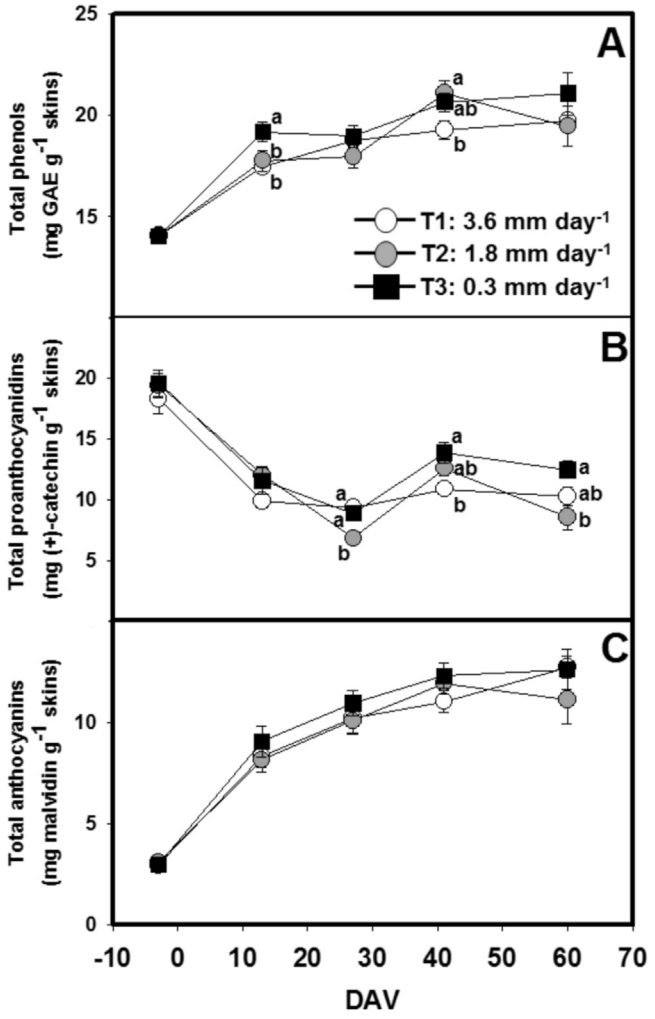

Fig. 3. Total phenols (A), total proanthocyanidins (B) and total anthocyanins (C) in Cabernet Sauvignon grape skins throughout ripening. Vertical bars indicate standard error of five biological replicates. Different letters denote significant differences among treatments ($P < 0.05$, Tukey's HSD test). DAV: days after *veraison*.

