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Stem starch reserves studied by on-solid reactions coupled with reflectance detections in water stressed grapevines

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Summary

Wine grape is usually grown under water deficit conditions that could negatively impact plant reserves, including the organic carbon storage in perennial organs such as woody stems. Assessing the carbohydrate status in woody organs is therefore of interest as it can influence vegetative growth in the successive season. In this study, we aimed to apply an on-solid colour reaction (using Lugol's iodine solution) coupled with reflectance spectroscopy detection to assess the grapevine canes' starch accumulation in response to short drought periods. We used two *Vitis vinifera* cultivars ('Cabernet Sauvignon' and 'Syrah') that were subjected to three different water conditions (well-watered; early water stress; late water stress) during the growing season as case study. We sampled woody stem tissue during winter rest. The results showed that water stress reduced the starch storage in 'Syrah', especially when imposed late and recovery time was not enough for carbon restoration, while 'Cabernet Sauvignon' was not affected. The results showed that the sensitivity of the method used here is adequate to assess starch accumulation differences due to drought treatments in grapevine canes. Moreover, the analytical approach appears fast, low cost, and promising for future physiological and agronomical research applications.

K e y w o r d s: drought; stain; wood reserves; starch-iodine complexation; water potential; non-structural carbohydrates.

Introduction

Differently from other crops, wine grape composition usually benefits from moderate water stress during ripening, provided that its intensity is not severely restricting leaf gas exchange for long periods (Fereres and Soriano 2007, Chaves *et al.* 2010). In fact, a number of studies have demonstrated positive effects of controlled water stress management on the ripening process and final composition of grapes (Ojeda *et al.* 2002, Sivilotti *et al.* 2005, Castellarin *et al.* 2007, Van Leeuwen *et al.* 2009, Bucchetti *et al.* 2011, Shellie 2014, Herrera and Castellarin 2016) and derived wines (Chapman *et al.* 2005, Ou *et al.* 2010, Herrera *et al.* 2015). However, water stress also affects other physiological aspects of the plant such as carbon assimilation and partitioning (Chaves *et al.* 2010). Water stress can modify the vine source/sink balance by reducing the gas exchange through the closure of stomata (Müller *et al.* 2011) and leaf abscission (Hochberg *et al.* 2017), leading to a reduced allocation of carbon in woody organs as reserves (Dayer *et al.* 2013, Herrera *et al.* 2015).

In perennial plants such as grapevines, the growth resumption after the winter rest is only sustained by the wood organic and mineral reserves (Holzapfel and Smith 2012). Even though different compounds could represent sources of stored chemical energy and carbon, carbohydrates (especially starch) are considered the main reserves in plants (Dietze *et al.* 2014)

Different chemical and microscopy methods could be used to characterize woody tissues (Marts 1950, Rustioni *et al.* 2016, De Bei *et al.* 2017). Recently Rustioni *et al.* (2017) proposed a new indirect index for the assessment of starch in grapevine canes based on on-solid colour reactions (starch staining) coupled by reflectance spectroscopy detection. In particular, the method is based on the well-known starch-iodine complexation reaction using the Lugol's iodine solution, commonly adopted in microscopy to highlight starch accumulation. The intensity of the purple coloration obtained by the starch-iodine complexation can be quantifiable by reflectance spectroscopy and used to measure starch accumulation in woody tissues (Rustioni *et al.* 2017).

Here we took advantage of an experiment carried out during the season 2016 aiming at the characterization of short but severe water stress applied in different periods during the growing season on two grapevine varieties ('Syrah' and 'Cabernet Sauvignon'). In winter time, we sampled woody stems from vines subjected to the different treatments with the aim of testing the applicability and sensitivity of the starch-derived index determined by on-solid reaction and reflectance spectroscopy of the assessment of eventual differences in the starch accumulation as affected by water stress.

