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Carbon partitioning to berries in water stressed grapevines: The role of active transport in leaves and fruits

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ABSTRACT

Although imposed water stress is a common agricultural practice worldwide, the physiological and molecular responses of grapevine leaves and fruits, and their interactions, in relation to carbon partitioning remain unknown. We have assessed, in field grown grapevines, the effect of four deficit irrigation regimes, from veraison through to the end of the season, on daily and seasonal non-structural carbon stocks and assimilation in leaves and sugar content in berries, along with the transcript profile for sugar transport proteins in leaves and berries. Average midday xylem water potentials along the season ranged from mild to severe water stress, i.e., −0.7 MPa to −1.05 MPa, respectively. In all the treatments, berries reached equal sugar concentration 20–35 days after veraison because of a proportional effect on berry volume and sugar content per berry. In berries, mild water stress accelerated the sugar accumulation increasing the abundance of *VvSUC27*, *VvHT3* and *VvHT5*, only strictly around veraison. Transcripts abundance in berries did not match sugar uptake rate since, *VvSUC11*, *VvSUC12*, *VvHT5*, as well as the cell wall invertase *VvCWI*, kept rising after berries were filled. In leaves, when berries reached maximal sugar content, export was transiently reduced resulting in starch accumulation. Water stress increased the gene expression for sucrose transporters known to code for mesophyll cell proteins in leaves, without affecting the transcript abundance for the phloem loading protein. The latter suggests that mild water stress triggers active sugar transport in the source tissues as a means for supporting the sugar accumulation in berries under depressed carbon assimilation by leaves.

1. Introduction

Carbon partitioning, the process by which reduced carbon is distributed from photosynthesizing leaves to heterotrophic plant organs and tissues, strongly determines plant growth and development, as well as crop yield (Genard et al., 2008). Carbohydrates resulting from the carbon fixation in leaf mesophyll cells are funneled to the various plant organs incapable of complete autotrophy, through the phloem vasculature. Regulatory mechanisms have been described along the carbon partitioning pathways, enabling the coordination in the growth and development of the various plant parts, as well as allowing the interaction between sink and source organs.

The first step in the process of energy delivery, right after photosynthesis, is phloem carbon loading where either active or passive mechanisms have been described. Among the former, one consist of sucrose and/or polyols channeling to the apoplast, from where they are actively taken by means of proton gradient mediated symporter proteins (Lalonde et al., 2004; Noiraud et al., 2001) and, the other, relays on the build up of carbon polymers from sucrose, incapable of returning back to mesophyll cells but to move forward to the sieve elements (Rennie and Turgeon, 2009). The passive, or so called downstream phloem loading, considered to be a more primitive mechanism, is achieved by the maintenance of high solute concentration in the mesophyll cells so that photosynthates diffuse through plasmodesmata to the sieve elements, and has been described mainly in tree species (Rennie and Turgeon, 2009; Turgeon, 2010). It has been observed that the capacity to adapt to environmental changes, particularly those increasing the potential for carbon supply, as it occurs upon increases in the light availability, is more readily accomplished in apoplastic phloem loader species (Amiard et al., 2005). Such observation suggests that the plasticity of the species to adapt the carbon partitioning to environmental cues might be limited in those relaying solely in anatomical pathways, as it occurs in passive symplastic phloem loaders (Fu et al., 2011). In fact, plasmodesmata

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density is already set in early developmental stages in leaves (Slewinski and Braun, 2010). Transcription of the sucrose symporter proteins (SUC) in leaves of apoplastic loaders has been proposed as a powerful mechanism to adapt not only to increasing potential for sucrose export, such as that observed upon increases in the light availability but, also, in the opposite, when sink demand decreases. The former, by increasing the transcript levels and the latter by reducing them, both mediated by sucrose as a signal molecule (Ransom-Hodgkins et al., 2003). It has to be stressed, however, that this hypothesis has been experimentally supported by work on apoplastic phloem loading species only (Ainsworth and Bush, 2011).

At the other end, in sink organs, plants have also evolved mechanisms for maintaining the proper carbon supply. The Munch theory explains that carbon delivery occurs by mass flow (Münch, 1930), which depends on the proper pressure gradient along the sugar transit pathway. This means that, besides the need to move sugars into the phloem in leaves, there has to be a capacity to unload it at the sink end, for the gradient to be sustained. Similar to that described in the source phloem side, active and passive strategies have developed in sink tissues and they occur irrespective of the one for phloem loading (Fisher and Oparka, 1996; Patrick, 1997). Besides, one plant organ may switch from one mechanism to other (Godt and Roitsch, 2006; Roitsch, 1999). Precisely, the "sink strength", interpreted as the capacity of a plant part to predominately attract sugars (Minchin and Thorpe, 1996), often results because of a transition from passive to active phloem carbon unloading. Such change, observed in some fruits, involves the increase in the abundance of transport proteins and H^+/ATPases in cell membranes, which strongly drops the solute concentration at the phloem end and favors a convenient hydrostatic pressure gradient between the source and the sink. Very importantly, this transitions from passive to active unloading have also been observed upon environmental constraints (Roitsch, 1999; Zhang et al., 2006), conforming a potential mechanism to favor the sugar filling of plant parts, relevant for the species dispersion.

A better knowledge about the mechanisms of adaptation to changes in sink to source ratio is relevant since many environmental factors strongly determine, on one hand the photosynthetic capacity at the canopy level and, on the other, the magnitude of the sink strength, both influencing crop yield and quality (i.e., sugar concentration in fruits). One such complex environmental condition is water stress. Its complexity lies in the fact that it concomitantly reduces photosynthesis and, up to same extent, the size of sink organs. Comparison of results from various reports on water stress effects on carbon partitioning in some species has been difficult due to variations in the time point, duration, and severity of the applied water stress (Roitsch, 1999). Also, most studies on water stress have been made on potted plants in controlled environments with light intensities far below that of ambient or have been focused on partial processes, mechanisms and/or plant parts, leading to hypothesis based on fragmentary obtained data.

Grapevines are of great economical importance, and imposed water stress is a worldwide common agronomical practice because of its convenient effect on red wine quality. Even though grapevines, depending on the variety, are known as water stress resistant plants, according to Flexas et al. (2002), a decline in photosynthesis due to either regulation or damage is observed under different water stress levels. Effects range from simple declines in stomatal conductance to non-reversible effects upon re-watering of plants (Flexas et al., 1999). Therefore, the extent of the water restriction will strongly determine the source to sink relation of plants during grape berry ripening. At the leaf level, despite being a species candidate for symplastic phloem loading, transcripts of genes coding for sucrose transport have been identified and thought to act as phloem carbon loaders (Davies et al., 1999). At the fruit level, grapevines are a well-characterized model species in terms of the strength of the berries as sugar sinks (Lemoine et al., 2013) where sucrose and hexose transporters, as well as cell wall invertases, have been identified (Afoufa-Bastien et al., 2010; Hayes et al., 2007). Berry growth follows a double-sigmoid curve with three distinct phases: (a) a first rapid growth phase where cell division occurs; (b) a lag phase with little change in berry size in which seed size and fresh weight reaches its maximum and; (c) a second rapid growth phase, whose transition from the lag phase is known as veraison, characterized by changes in colour, flesh softening, acidity and, most importantly, sugar accumulation, mainly as fructose and glucose (Coombe, 1992; Coombe and McCarthy, 2000). In grapes, a shift from a symplastic to an apoplastic phloem unloading pathway is known to occur around veraison leading to a massive import of hexoses (Zhang et al., 2006). In the present study, treatments of water stress were imposed right around veraison in order to restrict source and sink activities mainly to leaves and berries respectively.

Here we report on the water stress effect on carbon partitioning in field grown grapevines, simultaneously assessing carbon assimilation, storage and exportation at the leaf level, grape berry sugar filling kinetics and the transcript profile for sugar transporter proteins in leaves and fruits. Results are discussed in terms of the possible adaptations in both, sink and source organs, as well as their interaction.

2. Materials and methods

2.1. Plant material and experimental design

The experiment was carried out on 12 years old, own rooted, *Vitis vinifera* plants cv. Carmenere, in a commercial vineyard grown under a Mediterranean climate (with no summer rains) in the Maipo Valley in central Chile (33° 42′S, 70° 35′W), during the 2011–2012 growing season. Grapevines were trained to a vertical system with a Guyot double pruning method with nearly north–south oriented rows and a planting density of 5000 plants ha^{-1}. The vineyard was irrigated by conventional drip irrigation.

