Leaf carbohydrate metabolism in Malbec grapevines: combined effects of regulated deficit irrigation and crop load

S. DAYER, J.A. PRIETO, E. GALAT and J. PEREZ PEÑA

Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (INTA), Luján de Cuyo, Mendoza 5507, Argentina

Corresponding author: Dr Jorge Perez Peña, email perezpena.jorge@inta.gob.ar

Abstract

Background and Aims: Regulated deficit irrigation and crop load adjustment are viticultural practices used to improve grape and wine composition. Our objective was to evaluate the combined effect of irrigation and crop load levels on leaf photosynthesis, accumulation of non-structural carbohydrates and leaf carbon utilisation during the season.

Methods and Results: The trial started in 2006 in a *Vitis vinifera* L. Malbec vineyard in Mendoza. Two irrigation levels (100% and 25% of reference evapotranspiration) and two crop loads (20 and 10 bunches per vine, set at veraison) were studied. During the 2009/10 season, diurnal dynamics of leaf water potential, photosynthesis and carbohydrate concentration were determined at anthesis, veraison and harvest. Deficit irrigation reduced leaf starch concentration at veraison and increased soluble sugars. High-crop load reduced leaf starch concentration at veraison. Starch turnover was correlated with photosynthesis during the previous day.

Conclusions: Deficit irrigation had a greater effect on carbon allocation between soluble sugars and starch than on total carbohydrate production. Effects of deficit irrigation and crop load operated independently.

Significance of the Study: This experiment improved our knowledge of carbon assimilation and allocation during the season, which may assist the development of management practices to stabilise yield and fruit composition.

Keywords: *bunch thinning, deficit irrigation, photosynthesis, soluble sugars, starch*

Introduction

Regulated deficit irrigation has become a common practice in irrigated viticulture in arid and semiarid regions where water availability is scarce. Sustainable use of limited water resources in those regions is essential for grape and wine production. The effect of water deficits on grapevines has received considerable attention in the last 20 years, both in what are called 'traditional wine producing countries' (Rodrigues et al. 1993, Iacono et al. 1998, Flexas et al. 1999, Palliotti and Cartechini 2000, Flexas and Medrano 2002, Maroco et al. 2002, de Souza et al. 2003, Escalona et al. 2003, Medrano et al. 2003, Romero et al. 2010, 2012) and 'new wine producing countries' (Liu et al. 1978, Naor and Wample 1994, Murillo de Alburquerque and Carbonneau 1997, Dry et al. 2000, Dayer et al. 2013, Edwards and Clingeleffer 2013). Partly, this is because water deficits significantly affect important grapevine physiological processes, for example photosynthesis, grape composition, yield and wine composition.

Plant growth and grape yield are a function of photosynthetic carbon assimilation, allocation within the leaf and partitioning within the vine (Bota et al. 2004). Photosynthetic carbon assimilation occurs during day light, whereas growth and maintenance processes occur throughout the whole day–night cycle (Gordon 1986, Gibon et al. 2004). Under non-constraining conditions, carbon assimilation provides sufficient carbohydrates to support the immediate demand for growth and maintenance and for storage to be used during the night (Gordon 1986). Some evidence suggests the existence of a regulatory mechanism that balances the plant carbon gain with its use during the night (Smith and Stitt 2007). This implies that the rate of starch synthesis during the day is set by mechanisms that anticipate the amount of carbon required during the night, so the rate of starch turnover, that is the variation in starch content between the end of the day and the end of the night (Sulpice et al. 2009), leads to almost a complete utilisation before the sun rises the following day (Smith and Stitt 2007). When on-site rate of sucrose use and export out of the leaf is less than photosynthesis, photoassimilates are diverted into starch formation (Holzapfel et al. 2010). Environmental constraints, however, may reduce carbon assimilation needed for sustained growth over a whole day–night cycle (Smith and Stitt 2007). For example, a change in day length results in alterations in both the allocation of photoassimilates between starch and sucrose during the day, and starch turnover (Chatterton and Silvius 1979, 1980, Gibon et al. 2004, Lu et al. 2005). It is currently not known if the close match between starch synthesis and storage during the day, and starch degradation during the night, observed mainly in *Arabidopsis* sp., is also present in other plants (Graf and Smith 2011).

Water deficit has been shown to reduce leaf photosynthesis by diffusional limitations, that is reduction of stomatal and mesophyll conductance (Perez-Martin et al. 2009) and metabolic impairment. When water deficit is mild, photosynthesis is mainly limited by stomatal closure (Pou et al. 2008), whereas when the deficit is severe, limitation occurs mainly because of metabolic impairment (Lawlor and Cornic 2002) and reduction of mesophyll conductance (Flexas et al. 2009). Water deficit has also been shown to modify leaf carbon allocation between starch and soluble sugars (Quick et al. 1992). Under water deficit, with a lower rate of carbon assimilation, leaves maintained the concentration of soluble sugars at the expense of a reduction in starch concentration (Chaves et al. 2003). Some studies in grapevine have also shown that water stress caused a significant reduction in sugar export out of the leaves (Quick

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et al. 1992, Bota et al. 2004) and in starch synthesis (Düring 1984). Plant response to abiotic factors such as water deficit may be dependent upon the source–sink balance (Flore and Lakso 1990, Poni et al. 1993, Dayer et al. 2013). Limited knowledge exists about the combined effect of water deficit and crop load on field-grown grapevines, on leaf carbohydrate consumption or exportation to other sinks during a whole day–night cycle. In a previous study, we have shown that trunk starch concentration was affected by both factors, water deficit and crop load (Dayer et al. 2013). Although crop load affected trunk carbon reserves (long-term response), it did not affect photosynthesis (short-term response). Current literature concerning leaf carbohydrate metabolism in grapevine (*Vitis vinifera* L.) during the whole day–night period is scarce (Chaumont et al. 1994, Bota et al. 2004, Tarara et al. 2011). Information about leaf carbon utilisation, storage and transport during the whole day–night cycle may provide some clues to understand how assimilated carbon is partitioned among the different organs (Graf and Smith 2011), especially under water deficit conditions in relation to its use in respiration, storage in perennial organs, growth or berry maturity.