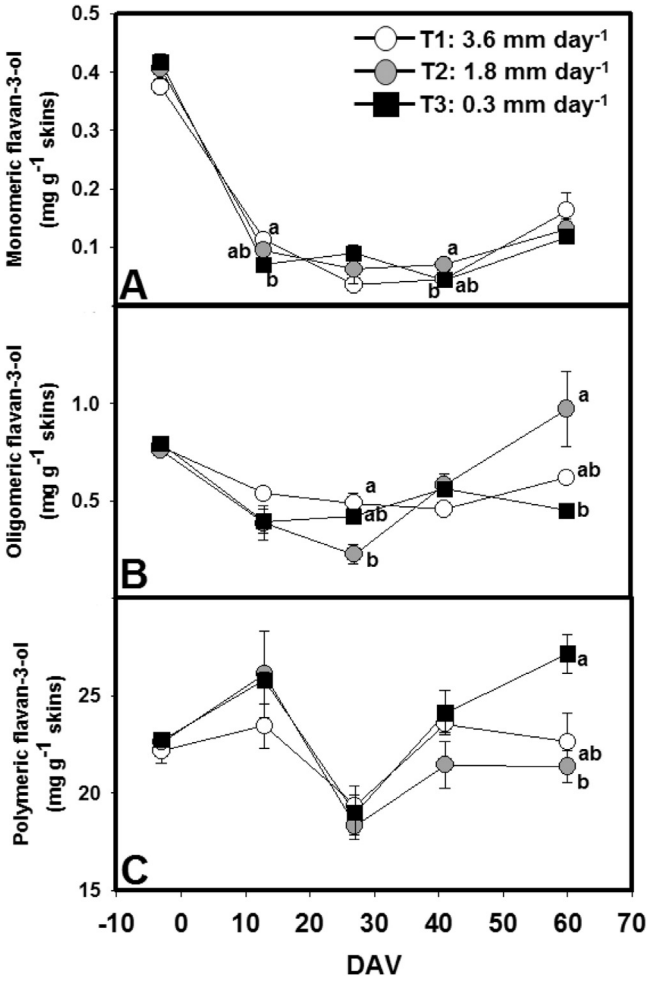

Fig. 4. Solid phase separation of proanthocyanidins by Sep-Pak tC_{18} cartridges: monomeric fraction (A), oligomeric fraction (B) and polymeric fraction (C) from Cabernet Sauvignon grape skins throughout ripening. Vertical bars indicate standard error of five biological replicates. Different letters denote significant differences among treatments ($P < 0.05$, Tukey's HSD test). DAV: days after *veraison*.

The mean degree of polymerization (mDP), the average molecular weight (aMW) and the percentage of galloylation (%G) are shown in Fig. 5. The mDP increased during ripening from average values of 2.95 for all the treatments to an average of 8.7 for T1 and T2 and to 14.32 in T3, a value significantly higher than the other two less restrictive treatments (Fig. 5A). Already at 30 DAV, the mDP value was significantly different among all the treatments, being higher in the more restricted treatment (Fig. 5A). As expected, the aMW value followed a similar pattern than mDP, increasing throughout ripening, with the highest values in T3 at 40 DAV and 60 DAV (Fig. 5B). As for %G, a general reduction was observed throughout ripening, with no significant differences among treatments (Fig. 5C).

The relative transcript abundance of *VvLAR2* was nearly maintained from veraison until 30 DAV, decreasing at 40 DAV and increasing again at 60 DAV (Fig. 6A). The water regimes affected the expression of *VvLAR2* in a way somehow proportional to the extent of the stress level, with higher values in T2 and T3. A similar pattern was observed for the *VvMYBPA1* transcripts, with higher values in the more restricted treatments at −3 DAV, 13 DAV and 27 DAV (Fig. 6B). In both cases, *VvLAR2* and *VvMYBPA1*, no significant differences in the transcript abundance were observed from 40 DAV until harvest. Regarding the transcription factor *VvMYBPA2*, an increase of the transcript abundance was observed from 10 DAV to 30 DAV, decreasing from then until harvest, with no significant differences among treatments during berry ripening (Fig. 6C). As for *VvMYB4a*, the gene expression slightly increased along ripening for all the treatments but reached significantly higher values in berries from the less restricted treatment, at harvest (Fig. 6D).

4. Discussion

Water scarcity has become an important focus in research and policy making because it will be a major limiting factor in food production in the near future. For viticulture, drought is likely to affect wine production globally and to be considerable threat to the quality (IPCC, 2007; Gerós et al., 2015). Grapevines are known to resist water scarcity because of their deep rooting ability and capacity to control water loss among many other physiological strategies (Deluc et al., 2009; Lovisolo et al., 2002). In many viticultural regions, controlled deficit irrigation is a common field practice because water limitation is known to improve the quality of the fruit and of the final wine.