There were five blocks, one per row, and each containing all the treatments. In each, row four separated groups of five homogenous vines were selected. Each group of plants corresponded to one treatment, in which every plant of the group was irrigated with the same combination of drip emitters. No rain occurred throughout the experiment.

2.2. Leaf water status and gas exchange measurements

Four irrigation treatments were established by means of using a combination of drip emitters with different water volumes, resulting in T1: 1 mm h^{-1}; T2: 2 mm h^{-1}; T3: 4 mm h^{-1}; T4: 6 mm h^{-1}. Irrigation regimes were applied from few days before veraison through the end of the season, twice a week, with water volumes corresponding to 15% (T1), 30% (T2), 60% (T3) and 90% (T4) of the ETc, respectively, and resulting in a seasonal average midday stem water potential ranging from −1.1 MPa to −0.7 MPa for the extreme treatments T1 and T4, respectively. Irrigation of the vineyard, before the beginning of the experiment, was scheduled to maintain midday stem water potential values of −0.9 MPa. Plant water status was monitored weekly by measuring midday stem water potential of fully mature leaves bagged with a plastic sheet and covered with aluminum foil for at least 2 h before measurements (Fulton et al., 2001).

Net carbon assimilation rate (A) were measured using a portable photosynthesis system CIRAS-2 (PP Systems Co. Ltd., USA). Measurements were made at 5, 17, 35, 50 and 65 days after verasion (DAV). Fully developed, mature leaves from the middle of both sides of the canopy were selected and marked early in the morning. Given the nearly north-south row orientation, illumination is variable and contrasting in the leaves from opposite positions. Therefore, measurements of photosynthesis were assessed from both sides of the canopy: East (E), illuminated in the morning, and West (W) illuminated during the afternoon. Measurements were made between 9:00 and 18:00 h on a sunny day. All measurements, for a time point, were usually completed within 1 h, and the interval between time points of the day was nearly 2 h. During each measurement, CO_2 concentration was maintained at 365 ± 5.0 ppm, by the CIRAS-2 portable photosynthesis system.

The daily totals of leaf CO_2 uptake were obtained by integrating the curve described by the variation of A with time-of-day for each replicate using the trapezoidal method (Rogers et al., 2004). Results were expressed as glucose equivalents.

2.3. Sugar content and concentration

Berry samples for carbohydrate analyses were taken at late afternoon on five days (5, 17, 35, 50 and 65 DAV). Grapes from all bunches of each replicate were randomly and carefully detached from the rachis and immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Five berries were deseeded and grounded under liquid nitrogen by mortar and pestle to obtain a fine powder. 1 g of the fine powder was transferred to tubes containing 10 mL of Milli-Q water and shaked for 30 min at 4 °C. Extracts were clarified by centrifugation for 10 min 4500 × *g* at 4 °C. 100 μL from the supernatant were mixed with 400 μL of Milli-Q water and 500 μL of acetonitrile centrifuged for 15 min 14,000 × *g* at 4 °C filtered through a 0.22 μm membrane.

Leaves samples for carbohydrate determination were taken in parallel with the diurnal measurements of gas exchange on five days (5, 17, 35, 50 and 65 DAV). Each leaf sampled was removed from the canopy, wrapped in foil, plunged immediately into liquid nitrogen and stored at −80 °C until analysis. Leaf samples were ground to powder in liquid nitrogen. For soluble solids 0.5 –1 g were transferred to tubes containing 10 mL of Milli-Q water and incubated at 60 °C for 1 h. Extracts were clarified by centrifugation for 10 min 4500 × *g* at 4 °C. 500 μL from the supernatant were mixed with 500 μL of acetonitrile, centrifuged for 15 min 14,000 × *g* at 4 °C and filtered through a 0.22 μm membrane.

For starch determination, 0.1 g of the powdered leaves were resuspended in 5 mL of ethanol 80%, incubated at 80 °C for 5 min, centrifuged for 10 min at 5000 × *g* at 25 °C discarding the supernatant, three times. The pellet was added 0.2 mL of ethanol 80%, 3 mL of water and 0.02 mL of α-amylase from *Bacillus licheniformis* (A4582, Sigma) mixed, incubated in boiling water for 5 min and cooled at room temperature. Then, the volume of the mix was bring up to 10 mL, and an aliquot of 1 mL was added to 1 mL amyloglucosidase from *Aspergillus niger* (S9144, Sigma), mixed and incubated for 15 min in a 60 °C shaking water bath. Finally, 100 μL of the solution was added 400 μL of Milli-Q water and 500 μL of acetonitrile centrifuged for 15 min 14,000 × *g* at 4 °C filtered through a 0.22 μm membrane.

Sugars were analyzed using an Agilent 1200 series HPLC system. Finally 20 μL of each sample were injected. Sugars were detected using a refractive index detector with the reference cell maintained at 45 °C. A Zorbax Carbohydrates column (4.6 mm × 150 mm, 5 μm) with a guard column cartridge Zorbax NH2 (4.6 mm × 12.5 mm, 5 μm) was used. The column was maintained at 35 °C with a thermostated column compartment. Samples were eluted with acetonitrile:water (78:22). The flow rate was 1.5 mL min^{-1}. Fructose, glucose and sucrose were detected by their retention time and quantitations were performed using the external standard method with commercial standards of D-(+) glucose, D-(−) fructose and sucrose (Sigma Chemical Co.).

2.4. Glucose equivalents exported from leaves

The potential rate of carbon export (E) along the day was calculated, as described by Zufferey (2000), as follows:

$$E = A - \Delta C$$

where *A* is the carbon fixed by net photosynthesis in a given time and ΔC is the variation in the total non-structural carbon content in leaves during the same period of time. Both parameters were expressed as glucose equivalents. The daily total of CO_2 uptake, carbon storage and carbon export of leaves, were obtained by integrating the curve described by the variation of *A*, ΔC and *E*, along the day, respectively, for each replicate using the trapezoidal method.

2.5. S-(cis)-ABA extraction and determination

For ABA assays, a sample of five berries randomly collected from different clusters per replicate from each treatment, and two leaves randomly collected per replicate, were immediately frozen with liquid nitrogen and stored at −80 °C until analysis. S-(*cis*)-ABA determination was performed by an indirect competitive ELISA analysis (Walker-Simmons, 1987) based on a monoclonal antibody (DBPA1) raised against S-(*cis*)-ABA (Vernieri et al., 1989). Briefly, leaves and deseeded berries were grounded to a fine powder in liquid nitrogen, of which 1.2 g were immediately extracted in distilled water (1:10 w/v) overnight at 4 °C in the dark. Plates were coated with 0.2 mL per well of ABA-4′-bovine serum albumin (BSA) conjugate and incubated overnight at 4 °C, then washed three times with 75 mM phosphate-buffered saline (PBS) buffer, pH 7.0, containing 1 g L^{-1} BSA and 1% Tween-20, keeping the third washing solution for 30 min at 37 °C. Next, a 0.1 mL ABA standard solution or sample and, subsequently, 0.1 mL of DBPA1 solution (lyophilized cell culture medium diluted in PBS buffer containing 10 g L^{-1} BSA and 0.5% Tween-20, at a final concentration of 50 μg mL^{-1}) were added to each well, and competition was allowed to occur at 37 °C for 30 min. Plates were then washed again as described above and 0.2 mL per well of Anti-Mouse IgG (whole molecule) – alkaline phosphatase (A4312, Sigma, USA) in PBS buffer containing 10 g L^{-1} BSA and 0.5% Tween-20, at a final dilution of 1:2000 was added and incubated for 30 min at 37 °C. Plates were washed again and 0.2 mL per well of *p*-Nitrophenyl phosphate (N2770, SIGMAFAST™, USA) were added and incubated for 30 min at 37 °C. Absorbance readings at 405 nm were obtained using an Epoch Microplate Spectrophotometer reader (BioTek Winooski, USA). All experiments were performed with five biological replicates and four technical replicates.

2.6. Preparation of RNA and cDNA synthesis

Five grape berries randomly collected from each plant were frozen in liquid nitrogen and stored at −80 °C, until processed. Deseeded berries were ground to a fine powder in 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 volumes of ethanol 96% (v/v), then sedimented, rinsed and dried. The pellet was resuspended in 0.5 mL of sterile water, mixed with 0.25 volumes of 10 M $LiCl_2$ and incubated all night 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, resuspended in 50 μL of sterile water.