In this experiment, we evaluated if water deficit imposed on the vine by reducing irrigation level changed the pattern of carbon allocation (i.e. utilisation, storage and transport) within the leaf on vines with high and low-crop load. We hypothesised that: (i) water deficit reduces leaf carbohydrate storage in high-cropped vines more than in low-cropped vines; (ii) assuming that crop load does not affect photosynthesis (Dayer et al. 2013), low-cropped vines will have a similar amount of leaf assimilated carbon as high-crop vines during the day available to be distributed to other sinks apart from the fruit (i.e. trunk, cordons and roots); and (iii) vines under water deficit and high-crop load will utilise during the night more of the stored carbon accumulated during the previous daylight period than low-crop vines.

Materials and methods

Experimental site and treatments

The study was undertaken during 2009/10 in a vineyard located in Luján de Cuyo, Mendoza, Argentina (32°59′ S; 68°52′ W, elevation 960 m asl). The climate is arid with dry and hot summers. Historical reference evapotranspiration [ETo Penman-Monteith, Allen et al. (1998)] from October to April is 775 mm and annual rainfall is around 245 mm occurring mainly in summer. *Vitis vinifera* cv. Malbec vines grafted on 101-14 rootstock were planted in 1998 into a deep clay soil, in north–south oriented rows at 2 m between rows and 1.5 m between vines. Vines were trellised in a vertical shoot positioned system, spur pruned to 14–16 buds per vine and drip irrigated. The vineyard was protected with anti-hail black net installed in a Grembiule system (Figure S1). Measurements for this study were conducted during the fourth season (2009/10) of an experiment commenced during 2006/07 described in detail in a previous paper (Dayer et al. 2013). For this particular study on leaf carbohydrate metabolism, measurements were taken only on the plots that combined two irrigation levels, FI (fully irrigated) and DI-3 (deficit irrigated), with two crop loads and three replicates. In each replicate, two leaves from two experimental vines were measured. Irrigation levels were applied between fruitset [stage 27 of the E–L scale modified by Coombe (1995)] and harvest maturity (E–L stage 38). Within that period, FI and DI-3 were irrigated at 100% and 25% of ETo [Penman-Monteith, Allen et al. (1998)], respectively. For the crop load treatment, after anthesis some inflorescences were removed, and all experimental vines were left with 22 inflorescences. At veraison [day of the year (DOY) 20, E–L stage 35], two crop load levels were established within each irrigation level by bunch thinning: a high-crop load (HC) with 20–22 bunches per vine, and a low-crop load (LC) with 10–11 bunches per vine. A detailed description of the experimental setup of the initial experiment and the treatments applied were provided in a previous paper (Dayer et al. 2013). Meteorological variables, such as global radiation, air temperature, relative humidity and rainfall, were obtained from an automatic meteorological station (iMetos II, Pessl Instruments, Weiss, Austria) located next to the vineyard. Before budburst (DOY 258), irrigation was applied to fill the soil profile to field capacity. Irrigation treatments were applied from fruitset until harvest. From budburst to fruitset, and after harvest, all experimental plots were equally irrigated at 60% of ETo. Experimental plots were irrigated twice a week. Measurements were performed on days with clear skies at anthesis, veraison and harvest maturity (stage 23, 35 and 38 E–L, respectively) on leaves located only on the east side of the canopy because of the size of the initial field experiment. Because of the number of plots and the distance between them, to measure leaves on both sides of the canopy would have taken much more than 2 h between the first and last measurement, and that would have limited reasonable comparisons. The effect of irrigation level on vine water status was evaluated by measuring leaf water potential (Ψ_L) at 0600 h [predawn water potential (Ψ_{PD})] and then Ψ_L at 1000 h, 1230 h and 1500 h. Only healthy and fully expanded mature leaves were measured with a pressure chamber (Modelo 4, Biocontrol, Buenos Aires, Argentina). Net photosynthesis (P_n), stomatal conductance (g_s) and transpiration (E) were measured on two leaves per vine at 0800 h, 1000 h, 1230 h and 1500 h at each phenological stage. An extra measurement was taken at 0530 h at veraison and at 1400 h at harvest. Leaf gas exchange was measured with a portable open-circuit infrared gas analyser (CIRAS-2, PP Systems International, Amesbury, MA, USA) equipped with an automatic cuvette [PLC6 (U), CRS121, PP Systems International] that enclosed 2.5 cm^2 of leaf area.

For assays of non-structural carbohydrates, eight leaf discs (6.4 mm diameter) were collected from the same leaf used for gas exchange measurements. In order to calculate the whole-day leaf carbon balance, samples were taken before sunrise (first sample) and after sunset (second sample) of the same day, and before sunrise the following day (third sample). Leaf discs were collected between veins with a hole puncher with a 1.5 mL microtube attached. When the leaf discs were cut by the puncher, they dropped into the microtube, which was immediately snapped frozen in liquid nitrogen. Disc samples were then stored at −80°C until analysis. Leaf discs were counted and weighed. Around 20, 1.25 mm diameter zirconia/silica beads (Glen Mills, Clifton, NJ, USA) were added to the microtube and snapped frozen in liquid nitrogen to facilitate grinding using a bead-beater type homogeniser for 45 s (Mini-BeadBeater-8, Glen Mills). After homogenisation, an aliquot of 1.25 mL of 80% v/v aqueous ethanol was pipetted into the microtubes and incubated for 15 min at 80°C in a water bath. After incubation, the ethanol was decanted into a 15-mL centrifuge tube and replaced with another 1.25 mL of 80% v/v ethanol. This step was repeated one more time, resulting in three extractions. An aliquot of 0.5 mL was pipetted from the 15-mL tubes after extraction of soluble sugars and mixed with 10 mg of activated charcoal (Sigma C3345, Sigma-Aldrich, St Louis, MO, USA) in a 0.45 μm cellulose acetate-microfilter tube assembly (Costar 8163-Corning, Sigma-Aldrich) and centrifuged at 2200 *g* for 3 min to produce a clear extract. The concentration of sucrose, D-fructose and D-glucose was determined according to the pro-