Water stress reduces the berry size and concentrates the skin phenolics, by conveniently increasing the skin to pulp ratio (Gil

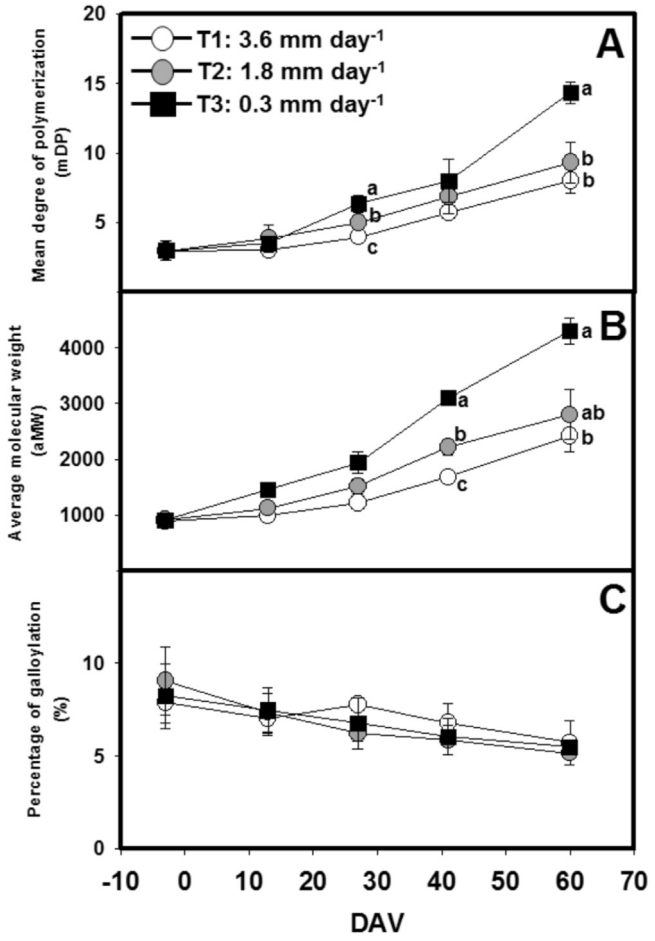

Fig. 5. Mean degree of polymerization (mDP) (A), average molecular weight (aMW) (B) and percentage of galloylation (C) in Cabernet Sauvignon grape skins throughout ripening. Vertical bars indicate standard error of five biological replicates. Different letters denote significant differences among treatments ($P < 0.05$, Tukey's HSD test). DAV: days after *veraison*.

et al., 2012; Kennedy et al., 2002; Thomas et al., 2006). Indeed, in our study, the more restricted irrigation regime, implemented immediately prior to veraison (second berry growth phase), resulted in an increase in both: the skin to total berry weight ratio (Fig. 2C), and in total phenols and proanthocyanidins (Fig. 3A and B). Increases in the skin proportion of the total berry weight are considered as a convenient feature since most phenolic compounds are found in the berry skin (Adams, 2006). However, there is still some debate regarding the basis of such increases, on one hand suggesting that it would be the result of smaller berries upon restrictions in water supply (Roby et al., 2004; Acevedo-Opazo et al., 2010) and on the other, the result of differential growth sensitivity in the cells of the mesocarp (Roby et al., 2004). In addition to a thicker berry skin, water stress promotes the metabolic pathway leading to anthocyanin and proanthocyanidins biosynthesis (Castellarin et al., 2007; Deluc et al., 2009; Genebra et al., 2014). In our results, even though a higher concentration of total phenols and total proanthocyanidins were found (Fig. 3A and B), no significant differences in total anthocyanins were observed between treatments (Fig. 3C). These, possibly because the extent of the water stress was milder than in other studies and/or the warmer climate condition in our study, which promotes anthocyanin degradation, might have mask eventual increases in their synthesis.

Proanthocyanidins, on the other hand, are known to accumulate up to a maximum concentration prior to veraison in grape berry skins, declining afterwards (Hanlin and Downey, 2009; Bogs et al., 2005). More recently, Villalobos-González et al. (2016) reported that proanthocyanidins resumed synthesis after veraison by observing increases in concentration and gene expression for catalytic enzymes involved in their synthesis in Carménère. We have also observed that proanthocyanidin concentration rises from a minimum value after 25 DAV in Cabernet Sauvignon, and is further increased by the controlled water deficit (Fig. 3), coinciding with the gene expression results. In fact, *VvLAR2*, which is known to code for leucoanthocyanidin reductase, the protein involved in the synthesis on flavan-3-ol monomers, specifically (+)-catechin (Bogs et al., 2005), and *VvMYBPA1* and *VvMYBPA2*, positive regulators in proanthocyanidin synthesis (Matus et al., 2009) increase their expression in berries from water stressed plants (Fig. 6).