For leaves, a mature leaf was selected from the west side of the row late in the afternoon, wrapped in foil, plunged immediately into liquid nitrogen and stored at −80 °C until analysis. Total RNA was extracted from leaves using PureLink® Plant RNA Reagent (Ambion®).

The quantity and quality of the total RNA were assessed by spectrophotometry (OD 260/280 and 260/240) and electrophoresis on 1.2% (w/v) formaldehyde-agarose gels. RNA was treated with DNase I, Amplification Grade (Invitrogen™) using the manufacturer's protocol. For cDNA synthesis, 1 μg of total RNA was reverse transcribed using the SuperScript™ III First-Strand Synthesis System SuperMix for RT-PCR (Invitrogen).

2.7. Quantitative real-time (qReal-time) PCR analysis

Gene expression analysis was carried out by real time – PCR using a SYBR Green method on a LightCycler instrument (Roche, Mannheim, Germany). The specific primers used are shown in Table 1 and were designed with Primer3 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 (Roche Basel, Switzerland) 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. For relative gene expression calculations, a previous standard quantification curve with five serial dilutions of cDNA was constructed for each gene to calculate amplification efficiency according to the Eq. (1):

$$E = \left[10^{(-1/\text{slope})}\right] - 1.$$

This value was then used to obtain an accurate ratio between the gene of interest (GOI) and the housekeeping gene expression, using the Eq. (2):

$$\frac{(1+E_{\text{GOI}})^{-\Delta\text{Ct}}}{(1+E_{\text{Actin}})^{-\Delta\text{Ct}}} = \frac{(1+E_{\text{GOI}})^{-(\text{CtGOI}-\text{CtGOIcalibrated})}}{(1+E_{\text{Actin}})^{-(\text{CtActin}-\quad\text{CtActincalibrated})}}$$

Gene expression levels were normalized to the expression of the first sample for the T4 treatment (6 mm h^{-1}), in order to obtain a calibrated ΔCt for each gene. The LightCycler® Instrument software v 1.1 was used in order to calculate cycle threshold values and observe melt profiles. Ct values for Actin did not varied more than one unit between all samples analyzed for each real time treatments along the experiment. All primer pairs amplified a single product of the expected size, which was confirmed by melt-curve analysis, agarose gel electrophoresis. All experiments were performed with five biological replicates and three technical replicates.

2.8. Grape berry allometrics

For grape berry mass and volume determination, a sample of ten berries were randomly collected from different clusters per replicate from each treatment. The ten berries mass was determined using an electronic scale, and the same berries were used for volume determination by means of a 100 mL measuring cylinder.

2.9. Statistical analysis

Data were statistically analysed by one-way randomized blocks ANOVA with $p < 0.05$. When significant differences were determined, treatments were compared by Tukey media analysis. Statistical analysis was performed by statistical software JMP®, Version 11 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Water relations

To reduce the xylem stem water potential (ψ_{ST}) of plants, irrigation was suspended at 30 days before veraison (DBV) until 10 DBV, i.e., the time when irrigation treatments begun. Different combinations of drip emitters per treatment, yielded substantial differences in the water volumes applied, from the beginning of the irrigation treatments until the end of the season at 65 DAV (Fig. 1B). The ψ_{ST} values ranged from approximately −1.4 to −1.2 MPa in the two extreme treatments during veraison, and increased during the season, reaching values of approximately −0.7 and −1.05 MPa for T4 and T1, respectively, at 60 DAV. Significant differences in the ψ_{ST} were observed between treatments, particularly when irrigation was resumed after veraison (Fig. 1A). Except for the last day of measurement, the most stressing treatment (T1) yielded significantly lower ψ_{ST} values from 0 to 50 DAV, compared with T2, T3 and T4.

3.2. Abscisic acid content in the grapes and leaves

The abscisic acid (ABA) concentration in grapes and leaves varied according to the water regime imposed, with different variations along the season depending on the organ. In leaves, the average ABA concentration was inversely proportional to the water availability on each date, but no significant differences were detected on the first sampling date after veraison (Fig. 2A). In

Table 1
Accession number of sequences and primers used for qPCR, concentration, efficiency and R^2 from standard curves.

Gene	NCBI	Primer sequence (5′-3′)		nM	E ± SD	R^2
	Accession no.	Forward	Reverse			
VvSUC11	AF182445.1	TGTGCCAATCTCAAGTCTGCC	CCTGGGCTGCTGTTATGCTT	500	1.99 ± 0.01	0.99
VvSUC12	AF021809.1	ACCAGCCTCACCATTTATCAGAC	ATTTCATAACTGCTCTCAGGGTTG	500	2.00 ± 0.03	0.97
VvSUC27	AF021810.1	TGCTTGGCACTGACGGTACT	GCTGTAGGTGATCGCAAGAGG	250	2.03 ± 0.01	0.98
VvHT1	Y09590.1	GCTGTTTGGATGTTGATTGTCGG	TGGAAGCCAATGTTGAGGGC	500	1.92 ± 0.02	0.99
VvHT3	AY854146.1	GGTATTGGCATTGGATTTGGCG	GATCATGTTTGCTGTGAAGATCCC	500	2.0 ± 0.04	0.98
VvHT5	AY538261.1	CTTTGGGATTTGGTAGTGATGCG	AGAGTAGACGGACACGAGGG	250	1.97 ± 0.01	0.99
VvCWI	AY538262.1	GGTTGAAGTTGGTGGAATAACAGC	TACTGATGCACCCCTTTGACTG	500	2.07 ± 0.04	0.99
VvACT	AF369524.1	AGCTGGAAACTGCAAAGAGCAG	ACAACGGAATCTCTCAGCTCCA	250	2.03± 0.04	0.99

addition, the maximal concentration was observed on 20 DAV compared with the previous date, although the ψ_{ST} was less negative (Fig 1A). Although minimum levels of ABA were observed at 40–50 DAV, an increase in this hormone was observed at the end of the season, and the concentration increased with increasing stress treatment. Similarly, in grape berries, a relationship between the ABA concentration and water restriction levels was detected, with significant differences observed immediately after veraison (Fig. 2B). Subsequently, a constant decrease in the ABA content was observed until 50–60 DAV, and coincidently, a slight increase in ABA was detected in the leaves at the end of the season, i.e., 68 DAV (Fig. 2A,B).

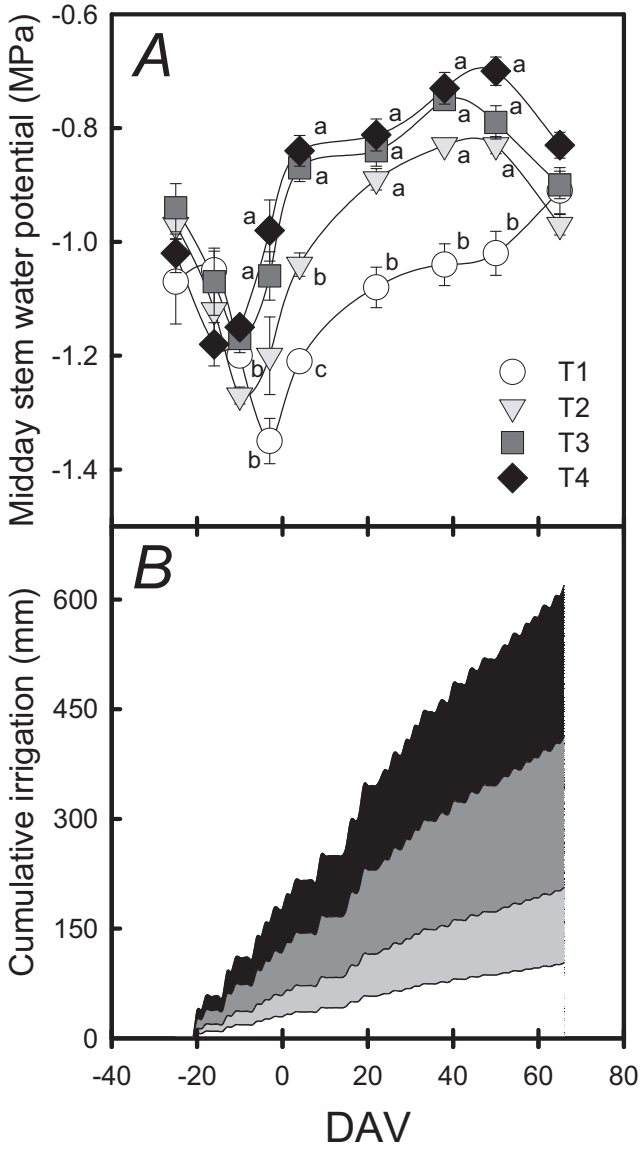

Fig. 1. Midday stem water potential and water supplied during season. (A) Midday stem water potential measured with a pressure chamber after enclosing the leaves for 2 h in an aluminized plastic bag along the season. Time is represented by the days after veraison (DAV). Treatments comprise plants irrigated with different drop emitters: 1 mm h^{-1} (T1), 2 mm h^{-1} (T2), 4 mm h^{-1} (T3) and 6 mm h^{-1} (T4). Different letters indicate differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 5$). (B) Cumulative water supplied per plant, during the season. Colours white, light gray, gray and black, corresponds to T1, T2, T3 and T4, respectively.