cedure outlined in the commercial enzyme assay kit used (K-SUFRG, Megazyme International, Bray, Ireland). For starch analysis, the remaining insoluble fraction was re-suspended in 200 µL dimethylsulfoxide and heated at 98°C for 10 min. Starch concentration was determined according to the procedure outlined in the commercial enzyme assay kit used (K-TSTA, Megazyme International). Briefly, 300 µL of thermostable α-amylase in sodium acetate buffer was added, mixed and incubated for 15 min at 98°C in water bath. After cooling, 10 µL of amyloglucosidase enzyme was added and incubated at 50°C for 60 min. The samples were mixed at 20-min intervals, and then centrifuged at 10 000 *g* for 2 min. A 20 µL aliquot of extract was placed in a microplate well together with 300 µL GOPOD reagent (a mixture of glucose oxidase, peroxidase and 4-aminoantipyrine in a potassium phosphate and *ρ*–hydroxybenzoic acid buffer), and the microplate was covered and incubated at 50°C for 20 min. Glucose concentration of the samples was then determined colorimetrically by reading the absorbance at 510 nm and the concentration of starch in the sample calculated. Starch turnover, that is the variation in starch concentration between the end of the day and the end of the night, was calculated as the difference between starch concentration at the beginning of the night (second sample) and before sunrise the following day (third sample).

Experimental design and statistical analysis

The experiment initiated in 2006/07 was laid out as a randomised block factorial design with two factors, irrigation and crop load, and five replicates. Blocking was based on initial vine pruning mass and trunk diameter measured in winter 2006. One replicate consisted of 36 vines arranged in three adjacent rows of 12, all receiving the same irrigation treatment. All measurements were made on the central eight vines of the central row, with the remaining vines being treated as 'guards'. Physiological variables and non-structural carbohydrates were measured on two central vines of the middle row of the experimental unit. All data were tested for normality using the modified Shapiro–Wilk test and for homogeneity of variance using Levene's test. Data were analysed by a two-way (irrigation x crop load) ANOVA using the general linear model procedure for randomised blocks. Means were compared by Fisher's multiple tests ($P \leq 0.05$), and significant interactions between treatments are indicated and described in the text. Time series data, such as Ψ_L and gas exchange values, were analysed as a repeated-measure design using multivariate statistics and Hotelling's multiple test ($P \leq 0.05$) for comparison of means between treatments. Statistical analysis was performed with Infostat software (version 1.5, National University of Córdoba, Córdoba, Argentina).

Results

Results presented below correspond to the last season of a 4-year trial which commenced in 2006.

Weather conditions

During 2009/10 total rainfall was around 100 mm for the whole season concentrated during the fruitset–veraison period (Figure 1). Total ETo from budburst to harvest was 810 mm, with a maximum daily average of 5.6 mm in January. During the veraison measurement, maximum temperature was 34°C, vapour pressure deficit (VPD) was 5.8 kPa, relative humidity 42% and ETo 6.8 mm (Figure 2e). During the measurements at anthesis and harvest, a similar mean temperature was recorded, but radiation and VPD were lower during harvest, and as a consequence lower values of ETo were registered (3.7 mm/day, Figure 2). Irrigation received by the vines from budburst to leaf-fall (from 20 August 2009 to 15 April 2010) was 864 mm and 416 mm for FI and DI-3, respectively (Figure 1), whereas during the period the treatment was imposed, from fruitset to harvest, irrigation applied was 574 mm and 143.5 mm for FI and DI-3, respectively.

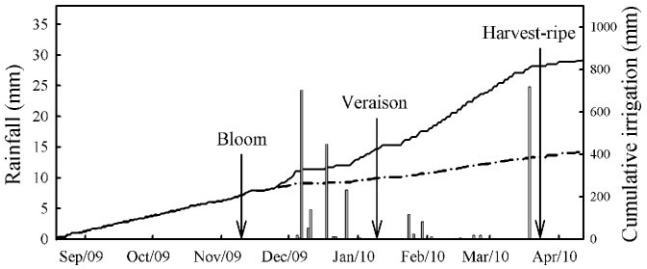

Figure 1. Seasonal evolution of rainfall (▩) and cumulative irrigation applied to full (FI) (—) and deficit (DI) (–•–) irrigated *Vitis vinifera* cv. Malbec grapevines from budburst to harvest during the season 2009/10, Luján de Cuyo, Mendoza.

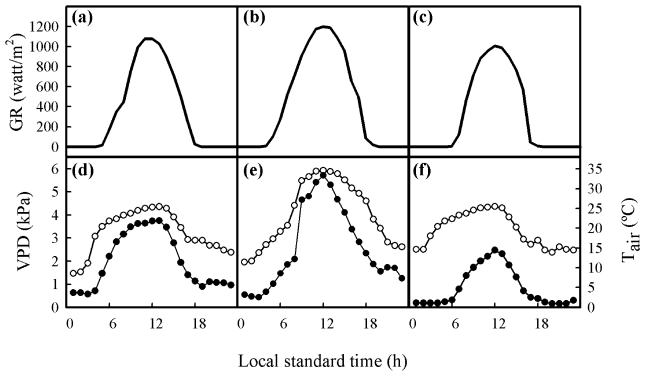

Figure 2. Hourly radiation (GR) (watt/m^2) (—), air temperature (T_{air}) (○), vapour pressure deficit (VPD) (●) and total reference evapotranspiration (ETo) during the days measurements were taken at (a,d) anthesis [day of the year (DOY) 315, 2009, ETo 5.4 mm], (b,e) veraison (DOY 20, 2010, ETo 6.8 mm) and (c,f) harvest (DOY 82, 2010, ETo 3.7 mm) in *Vitis vinifera* cv. Malbec grapevines.