The transcript abundance of *VvMYB4a*, a negative regulator of the synthesis of low weight phenols, was observed to increase during ripening, in a concomitant way with the PAs concentration (Fig. 3B). This observation is in agreement with the reports of Cavallini et al. (2015) and Villalobos-González et al. (2016). As *VvMYB4a* regulates negatively anthocyanin synthesis (Matus et al., 2009), it is suggested that it act as a biochemical valve diverting the substrates from the phenylpropanoid pathway to other end products such as PAs.

A severe water stress condition may imply that the nutrient uptake, including nitrogen (N), is reduced in plants. N has been suggested to affect the secondary metabolism in grape berries (Keller et al., 2005), although the work of Soubeyrand et al. (2014) shows that the reduction of N fertilization in grape vines have resulted in an up-regulation of genes involved in anthocyanins synthesis, with no effect on the transcript levels of *VvMYBPA1* and *VvMYBPA2* (Soubeyrand et al., 2014). Considering also that N uptake in vines occurs mainly from budbrake to bloom (Hanson and Howell, 1995), it is unlikely that the irrigation treatments in our study, would have a mediated effect on the PAs concentration.

Among other factors, the organoleptic quality of wines is closely related to the presence of PAs, known to confer a convenient mild sensation of astringency, bitterness, and body mouth. These sensory properties depend not only on the concentration of PAs but also on characteristics such as size and composition (Brossaud et al., 2001; Vidal et al., 2003; Chira et al., 2012). Few studies have explored the effect of water stress on the different proanthocyanidin fractions in the berry skins. As previously described (Kennedy et al., 2001; Kontoudakis et al., 2011), an increase of the PAs mDP during grape berry ripening was observed in our study but to a higher extent in berry skins from the more water stressed plants, together with a higher molecular weight average of the PAs after veraison (Fig. 5A and B) and a higher increase in the polymeric flavan-3-ol fraction in skins (Fig. 4C). The ways that water supply levels may affect PAs polymerization are difficult to determine, mainly because the mechanisms by which proanthocyanidins are polymerized are still not clear, with models suggesting enzymatic and non-enzymatic mechanisms (Dixon et al., 2005; Zhao et al., 2010).

From an oenological point of view, an increase in the size of PAs could produce an increase in the perceived astringency (Vidal et al., 2003; Chira et al., 2015), a sensory property that could be enhanced as a coarse perception by a high percentage of galloylation (Vidal et al., 2003). In our results, the irrigation regime affected the size of PAs, as observed from degree of polymerization, mDP and aMW, likely altering the astringency of PAs but with no increases on the percentage of galoylation (Fig. 5C), a positive trait for wine making (Vidal et al., 2003).