3.3. Grape berry allometrics

In response to treatments T3 and T4, the berry weight and volume (Fig. 3A,B) reached the highest values simultaneously at 25 DAV, but these parameters rapidly increased after veraison when irrigation was resumed. Similarly, the more stressing treatments T1 and T2, generated similar values for the weight and volume of berries, but the highest averages were observed with treatment T2 compared with the two more irrigated treatments. However, for treatment T1, nearly two more weeks were required to generate a peak in the weight and volume of the berries (Fig. 3A,B). Both T1 and T2 reduced nearly 30% of the weight and volume of berries compared with T3 and T4.

3.4. Sugar content in the grape berries

The sugar concentration in the grapes was assessed as the concentrations of glucose and fructose, the major forms of reduced carbon in grape berries, and the non-reducing sugar sucrose, representing less than 10% of the total soluble solids. Fig. 4A shows the total sugar concentration expressed as glucose equivalents (see materials and methods), in which the intermediate water stress treatments T2 and T3 induced accumulation at 5 DAV, with significantly higher concentrations than in the less and more irrigated treatments, T1 and T4, respectively. However, from 25 DAV until the end of the season, the final concentration of glucose equivalents in the grape berries was the same for every treatment (Fig. 4A). As shown in Fig. 4B, the higher average sugar content at 5 DAV was observed in the intermediate stress treatments, while T1 generated the lowest content. All treatments generated high glucose equivalents between 25 and 37 DAV, but the two most irrigated treatments generated 20% more sugars than treatments T1 and T2 (Fig. 4B). The differences in the content of glucose equivalents at 5 DAV (Fig. 4B) was confirmed through the sugar import rate, with higher averages obtained for intermediate irrigation treatments T2 and T3, and the lowest averages observed for the less irrigated treatment T1 (Fig. 4C). Moreover, the sugar accumulation was strongly reduced after 20 DAV, irrespective of the water regime (Fig. 4C).

3.5. Transcript profiles for sugar transporters in grape berries

The transcript profile of the genes for hexose transport (*VvHT1*, *VvHT3* and *VvHT5*) and sucrose transport (*VvSUC11*, *VvSUC12* and *VvSUC27*) in grape berries revealed a similar pattern of expression, with only minor differences among the water regimes (Fig. 5). There was a reduction on *VvHT1* and *VvHT3* expression from veraison until the end of the season. The highest *VvHT1* expression was observed in T1, T2 and T3 treatments whereas the *VvHT3* expression was high in the T2 and T3 treatments at veraison (Fig. 5A,B). *VvHT5* transcripts were increased from veraison until 55 DAV, and a slightly higher increase in *VvHT5* expression was observed until 65 DAV for the two more stressing treatments T1 and T2, whereas decreased expression was detected in T3 and T4 (Fig. 5C). Two distinctive patterns were observed for the transcripts of the sucrose transporter proteins, showing an increase in *VvSUC11* and *VvSUC12* expression from veraison until the end of the season and a reduction in *VvSUC27* expression during the same period (Fig. 5D–F). Interestingly, the expression of *VvSUC11* transcripts rapidly increased for the more irrigated treatments after veraison, and similar results were observed for VvSUC12, except the expression level was significantly higher for the more restricted treatments at 65 DAV (Fig. 5D,E). In general, *VvSUC27* gene expression was reduced from veraison onwards, and slight, but significant, differences occurred among treatments. Although, the two more stressing water regimes, T2 and T1, resulted in higher

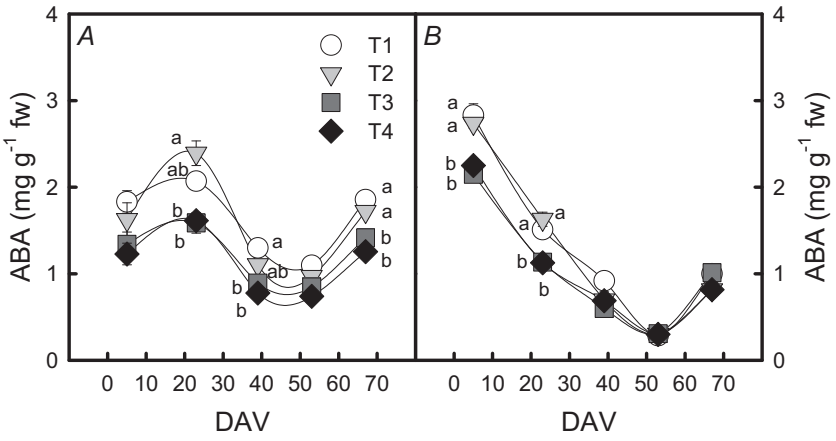

Fig. 2. ABA concentration in leaves (A) and berries (B) during season for the different irrigation treatments. Symbols as in Fig. 1. Different letters indicate significant differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 20$).

transcript expression immediately after veraison and at 5 and 20 DAV (Fig. 5F), this trend was reversed at the end of the season, where the two more watered regimes generated slightly higher transcript abundance compared with the more stressed treatments (Fig. 5F).

Moreover, in berries, the transcripts for the cell wall invertase, *VvCWI*, were slightly reduced from veraison until 20 DAV, with increased transcript abundance towards the end of the season, reaching significantly higher levels for the two more stressing treatments compared with treatments T3 and T4 (Fig. 6), similar to that observed for *VvHT5* and *VvSuc12* (Fig. 5C,E).

3.6. Carbon assimilation and content in grapevine leaves

The photosynthetic capacity of the vines was reduced over time from veraison until the end of the season, reaching lower values for the more stressed treatments (Fig. 7A,B). Similar differences were evident between both sides of the canopy. Particularly during veraison, the averages for the total daily assimilated carbon were higher for the W side. While this parameter steadily declined for the E side from 40 to 65 DAV, a transient increase occurred from 40 to 55 DAV on the W side of the canopy, particularly for the more irrigated treatment. For both sides of the canopy, a clear proportionality was observed between the total glucose equivalents assimilated during the season (Fig. 7A,B) and the carbon exported (Fig. 7C,D). The W side of the canopy showed a transient increase in carbon exportation from 40 to 55 DAV (Fig. 7D) as observed in assimilation on the same date (Fig. 7B). No significant differences in the leaf glucose equivalents were observed between treatments for the E side of the canopy, except for the last day, late in the season, with highest amounts in T4 (Fig. 7E). In the W side, there was an inverse trend between exported and stored carbon at 40 and 55 DAV (Fig. 7D,F). Moreover, at 20 and 40 DAV, the most significant differences in stored carbon were found between T4 and T1 treatment.