Leaf water potential and gas exchange measurements

Diurnal patterns of Ψ_L differed between vines once irrigation treatment was established (Figure 3b,c), but no difference between treatments was observed at anthesis (DOY 315, Figure 3a). At veraison (DOY 20), DI-3 presented Ψ_L values 40% lower than those of FI, with a minimum of −1.7 MPa at 1230 h. The difference between irrigation levels was still present at harvest (DOY 82), although DI-3 vines showed Ψ_L values higher than those recorded at veraison, indicating that they were less water stressed. No interaction was found between irrigation and crop load levels for Ψ_L. Moreover, Ψ_L was not affected by the crop load treatment (data not shown).

At anthesis, before the irrigation treatment was established, stomatal conductance and rates of net CO_2 and H_2O exchange were similar among all vines (Figure 3d,g,j). At this stage, maximum P_n [10.3 µmol CO_2/(m^2 · s)] was reached at 0800 h and declined to 7 µmol CO_2/(m^2 · s) around 1500 h in the afternoon. Stomatal conductance also attained its maximum value at 0800 h [235 mmol/(m^2 · s)], while E increased from early morning to 1300 h. Since measurements were performed only on east-exposed leaves, net CO_2 exchange declined to zero after midday in all developmental stages.

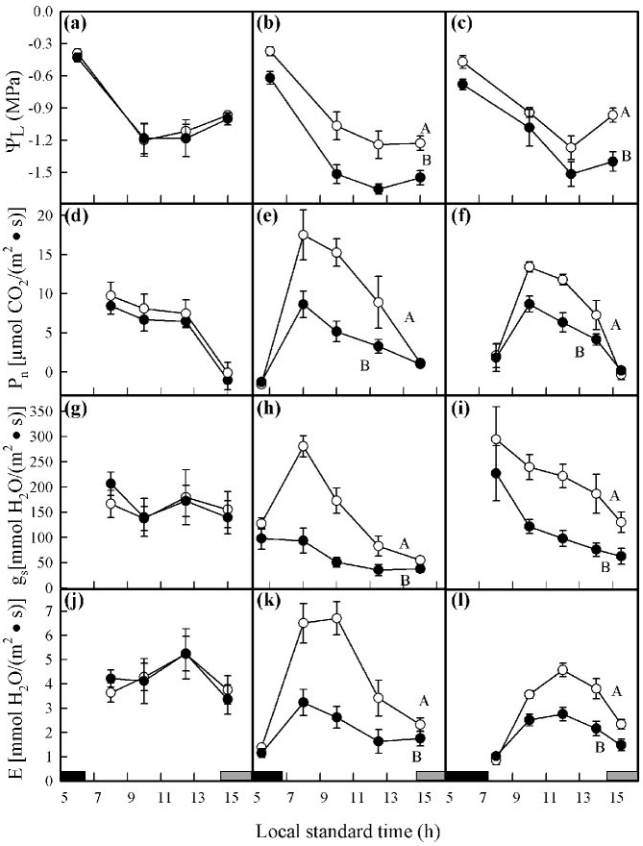

Figure 3. Diurnal evolution of (a,b,c) leaf water potential (Ψ_L), (d,e,f) net photosynthesis (P_n), (g,h,i) stomatal conductance (g_s) and (j,k,l) transpiration (E) of *Vitis vinifera* cv. Malbec grapevines irrigated at 100% (fully irrigated) (○) and 25% (deficit irrigated) (●) of reference evapotranspiration registered at (a,d,g,j) anthesis, (b,e,h,k) veraison and (c,f,i,l) harvest during the 2009/10 season. Black and grey bars in the x axis indicate night and shade conditions, respectively. Data points represent mean values ± confidence interval at $P = 0.05$ ($n = 6$). Different capital letters indicate a significant difference between treatments at $P \leq 0.05$ by Hotelling's test.

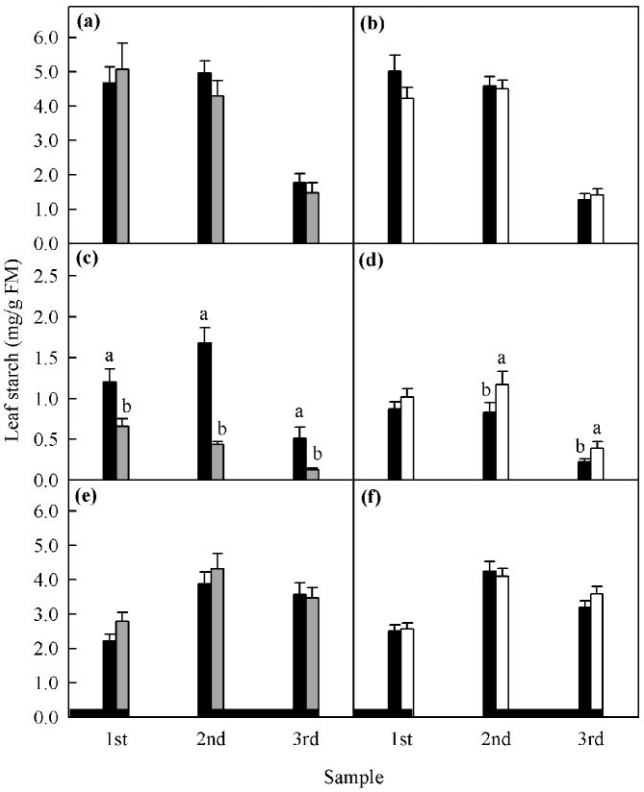

Figure 4. Day–night evolution of leaf starch concentration in *Vitis vinifera* cv. Malbec grapevines (a,c,e) irrigated at 100% (fully irrigated) (■) and 25% (deficit irrigated-3) (▩) of reference evapotranspiration carrying (b,d,f) high (HC) (■) or low (LC) (□) crop loads at (a,b) anthesis, (c,d) veraison and (e,f) harvest during 2009/10 season. Black bars in the x axis indicate night hours. The 1st sample and 2nd sample were taken before sunrise and after sunset of the same day, the 3rd sample before sunrise of the following day. Different small letters indicate a significant difference between treatments at $P \leq 0.05$ by Fisher's LSD test.