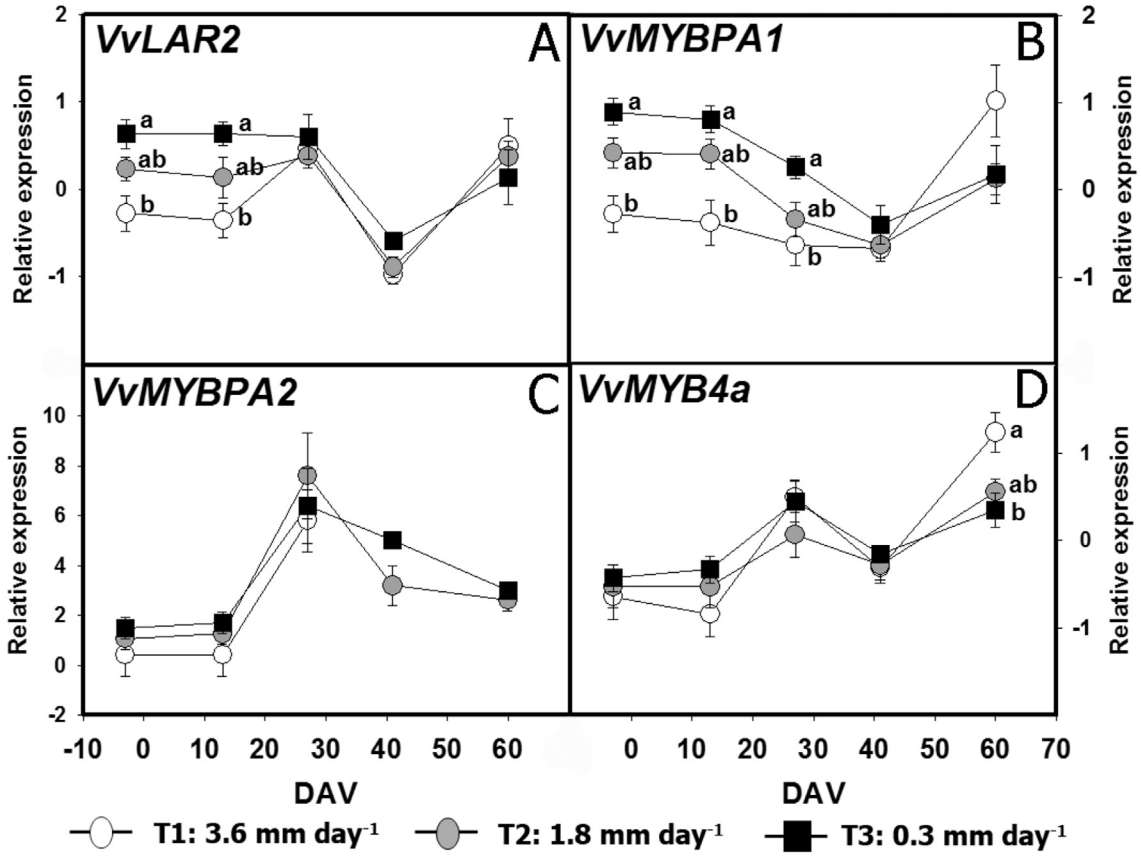

Fig. 6. Expression profile of key genes involved in proanthocyanidin synthesis and MYB regulators in Cabernet Sauvignon grape skins throughout ripening. Vertical bars indicate standard error of five biological replicates. Different letters denote significant differences among treatments ($P < 0.05$, Tukey's HSD test). DAV: days after *veraison*.

5. Conclusions

Controlled water deficit is a common viticultural practice worldwide. This is due to the well-known effects on grape berries for wine making, including grape berry size, microclimate of the fruiting zone and the secondary metabolism. Less attention has been paid to the effects of the water stress extent on PAs concentration, degree of polymerization and composition. From our data, we conclude that previous observations reporting the resuming of PAs synthesis after veraison in Carménère is also occurring in Cabernet Sauvignon. Additionally, moderate water stress further enhances PAs accumulation after veraison, as observed in both PAs concentration and in gene expression for proteins involved in PAs synthesis. Also, PAs properties are affected by water availability, with moderate controlled water deficit leading to higher degrees of polymerization without affecting the percent of galloylation, aspects that may contribute to more convenient sensory properties of the final wine.

Contributions

Alejandro Cáceres Mella was responsible for field experiment, water treatments, sampling, discussion, statistical analysis and writing.

M. Inmaculada Talaverano was involved in berry sampling and support in field experiment.

Luis Villalobos González was responsible for analysis of gene expression.

Camila Ribalta Pizarro was involved in berry sampling, stem water potential measurements and extraction of phenolic compounds.

Claudio Pastenes was involved in planning, discussion and guidance of work and final writing.

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