In both sides of the canopy, the sucrose content was higher in T3 and T4 (Fig 8A,B). The starch content was also increased in the most irrigated vines in both sides for the most of sampled date (Fig 8C, D). In addition, during the season, primarily reflecting the increase in the glucose polymer content, starch was relatively more abundant than sucrose during the day, strongly influencing the total non-structural carbon content in the leaves (Fig. 8E,F). Furthermore, when plotting the sucrose to starch content ration in the leaves on each date (Fig. 8G,H), a strong decrease in the values

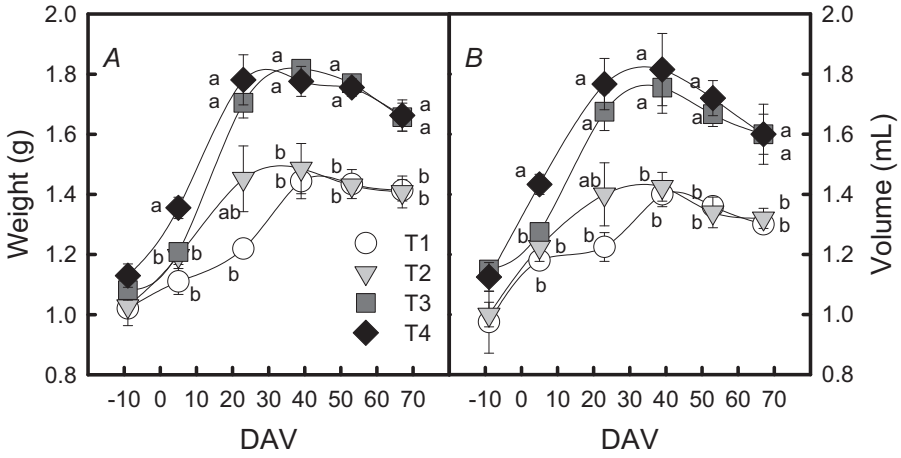

Fig. 3. Berry allometrics. Berry weight (A) and volume (B) during the season, for all the irrigation treatments. Symbols as in Fig. 1. Different letters indicate significant differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 5$).

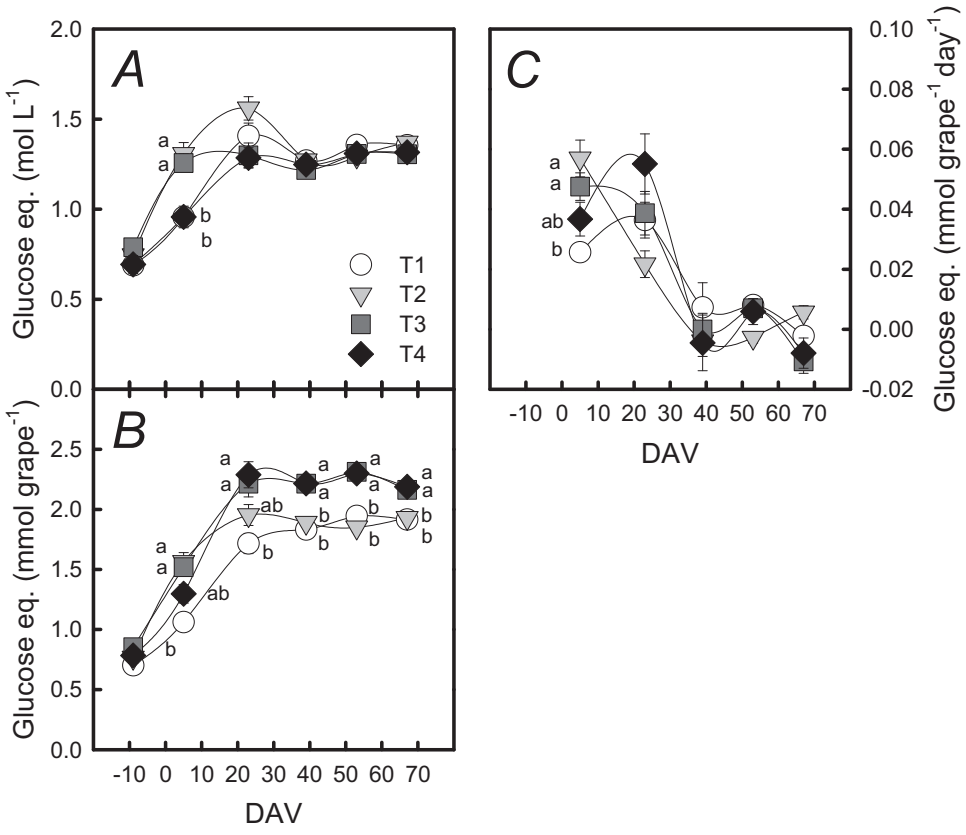

Fig. 4. (A) Concentration of sugars, as glucose equivalents, results from the addition of sucrose, glucose and fructose concentration in berries along the season. (B) Sugar content in berries (C) The sugar import rate, calculated by subtracting the sugar content in berries on each measuring day from the content on the previous measuring day. Symbols as in Fig. 1. Different letters indicate significant differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 5$).

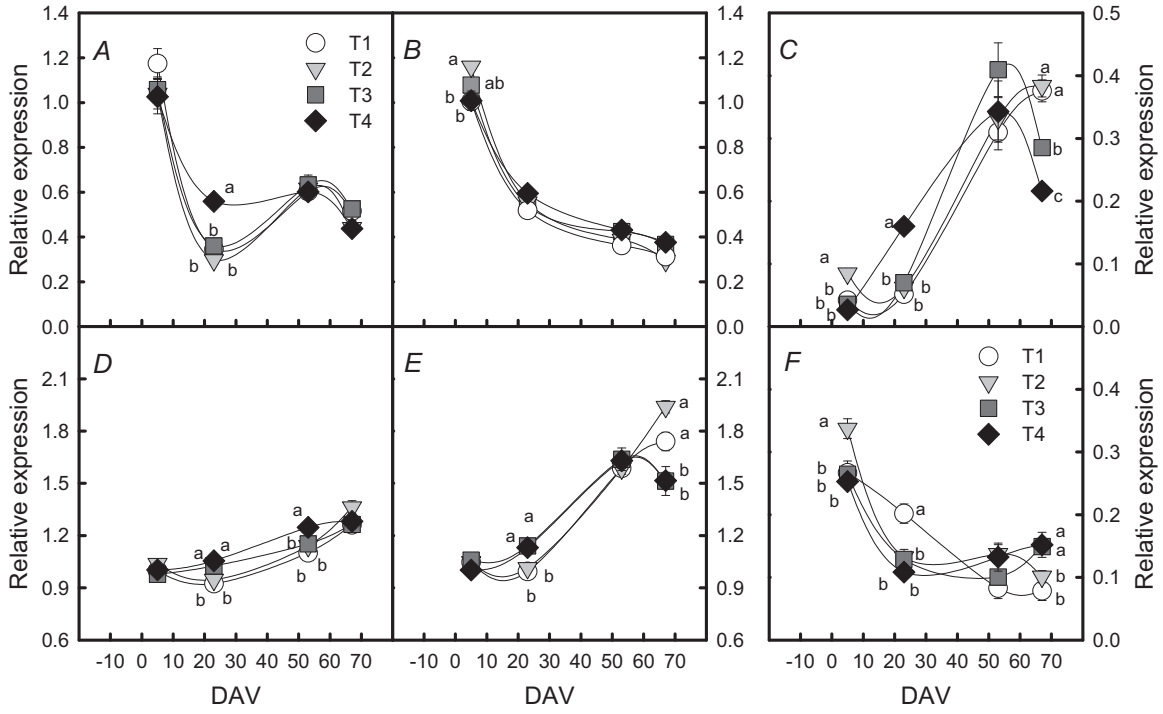

Fig. 5. Expression profiles for sugar transporters in berries. Relative expression of the genes encoding the hexose transporters *VvHT1* (A), *VvHT3* (B) and *VvHT5* (C) and the sucrose transporters *VvSUC11* (D), *VvSUC12* (E) and *VvSUC27* (F) in grape berries during the season. Symbols as in Fig. 1. Different letters indicate differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 15$).

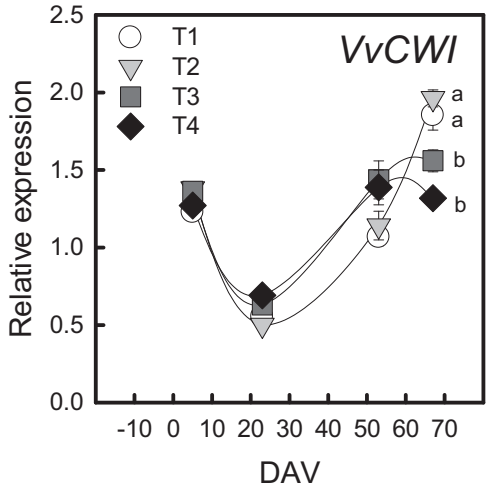

Fig. 6. Expression profile for cell wall invertase. Relative expression of the genes encoding the cell wall invertase *VvCWI* during the season. Symbols as in Fig. 1. Different letters indicate significant differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 15$).

was observed, reflecting an increase in the starch content (Fig. 8C,D) consistent with a slight reduction in the sucrose content, as observed from 25 to 40 DAV (Fig. 8A,B).