At veraison, we observed a significant difference in Ψ_L, P_n, g_s and E between treatments (Figure 3e,h,k). Deficit irrigated vines had a lower rate of P_n (50% less throughout the day) than that of FI vines (Figure 3e), which were mainly associated with lower stomatal conductance (Figure 3h). Maximum rate of P_n was attained at 0800 h for both irrigation levels, 17 and 9 μmol CO_2/($m^2 \cdot s$) for FI and DI-3, respectively, and decreased steadily thereafter. Fully irrigated vines presented a higher transpiration rate during the measurement period than DI-3 vines (Figure 3k). At harvest, gas exchange rates declined from 1000 h until 1500 h, when leaves were no longer exposed directly to the sun (Figure 3f,i,l). Vines under deficit irrigation presented a lower rate of P_n, g_s and E than that of FI vines during almost all measurement time-points. Photosynthesis in FI vines at harvest was lower than that observed at veraison. Similarly to Ψ_L, no difference between crop load treatments was found for CO_2 and H_2O exchange at any date of measurements (data not shown), and no interactions (irrigation x crop load) were found for gas exchange measurements.

Leaf non-structural carbohydrates

Diurnal dynamics of soluble (sucrose, glucose and fructose) and insoluble (starch) non-structural carbohydrates in the leaves were related with leaf net photosynthesis and phenological stage. At anthesis, no difference between treatments was observed in coincidence with Ψ_L and P_n measurements. The concentration of soluble sugars at anthesis ranged from 30 to 40 mg/g fresh mass (FM), while starch concentration ranged from 1.5 to 5.0 mg/g FM (Figures 4 and 5a,b). Leaf starch concentration in all treatments was 70% lower at the second sampling day before sunrise than that of the previous day after sunset. At veraison, starch was significantly reduced in DI-3 vines at the three sampling times (Figure 4c). Starch concentration was correlated with the minimum Ψ_L and maximum leaf P_n measured the same day (Figure 6a,b). Moreover, leaf starch turnover during veraison was significantly lower in DI-3 vines (Table 1) and was also correlated with maximum leaf P_n (Figure 7). At the same time, DI-3 vines presented a higher concentration of total soluble sugars than that of well irrigated vines in both predawn samplings (first and third samples, Figure 5c). Crop load affected starch accumulation; HC vines had a lower starch concentration than that of the LC vines, but no difference was found for total soluble sugars (Figures 4d, 5d). At harvest, no significant difference was found in carbohydrate concentration at any sampling time between irrigation or crop load levels. Starch concentration ranged from 2.2 to 5.0 mg/g FM and night starch utilisation was similar in all vines (Figure 4e,f, Table 1). Similarly, at this stage we found no difference in total soluble sugars among treatments at any time during the day samples were collected (Figure 5e,f).

Discussion

Grapevine leaf carbohydrate dynamics were studied during 2009/10 growing season on field-grown Malbec grapevines (the last growing season of an experiment commenced in 2006).

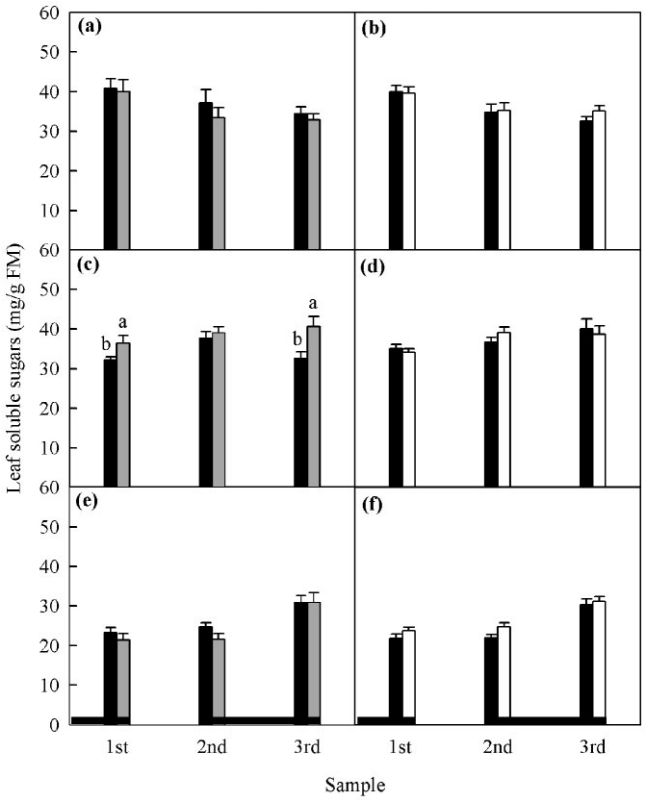

Figure 5. Day–night evolution of the concentration of leaf total soluble sugars in *Vitis vinifera* cv. Malbec grapevines (a,c,e) irrigated at 100% (FI) (■) and 25% (DI-3) (▩) of reference evapotranspiration and carrying (b,d,f) high (HC) (■) or low (LC) (□) crop loads at (a,b) anthesis, (c,d) veraison and (e,f) harvest during 2009/10 season. Black bars in the x axis indicate night hours. The 1st sample and 2nd sample were taken before sunrise and after sunset of the same day, the 3rd sample before sunrise of the following day. Different small letters indicate a significant difference between treatments at $P \leq 0.05$ by Fisher's LSD test.

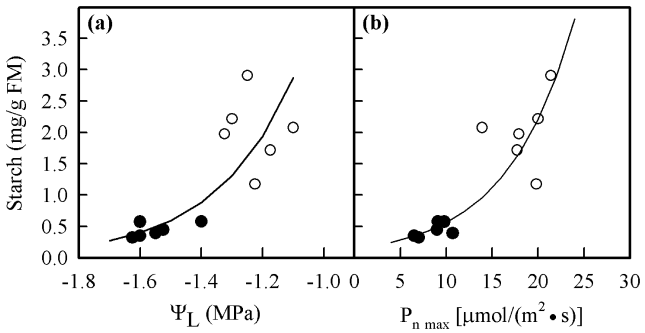

Figure 6. Response of leaf starch concentration to (a) minimum leaf water potential (Ψ_L; $r^2 = 0.78$, $P \leq 0.0001$) and (b) maximum net photosynthesis ($P_{n\,max}$; $r^2 = 0.83$, $P \leq 0.0001$) registered at veraison in *Vitis vinifera* cv. Malbec grapevines irrigated at 100% (○) and 25% (●) of reference evapotranspiration. Each point is a mean of two leaves per replicate.

During this growing season, we measured some physiological variables on single leaves one day during anthesis, veraison and harvest. We found that the irrigation and the crop load treatments affected leaf carbohydrate metabolism independently. Water deficit affected diurnal dynamics of leaf water potential, gas-exchange and non-structural carbohydrates, while crop load affected only leaf starch concentration at veraison.

Cumulative irrigation water received by the experimental vines at anthesis was around 200 mm; by veraison, FI and DI-3 vines had received 420 mm and 300 mm, respectively, and by harvest FI and DI-3 vines had received 800 mm and 400 mm, respectively. This difference affected predawn and Ψ_L accordingly. During anthesis, before irrigation and crop load treatments were applied, no difference was found between vines coming from FI and DI-3 treatments from previous seasons in Ψ_L, photosynthesis, stomatal conductance nor transpiration. This might suggest that in our experiment, treatments imposed during the three previous growing seasons did not affect any of these variables measured at anthesis. During these anthesis measurements, average midday Ψ_L was about –1.2 MPa, which has not been considered a high level of water stress (Schultz and Matthews 1988, Choné et al. 2001, Williams and Araujo 2002, Glenn et al. 2010, Romero et al. 2010). If conditions at this phenological stage would have been more stressful in terms of evapotranspiration demand, we might have found some difference between FI and DI-3 vines in these variables. It has been shown that previous plant water status conditions could modify some of the variables related to plant hydraulic architecture, such as shoot hydraulic conductivity, vessel transactional area and vessel diameter distribution (Lovisolo and Schubert 1998). Water deficit applied to our experimental vines during previous seasons reduced other features, such as trunk starch concentration and the number of flowers per inflorescence (Dayer et al. 2013).

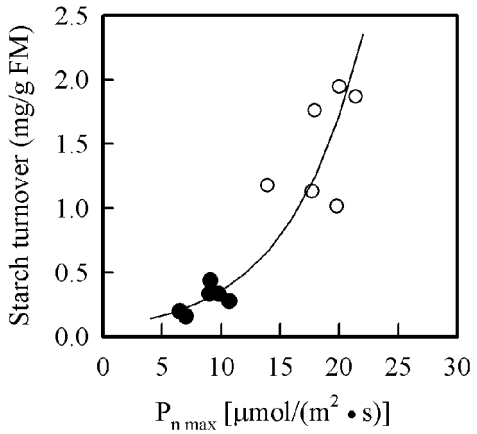

Figure 7. Response of starch turnover to maximum net photosynthesis ($P_{n\,max}$; $r^2 = 0.88$, $P \leq 0.0001$) registered at veraison in *Vitis vinifera* cv. Malbec grapevines irrigated at 100% (○) and 25% (●) of reference evapotranspiration. Each point is a mean of two leaves per replicate.

Table 1. Night starch consumption in *Vitis vinifera* cv. Malbec leaves at anthesis, veraison and harvest during season 2009/10, Mendoza, Argentina.

Treatment	Night starch consumption (mg/g FM)		
	Anthesis†	Veraison‡	Harvest§
Irrigation	NS	*	NS
Fully irrigated (FI)	3.21 ± 0.79	1.17 ± 0.18a	0.30 ± 0.50
Deficit irrigated (DI-3)	2.82 ± 1.03	0.31 ± 0.03b	0.85 ± 0.55
Crop load	NS	NS	NS
High crop (HC)	3.35 ± 0.97	0.61 ± 0.10	1.07 ± 0.27
Low crop (LC)	3.13 ± 1.08	0.79 ± 0.15	0.53 ± 0.33
Interaction¶	NS	NS	NS

*, significant at $P \leq 0.05$. †Day of the year (DOY) 315; ‡DOY 20; §DOY 82. ¶Irrigation × crop-load interaction. Means within columns followed by different letters differ significantly at $P \leq 0.05$ by Fisher's multiple range tests. NS, not significant.

Root biomass is also affected by deficit irrigation strategies. More than 40% reduction in root biomass in field-grown vines has been found between vines under prolonged water deficit compared to those well irrigated (Edwards and Clingeleffer 2013). In our experiment, DI-3 vines were under this irrigation strategy for three previous growing seasons and might have had a root biomass smaller than FI vines.