3.7. Transcripts for sucrose transporters in grapevine leaves

Similar to the pattern observed in the carbon assimilated and exported in leaves (Fig. 6A,B,D,E), the transcript abundance for *VvSUC27* was continuously reduced during the season from veraison onwards; however, this reduction was more rapid, reaching lower values for the more restricted treatments (Fig. 7A). However, right after veraison, the *VvSUC11* and *VvSUC12* expression was higher in T1 and T2 as compared to T3 and T4 (Fig. 7B,C). The *VvSUC11* transcript abundance in T1 and T2 was three-fold higher than T3 and T4 (Fig. 7B) at 20 DAV, while the abundance of *VvSUC12* for T1 was nearly eight times higher than T3 and T4 (Fig. 7C).

4. Discussion

Water stress is a serious environmental factor limiting crop yield, and has been identified as critical for primary productivity due to global environmental change (Fischer, 2001). Mechanisms for resistance have been described in plants involving morphological as well as metabolic changes mediated by altered gene expression (Bohnert and Sheveleva, 1998). The main focus of interest in research has been photosynthesis, considering that this process is notably inhibited by water shortages. However, rather than any single processes, the integrated response at the whole plant level, from photosynthesis to carbon partitioning and the capacity for reproductive development, has been long considered to be relevant for the successful species survival upon environmental constraints (Chaves et al., 2002).

Various crop species have been classified as isohydric and anisohydric according to their responses to drought at the leaf level in which, the former type, would induce a closure of stomata with the consequent reduction in transpiration, maintaining higher leaf water status, as compared to the latter, which behaves the opposite (Maseda and Fernández, 2006). In grapevines, variability exists among varieties as to the responses exhibited upon water stress (Schultz, 2003; Chaves et al., 2010; Lovisolo et al., 2010). The fact that the water status of the different irrigation regimes is mostly similar despite the differences in the amounts of the water

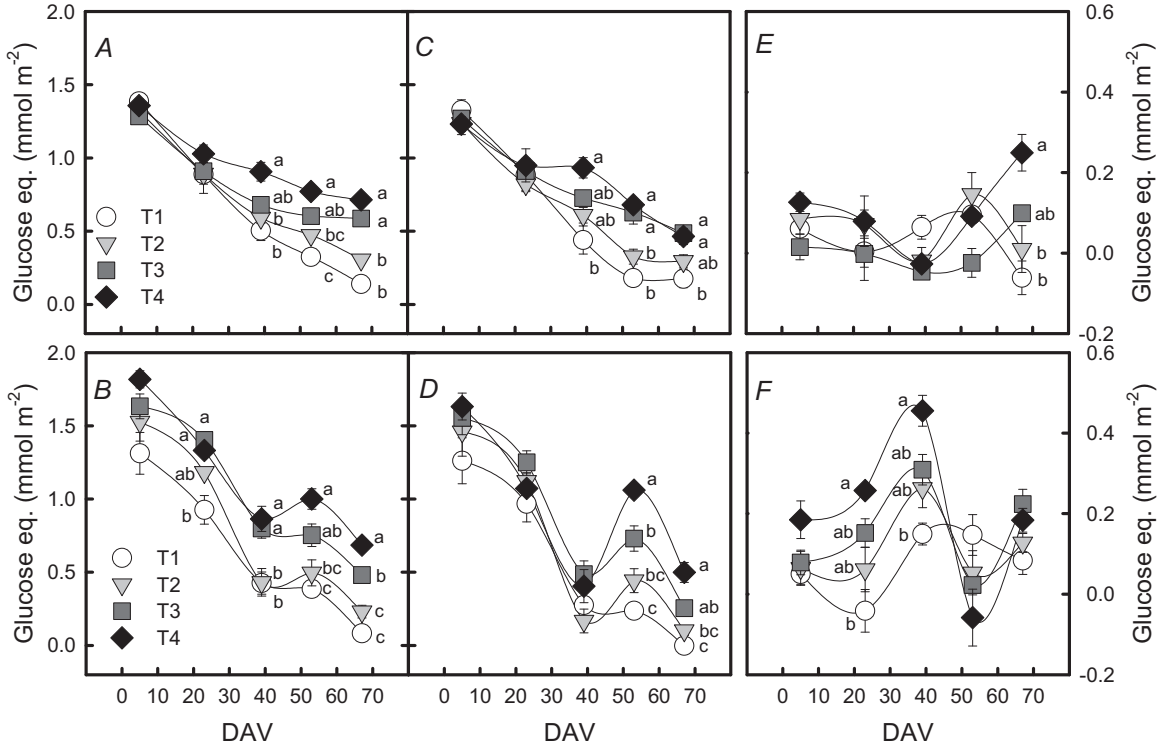

Fig. 7. Daily Carbon Assimilation, carbon export and carbon stored in leaves. Net photosynthesis as glucose equivalents of the carbon assimilated (A, B), glucose equivalents of the carbon exported (C, D) and the glucose equivalents stored in the leaves (E, F). The measurements and calculations were obtained separately for the leaves positioned on the East (A, C, E) and West (B, D, E) side of the vertical trellising system. Symbols as in Fig. 1. Different letters indicate differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 5$).

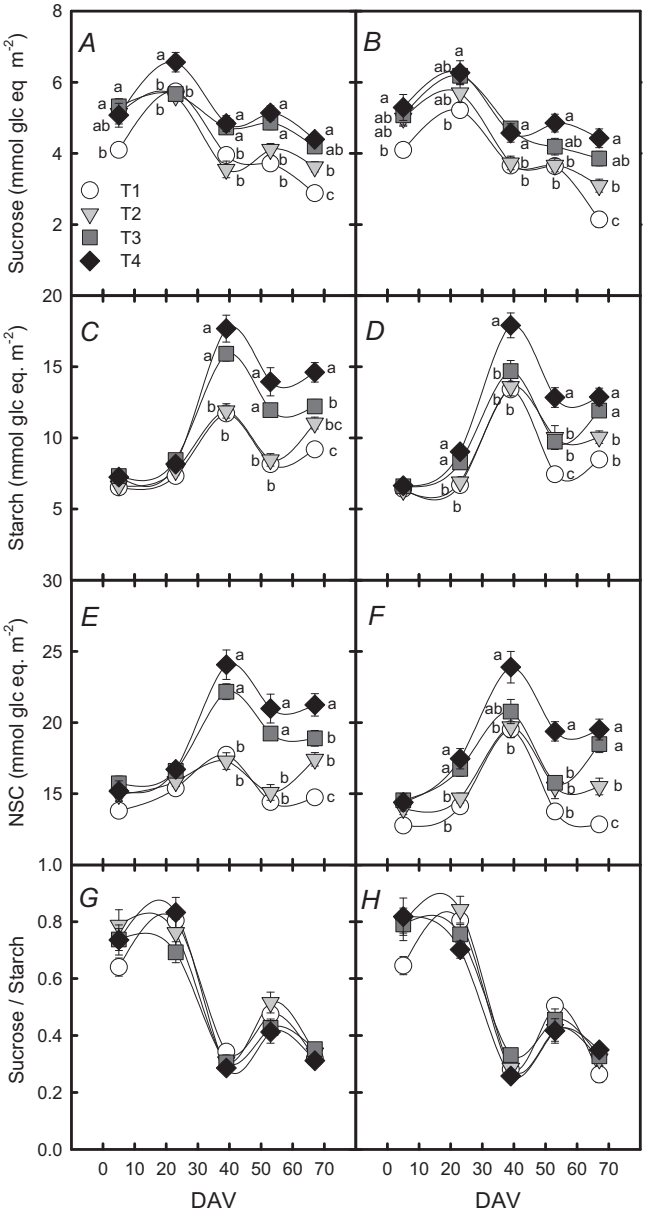

Fig. 8. Average daily leaf content of sucrose (A, B) and starch (C, D), and the non-structural carbon content resulting from the sum of sucrose and starch (E, F) and the ratio between sucrose to starch (G, H) on the East side of the canopy (A, C, E, G) and the opposite West side (B, D, F, H). Symbols as in Fig. 1. Different letters indicate differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 20$).

supplied from veraison through to harvest (Fig. 1), strongly suggest that Carmenere behaves as an isohydric varitey. It has to be said, however, that in spite of the close average stem water potential values, they range from what is considered weak water deficit to moderate/severe water deficit (Van Leeuwen et al., 2009)