During veraison (irrigation treatment started at fruitset) weather conditions of high evaporative demand prevailed (high VPD and temperature) and a significant difference of Ψ_{PD} and Ψ_L between FI and DI-3 vines was observed. Leaf CO_2 assimilation and transpiration rates were 50% lower in DI-3 vines compared to that of the FI vines, and showed a gradual decline during the day until the afternoon. This difference in gas exchange was caused by stomatal closure, as evidenced by the stomatal conductance that reached in DI-3 vines values as low as 25 mmol/($m^2 \cdot s$). At these low values of stomatal conductance, other metabolic processes, such as the electron transport rate or the apparent carboxylation efficiency, may also be downregulated contributing to some extent to the reduction in photosynthesis (Medrano et al. 2002, Bota et al. 2004). Reduction in stomatal conductance has been shown to result from hydraulic and chemical signals (Lovisolo et al. 2010). Reduction of vessel areas and increase in small-diameter vessel frequency in grapevines under water stress have been related with a lower value of shoot hydraulic conductivity (Lovisolo and Schubert 1998). Our experimental vines were under different irrigation strategies during the current and the three previous growing seasons and, although not measured in this study, DI-3 vines might have had lower hydraulic conductivity and smaller vessel diameters than that of the FI vines.

At harvest, more subtle differences between treatments were found than those observed at veraison, especially during the day. This could be explained first, by the environmental conditions registered at harvest that were less demanding (lower VPD and temperature) than at veraison, and second, because a rainfall of 25 mm occurred 3 days before measurements were taken. In spite of this, net photosynthesis and transpiration rate were significantly reduced on DI-3 vines, although no difference was found in leaf soluble sugars.

At anthesis, accumulation of leaf carbohydrates was not affected by the treatments. At this stage, accumulation of leaf carbohydrates does not rely on supply of trunk reserves anymore but on current photosynthesis. As we stated before, photosynthesis was not affected by irrigation treatments imposed during the three previous seasons and therefore a similar response in accumulation of carbohydrates was expected.

Accumulation of leaf carbohydrates at veraison, mainly starch, was impaired on DI-3 vines because of the reduction of net CO_2 assimilation. Previous research on grapevine had also shown a reduction in total non-structural carbohydrates because of lower leaf net CO_2 assimilation (Chaves 1991, Rodrigues et al. 1993, Chaumont et al. 1994) with its magnitude depending on the severity of the water deficit imposed (Patakas and Noitsakis 2001, Patakas et al. 2002). Results obtained by other authors may also differ depending on the timing and mode of imposition of the water deficit (Hummel et al. 2010). In contrast to many other studies concerning the response of carbon balance to water stress in potted or greenhouse vines (Düring 1984, Quick et al. 1992) or other species (Wang and Stutte 1992, Hummel et al. 2010), our study was carried out under field conditions, where water deficit was gradually imposed during the season. Also at veraison, we observed that leaf soluble sugars increased in DI-3 vines in parallel to a decrease in starch even though the concentration of total carbohydrates (soluble sugars + starch) was not different between both irrigation levels. Accumulation of leaf soluble sugars during water stress contributes to plant functioning by maintaining cell turgor by osmotic adjustment (Düring 1984, Wardlaw 1990, Chaves 1991, Wang and Stutte 1992, Clifford et al. 1998). In general, plants accumulate inorganic ions, such as potassium, organic acids or proline, in order to decrease the osmotic potential to maintain cell turgor above a critical level for cellular expansion (Patakas and Noitsakis 2001). The accumulation of sugars for active osmotic adjustment is a mechanism that represents a cost for the plant in terms of fixed carbon (Patakas and Noitsakis 2001). This accumulation of soluble sugars under water stress, instead of starch synthesis, is generated by a change in the allocation pattern of recently fixed carbon (Vassey and Sharkey 1989, Quick et al. 1992). Even when the water deficit reduces the rate of CO_2 assimilation, a priority is given to the synthesis of soluble sugars in the cytosol in order to maintain the plant metabolic activity (Quick et al. 1992). When discussing carbohydrate concentration, it is important to consider vine size to avoid misleading conclusions. Total vine leaf area was greater in FI than in DI-3 vines (Dayer et al. 2013). Also pruning mass was greater in FI than in DI-3 vines: 0.77 and 0.93 kg/plant in FI vines in winters 2009 and 2010, respectively, and 0.61 and 0.69 kg/plant for DI-3 vines the same years (Dayer et al. 2013). This suggests that although total leaf carbohydrate concentration was similar for both irrigation treatments, when considering vine size (e.g. total leaf area and pruning mass), FI vines showed higher carbohydrate content per plant. Another difference in carbohydrate content could have been found in roots because of differences in carbon concentration and root size (Holzapfel and Smith 2012, Edwards and Clingeleffer 2013), but this was not measured in our experiment. Furthermore, in this study, we measured only on east-exposed leaves. A complete study with east and west-exposed leaves should be undertaken in order to cover the difference in their environmental growing conditions (mainly VPD and temperature).

Coincident with the other variables measured at anthesis, when treatments were not imposed yet, starch turnover was similar for all vines. A difference in starch turnover at this stage would have been an effect of previous years. Similarly, leaf starch concentration observed between the first and the second sample does not mean that there was no starch accumulation during that day. Leaves were exposed to radiation until midday, but later they remained in shade until the second sample (2100 h).

At veraison, deficit-irrigated vines showed less starch turnover, indicating that leaf starch consumed during the night by DI-3 vines was lower than that in FI vines. This was probably because of a lower respiration rate, more delayed metabolism (Azcón-Bieto and Osmond 1983) and less sink strength given by a reduction in plant growth in DI-3 vines. It is known that vegetative growth is the first function impaired by water deficit (Schultz and Matthews 1988, Hsiao 1993, Keller et al. 2008). We found that, irrespective of the irrigation level, total starch concentration was not completely depleted during the night, suggesting that leaf carbohydrate production during the day matched with night consumption or export out of the leaf (Graf et al. 2010). These results are in line with those obtained by Hummel et al. (2010) in Arabidopsis where water deficit did not lead to complete carbon depletion. We found a close relationship between leaf maximum photosynthesis during the day and starch turnover the following night, which also suggests that an internal regulation system exists that allows the fine tuning of

the night consumption with the daily carbon assimilated (Stitt and Zeeman 2012). Also in Arabidopsis, it has been suggested that starch turnover is set by the circadian clock, which 'measures' (Graf and Smith 2011, Stitt and Zeeman 2012) the amount of starch in the leaf at the end of the day in order to maintain a constant supply of carbon coming from starch degradation throughout the night (Gordon 1986, Smith and Stitt 2007). Other mechanisms besides the circadian clock, however, could regulate starch mobilisation. It was shown that interrupting sugar export from leaves, or that feeding sugars to leaves in the dark, slowed starch breakdown (Stitt and Zeeman 2012). Although we observed this trend at veraison on both irrigation levels, starch turnover on leaves of FI vines was greater than those of DI–3 vines. This is coincident with more demanding growing sinks in FI vines, especially at this phenological stage when grapes start to accumulate sugar and become a priority sink for carbohydrates (Schurr et al. 2006).

During harvest measurements, no difference in leaf carbohydrate concentration between irrigation levels was found. Similarly, starch turnover did not show any difference between FI and DI-3 vines. Few studies have suggested that phloem transport and consequently carbohydrate translocation are less affected by deficit irrigation than carbon assimilation (Bota et al. 2004). This was the case in our study at harvest. It is interesting to note that in spite of the difference in the amount of water received during the whole season and that observed in gas exchange, no difference was found in carbohydrate concentration and starch turnover. One possible explanation is that the lower night temperature at this time of the season reduced substrate respiration and thus hid any possible effect on the concentration of carbohydrates (Azcón-Bieto and Osmond 1983). Gas exchange was shown to be more sensitive to water deficit than carbohydrate accumulation and turnover, which are not easily modified (Bota et al. 2004). Another possible explanation may be related to changes in the source–sink balance throughout the season. Close to harvest berries are almost functionally disconnected from the vine, and the plant has less active sinks and a lower rate of sugar exportation out of the leaf (Hunter et al. 1995). Consequently, higher starch concentration may be found at the end of the night (Hunter et al. 1995) which contrasts with that observed at veraison when starch was almost totally consumed before dawn. Even if this is a limited study, it is worth noting the importance of field experiments to understand better the vine metabolic and physiological responses to vineyard management practices, for example irrigation and crop load.

No interaction was found between irrigation and crop load for leaf CO_2 assimilation; crop load did not affect leaf CO_2 assimilation but affected leaf starch concentration at veraison. Total vine leaf area developed by HC and LC vines was 5.65 and 6.23 m^2/vine, respectively, while their yield was 5.30 and 3.01 kg/vine, respectively (Dayer et al. 2013). According to these values, leaf area to fruit ratio in HC and LC vines was 10 and 21 cm^2/g, respectively; they developed enough canopy leaf area to ripen the fruit (Kliewer and Dokoozlian 2005), and therefore we did not expect to find a source limitation. Most likely, a feedback limitation of photosynthesis would have occurred through triose phosphate accumulation in the chloroplast (Azcón-Bieto 1983). Differences found in starch concentration between HC and LC vines were maintained the next morning, suggesting similar starch turnover. Considering that leaf net photosynthesis was similar between both crop loads, the source–sink ratio may have changed the carbohydrate partitioning pattern. Thinned vines had less fruit sinks for photoassimilates than non-thinned vines, and consequently, carbohydrates accumulated in their leaves as starch. This transitory starch accumulation instead of sucrose export might also be considered a result of a limited transport capacity and indicates that starch synthesis proceeds independently of sink demand for sucrose (Hunter et al. 1995). In contrast to these results, a different response was found at harvest. Even when photosynthesis was similar between crop load levels, crop load did not affect leaf carbohydrate accumulation or starch utilisation, indicating that transitory pools of starch were constantly filled because of slow turnover despite the different crop loads. As stated before, at harvest, grapes are at maturity and other plant organs (i.e. permanent structures) become sinks for carbohydrates. Previous studies in potted vines have shown that vegetative sinks can create a large demand for carbohydrates in the absence of fruiting sinks (Edson et al. 1993). In an extensive study on the influence of crop load on photosynthesis and dry matter partitioning, these authors observed that grapevines represent a balanced system, responding to changes in sink demand by assimilate allocation to meet reproduction or survival carbon demands. These observations match our previous results in the same experimental vines (Dayer et al. 2013), in which thinned vines had greater starch concentration in the trunk during the winter than that of those not thinned. As discussed before, it is important to consider vine size to achieve a correct interpretation of carbohydrate metabolism based on single leaf measurements when it is not possible to measure whole plant CO_2 assimilation and/or biomass including roots.

Conclusion

The present study was conducted during the last season of a larger experiment and since measurements were taken only on 3 days during the season, conclusions have to be interpreted with caution. Results show that severe deficit irrigation can affect photosynthesis and leaf carbohydrate accumulation and exportation during a day–night cycle. Differences in plant water status between irrigation levels were greater during conditions of high evaporative demand and were most likely masked when evaporative demand was low. Deficit irrigation caused a significant reduction in leaf CO_2 assimilation, starch concentration and its utilisation during the day–night period (turnover). It also affected the allocation of fixed carbon, shown by an increase in soluble sugars and a reduction in starch accumulation. This indicates that for field-grown grapevines under water deficit, sucrose synthesis is favoured, and this allows vines to maintain or increase the pool of soluble sugars in their leaves. High-crop load reduced leaf starch concentration at veraison when the grape demand for carbohydrates was high. Leaf carbohydrate concentration, however, was similar in both crop load levels as other vegetative organs became more important sinks. Additional knowledge of carbohydrate dynamics at the leaf level is necessary to achieve a better comprehension of viticulture sustainability, yield and fruit composition when common viticultural practices such as deficit irrigation and crop load regulation are used.

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Supporting information

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Figure S1. Malbec vineyard protected with anti-hail net installed in a grembiule system.