Among others, water stress is a common practice in grapevine for wine purposes because it reduces cell division and elongation in fruits, resulting in smaller grape berries, with a higher skin to pulp ratio (Roby et al., 2004). In the present study, there is no clear proportionality between water supply and final berry weight and volume since, from the four contrasting water regimes only two final sizes were distinguishable (Fig. 3). The impact of water stress on berry size is more marked when imposed between flowering and veraison rather than, as in the present study, in post-veraison through to harvest (Becker and Zimmermann, 1984; Hardie, 1976; Van Leeuwen and Seguin, 1994). Berry volume is important since, combined with the capacity for sugar supply from leaves, it will result in any given sugar concentration. In fact, water stress effects on berry sugar concentration has been controversial, with reports suggesting decreases (Castellarin et al., 2007; Esteban et al., 1999; Wang et al., 2003), increases (Roby et al., 2004; Santesteban and Royo, 2006) and no changes (Sivilotti et al., 2005). In our study, all the water regimes led to the same sugar concentration in less than 30 DAV (Fig. 4A) as a result of the combination of sugar accumulation capacity and berry volume, with the more restricted conditions resulting in smaller berries (Fig. 3) with lesser glucose equivalent contents (Fig. 4A,B).

Of course, grape berry sugar filling results not only from the extent of the available reduced carbon from photosynthesis but, also, from the sink strength. In grape berries, veraison involves a transition from passive to active sugar unloading (Zhang et al., 2006) time from which sugars start to accumulate (Famiani et al., 2000). The strong sugar accumulation sometimes is accelerated by water stress (Roby et al., 2004), and the response is thought to be mediated by abscisic acid (Davies et al., 1997; Deluc et al., 2009), a known water stress induced plant growth regulator. In the present study, after water stress was imposed, a transient acceleration in sugar import into the berries was observed immediately after veraison, but only in the intermediate stress treatments (Fig. 4C). Sugar and ABA signaling pathways interact synergistically in the control of sugar transport in grape berries, as seen in ABA-, stress-, and ripening-induced proteins (ASR) involved in the up-regulation of the expression of berry hexose transporters (Cakir et al., 2003; Conde et al., 2006; Lecourieux et al., 2010). Also in grape berries, both, ABA and sucrose must be present over some concentration threshold for the induction of anthocyanin synthesis (Gambetta et al., 2010). Our data suggest that, under extreme water stress conditions, the acceleration of grape berry sugar accumulation fail because, even though there are high grape berry ABA concentrations (Fig. 2B), there is a photosynthetic limitation for the required sugar concentration threshold.

The abovementioned acceleration of the sugar uptake in berries with high sugar and ABA concentrations upon mild water stress (Fig. 2B and Fig. 4A), is explained by a higher transcript abundance of *VvHT3*, *VvHT5* and *VvSUC27*, but occurring only right after veraison. Considering that the differences in expression were only minor at the first sampling date 5 DAV (Fig. 5), it is possible that the up regulation of sugar accumulation resulted from previously coded proteins, right after imposing the irrigation treatments before veraison. Transcripts for *VvSUC27* were less abundant as compared to the other two sucrose transporters assessed, i.e., *VvSUC11* and *VvSUC12*. Even though there are still some discrepancies, if *VvSUC27* is, as described, a low affinity/high capacity transporter (Zhang et al., 2008), its higher expression right after veraison might partly explain the higher sugar import rate upon mild water stress. Besides, *VvSUC27* expression was also higher, but nearly 20 days later, due to a slower decay, in the most extremely water stressed samples (Fig. 5), a time when those berries were still increasing their sugar content (Fig. 4B).

In general, the pattern of expression of the sucrose transporters coincides with previous northern blot observations (Davies et al., 1999) in which *VvSUC27* progressively decreases after pre-veraison, contrary to *VvSUC11* and *VvSUC12* that increased their expression from veraison onwards (Fig. 5). Also, the expression pattern of the monosaccharide transporter genes *VvHT1* and *VvHT3*, declining from early stages of berry growth (Fig. 5), is similar to that previously reported in Cabernet Sauvignon (Deluc et al., 2007; Hayes et al., 2007) except that, in our study, no increases of the latter was observed after veraison. It is interesting to note that, even though grape berries were already at their maximal sugar

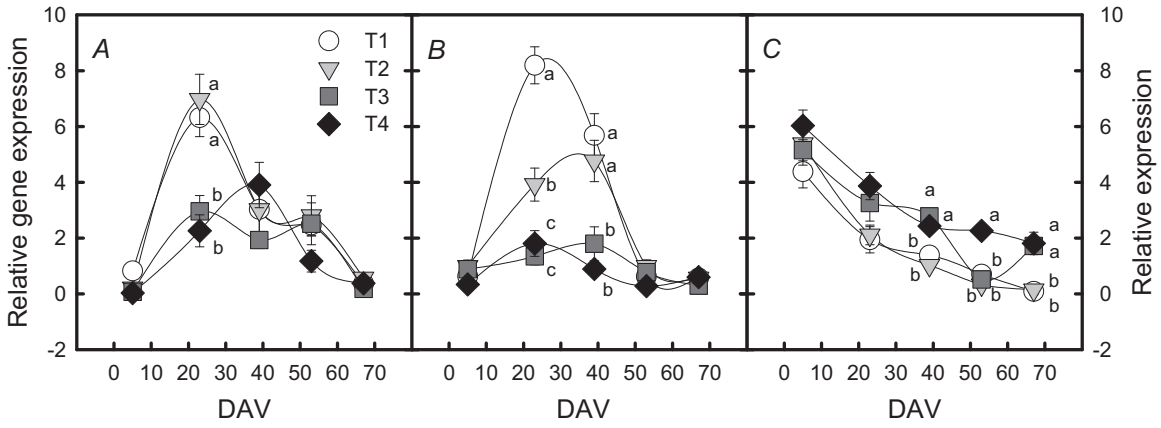

Fig. 9. Expression profile for sucrose transporters in leaves. Changes in the relative expression of genes encoding the sucrose transporters *VvSUC11* (A), *VvSUC12* (B) and *VvSUC27* (C) in leaves during the season. Symbols as in Fig. 1. Different letters indicate significant differences between treatments for each date ($p < 0.05$). Values represent means ± SE ($n = 15$).

concentration nearly 20 DAV to 30 DAV (Fig. 4A), expression of *VvSUC11* and *VvSUC12* kept rising until 65 DAV (Fig. 5). That was also the case for *VvHT5* but with a substantially low expression levels, similar to previous observations in Chardonnay and Cabernet Sauvignon grapes (Afoufa-Bastien et al., 2010; Hayes et al., 2007). It has been suggested that the importance of this hexose transporter in post-veraison sugar accumulation can not be ruled out, simply because of its expression level, until knowing its precise location within the grape berry. This is particularly important considering that its expression is generally assessed, like in the present study, in berry tissues as a whole (Hayes et al., 2007). Therefore, results are referred only as an average value, with the risk of under estimating its real importance, for instance as if it were concentrated in phloem unloading berry sites. Besides, its pattern of expression was very similar to that of the cell wall invertase *VvCWI* (Fig. 6).

The increases in expression of *VvSUC11* , *VvSUC12* , *VvHT5* and *VvCWI* after the grape berries were already at their maximum sugar content are confusing. One possibility is that they are involved in between and within cell sugar movement later than 35 DAV but further studies on the subcellular location of the proteins are necessary to be conclusive. On the other hand, the need to continue the sugar uptake in order to replace glucose equivalents taken by cell processes consuming either energy or carbon equivalents, can not be ruled out. In fact, according to Famiani et al., (2014) and Ollat and Gaudillère, (2000), nearly 13% of the sugars present in the pericarp are respired during ripening. If grape berry sugar concentration ought to be maintained long after reaching its maximal concentration, further sugar import for respiration is needed. Finally, there is the possibility that such continuous expression does not respond to a need for sugar import into the berries, but it is simply induced by the presence of sucrose at the phloem end, explaining the higher transcript abundance of *VvSUC11* and *VvSUC12* in berries from the more irrigated plants (Fig. 5).

Differences in expression levels between irrigation regimes very late in the season were also observed, such as those for the sucrose transporters *VvSUC12* and *VvSUC27*, the hexose transporter *VvHT5* , and the cell wall invertase *VvCWI* (Figs. 5 and 6) Such differences do not necessarily result from a direct long term molecular and metabolic effect of irrigation on the berries but, rather, might be associated with the microclimate of the fruiting zone. For the more restricted treatments, premature leaf senescence occurs at that time, leading to more illuminated, and hence warmer, conditions for the clusters. Grape berry ABA concentration at the end of the season also differs between irrigation treatments (Fig. 2B) but, then again, external conditions related to the grape berry microclimate might be the cause.

Daily carbon assimilated by photosynthesis was reduced along the season, right from veraison onwards, in an extent proportional to the stressing condition of the vines. The general decline might be the result of the reduction of the global radiation which is known to begin to decrease from February at the latitude of the experimental site, but also because the maximal CO_2 assimilation rate in both sides of the canopy is reduced from veraison through to the end of the season. Interestingly, at the time when the berries reached their maximal sugar content, from 20 to 30 DAV (Fig. 4), there was an increase in the total non-structural carbon being stored in the leaves during the day (Figs. 7E,F), indicating that the vines sink capacity was transiently reduced at that time, irrespective of the irrigation regime. In fact, when maximum berry sugar content was reached, the glucose equivalents in the leaves totaled nearly 40% of that assimilated by photosynthesis on a daily basis, irrespective of the water regime, much higher than the merely none to 13% observed earlier at 20 DAV, when berries were an active sink (Fig. 7). Further supporting a reduced vine sink capacity is the abrupt drop of the sucrose to starch ratio in leaves, from 20 to 40 DAV, actually resulting from increases in leaf starch contents (Fig. 8). It is well known that photosynthetic carbon partitioning between sucrose and starch is modulated by carbon intermediates in the cytosol (Paul and Pellny, 2003). An excess of sucrose in leaves, as that resulting from a reduction in sink demand, leads to an inhibition of sucrose phosphate synthase and, also, to increases in the concentration of phosphate containing intermediates in the cytosol (Pieters et al., 2001). A straight consequence is that reduced carbon molecules would accumulate in the chloroplasts, diverting to starch synthesis, exactly as that observed in our study (Fig. 8). Besides, this effect might be partly responsible for the observed reduction of photosynthesis due to feedback inhibition from 20 to 40 DAV (Fig. 7A,B).

The fact that a transient and slight increase in the daily carbon assimilation occurred late in the season (Fig. 7B), simultaneous with an increase in the sucrose to starch ratio in the leaves (Fig. 8G,H), is an indication that a new sink was activated and, possibly, that the decline in carbon assimilated by photosynthesis along the season partly results from sink limitations. In fact, such transient increase was observed in the W side of the canopy, with the highest daily CO_2 assimilation capacity. Reductions in sink demand have been associated to reductions in photosynthesis (Pieters et al., 2001) and restoration of such demand induces an increase in

photosynthesis, even in leaves that had engaged the senescence pathway (Hodgkinson, 1974). One such possible sink is carbon storage as reserves in roots and wood, known to happen late in the season in grapevines (Zapata et al., 2004).

It has been a long time since studies on sink to source relationship have been carried out in grapevines, assessing the photosynthetic responses to changes in sink organs, but always manipulating the crop load by cluster thinning or fruit removal (Foyer et al., 1995; Iacono et al., 1995). Our results indicate that such interactions also occur naturally, independent of the carbon export capacity, as phenological development proceeds, specifically as a feedback negative effect on carbon export upon berries sugar completion. Furthermore, the observed oscillations in carbon export, together with the changes in the sucrose to starch ratio, as well as carbon assimilation, is an indication that the various plant sinks needs a time to be induced and that a mismatch in their coordination exists. This would be the case, at least during the phenological stages spanned in the present study, if sucrose were needed to build up in the phloem for some time after berries finished the sugar intake, in order to induce transport protein genes elsewhere. As previously suggested, one such potential sink is the root system, which would actively uptake sugars but only after the sucrose concentration at that phloem end would rise inducing transport proteins. This trait would be convenient in order not to induce an abrupt competition between sinks if some of them were to be negatively affected by environmental constraints during a short period of time. Also, it is evident that the transient reduction of sugar export, concomitant to the increases in starch content compared to sucrose in leaves, appears in the West side of the vertical trellising system (Figs. 7C,D). The vineyard row orientation and the mountains to the east, resulted in longer direct light exposures in the west side of the canopy, with higher daily assimilated glucose equivalents compared to the east. Clearly then, the sink to source relationship discussed so far results from a cumulative effect of light exposure in leaves.

Among the three carbon phloem loading mechanisms described until now (Rennie and Turgeon, 2009; Slewinski and Braun, 2010), the one occurring in grapevine leaves is still not certain. Tall trees, as naturally occurring in grapevines, have been found to be passive phloem loaders, even though it has been suggested that some species are able to use more than one mechanism (Slewinski et al., 2013). Besides its convenience for maintaining low carbon stocks in leaves, active phloem loading has been described as a mechanism better suited for balancing photosynthetic activity in the leaves with photoassimilate utilization and storage in sink plant organs (Ainsworth and Bush, 2011). It is difficult to conclude active phloem loading from transcripts expression only, since even in species where loading is simplastic, sucrose transporters have been described, but interpreted as a need to retrieve leaked sugars (Turgeon, 2010). Our results show that, irrespective of the water status of the vines and, at least for the *VvSUC27* transcripts, its abundance is closely related to the daily carbon assimilation, dropping along the season. It has been hypothesized that phloem loading capacity is regulated by the sucrose concentration in the source phloem in a way that, as sugar demand in sinks is high, sucrose levels are low, triggering a signal cascade for symporter transcription (Ransom-Hodgkins et al., 2003). This was not the case for *VvSUC27* since its abundance dropped even after veraison (Fig. 9C), when the grape berries were actively accumulating sugars, suggesting that either other proteins are involved and/or phloem loading is passive in grapevines.

If grapevines are a predominately passive phloem loading species, the maintenance of a sucrose gradient along the carbon partitioning pathway is necessary, starting from the mesophyll cells. Water stress, even though it reduced the berry volume and the need for sugars to reach the maximal concentration, strongly affected the capacity for carbon assimilation and, therefore, the average non structural carbon content in leaves (Figs. 7 and 8). Interestingly, *VvSUC11* and *VvSUC12* , which are only expressed in mesophyll cells (Santi et al., 2013), were 3–4 times more abundant in leaves from the more stressed vines (Fig. 9) at the time of fast sugar accumulation in grape berries. Whether this results from a low sucrose concentration in the phloem, in the cytoplasm of mesophyll cells, the high ABA content in leaves or a combination of some or all of them, has yet to be elucidated. Expression of sucrose transporters with low capacity but high affinity, as it is the case for *VvSUC11* and *VvSUC12* (Manning et al., 2001) in water stressed plants, might be interpreted as a mechanism for efficient retrieval of leaked sucrose, back to mesophyll cells, with low concentrations of the disaccharide, in order to maintain a convenient symplastic concentration gradient for phloem loading.

From our results, clear water stress effects and responses occur in the carbon gain and export at the leaf level as well as in the carbon gain in grape berries. Such changes are not simply resulting from limitations in the capacity for carbon reduction in leaves or source induced limitations for sugar accumulation in berries. Molecular responses are triggered in both, source and sink organs, which can be interpreted as mechanisms for reinforcing carbon export in the former, and import in the latter, upon water restrictions. Such responses are occurring independently at both ends. Interestingly, in spite of the water status of plants, coordination between reproductive sink carbon demand and source carbon assimilation and export occurred and was orchestrated by sugar transporters in sinks and sources. Such coordination involved the triggering of active sugar transport in leaf mesophyll when carbon assimilation was reduced by water stress and a sugar transporter mediated enhancement of the sink strength of fruits which allowed conserving a similar fruit sugar concentration across a wide range of irrigation regimes. The active sugar transport in the leaves triggered by water stress might enhance the transport of sugars within the mesophyll towards the cells in the vicinity of minor veins, hereby increasing sugar concentration in cells surrounding the phloem, under depressed carbon assimilation conditions. Further investigations are needed in order to know if the observed sugar concentration in berries from stressed plants, similar to those from well watered, would be achieved if the size of the grapes were not affected by water constraints. From an ecological point of view, this result indicates that the water stress response of grapevines seeks to achieve a target fruit sugar concentration which ensures the dispersal of its seeds.

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