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Material and Methods

Plant material and experimental conditions: the experiment took place at the University of Udine experimental station "A. Servadei", located in the Friuli Venezia Giulia Region (north-eastern Italy; 46°02′ N, 13°13′ E; 88 m a.s.l.). 40 cuttings of *Vitis vinifera* 'Cabernet Sauvignon' and 40 cuttings of *Vitis vinifera* 'Syrah', both grafted on SO4 rootstock, were planted in March 2016 in 7 L pots filled with commercial potting media (Gebr. Brill Substrate Type 1, Georgsdorf, Germany) supplemented with 20 % perlite. Budbreak occurred on April 10th, 2016, and only one shoot per plant was left to develop vertically. For each cultivar, pots were arranged in 4 plots of 10 vines each in a fully randomized scheme encompassing 2 rows. No hedging was performed to the shoots and when the shoot length surpassed the height of the trellising system (2.1 m) the shoot was positioned horizontally in the last catching wire. To fully control irrigation and to avoid precipitation, pots were positioned under a covering structure described in Herrera *et al.* (2015).

Water was supplied by a drip irrigation system with one emitter per pot (PCJ 2 $L \cdot h^{-1}$, Netafim, Israel). All vines were daily irrigated twice (at 14.00 and at 23.00 h) to saturation (well-watered; WW) until the imposition of water stress. Sustained water stress (WS) condition consisted of withholding irrigation completely for a period of 12 d; after that period irrigation was restored to saturation (*i.e.* as in WW) until autumn leaf-fall (November 1st, 2016). Water stress was imposed on two different periods: (i) early WS, imposed in July 18th and (ii) late WS, imposed in September 12th. For each water stress treatment, a group of randomly selected 10 vines for each cultivar was used, so the plants experienced water stress only in one short period of their lives. On the other hand, the remaining 20 vines per cultivar served as WW control treatment and were randomly divided in two groups of 10 vines each used as control during early and late WS, respectively.

Leaf area (LA) was assessed before the beginning of each trial to ensure similar plant size among the treatments. LA was calculated by measuring the leaf length of all the leaves in the shoots and using a regression between leaf length and leaf area previously determined using a leaf area meter (LI-3100C, LiCor, Inc., NE, USA).

Stem water potential (Ψ_S), stomatal conductance (g_s) and net assimilation (A_N) where measured at midday and during clear sunny days, every 2-3 days during the periods of water stress. For the determination of stem water potential (Ψ_S), young fully expanded leaves (mid position on the shoot) were bagged and covered with aluminium foil 1 h before the measurement, and then excised with a razor blade. Then the leaves were placed in a pressure chamber (Soil Moisture Co., Santa Barbara, CA, USA) and the stem water potential assessed within a few seconds. At each sampling point, one leaf of three different plants per treatment was used and no more than 2 leaves per vine were used during the whole period of WS. Stomatal conductance and net assimilation were measured with an infrared gas analyser (LI-6400, LiCor, Inc., NE, USA), using a constant light intensity (1000 µmol m^{-2} s^{-1}) and CO_2 concentration (400 µmol mol^{-1}).

Wood sampling and spectroscopy analyses: Samples of cane wood were collected from WW, early WS, and late WS vines during dormancy (January 2017). In details, the canes of three randomly selected vines from each combination (cultivar x treatment) were sampled at different positions. For each cane, the 4th, the 9th and the 14th internode were selected. 10 sections/internode were obtained by using a penknife and separately analysed. A total of 540 sections (54 internodes) were considered.

Analyses were carried out by using a Jaz System spectrometer (Ocean Optics, B.V., Dunedin, USA), completed with a channel with a DPU module and an ILX511b detector, an OFLV-3 filter, an L2 lens, and a 50 µm slit as installed options. A reflection probe QR600-7-VIS125 consisting of a tight bundle of seven optical fibers (600 µm in diameter), in a stainless-steel ferrule (six illumination fibers around one read fiber), was coupled to the spectrophotometer. A probe holder was included to ensure the analytical reproducibility: the distance between the sample surface and the probe was fixed at 12 mm. The instrument was set up with a near infrared-visible (NIR-vis) light source (Ocean Optics) 4095 power setting and an integration time automatically corrected by the instrument. Collected spectra ranged between 341 and 1025 nm and had a spectral resolution of about 0.3 nm. Each spectrum was set up to be the average of 15 spectra, which were directly calculated by the instrument. The spectra were converted into percentage of reflectance after a calibration with a blank, obtained by using a polytetrafluoroethylene (PTFE) diffuse reflectance standard (Ocean Optics B.V.).

Reflectance spectroscopy analyses were carried out considering the xylem region. For each internode considered, 5 woody slides were obtained by a penknife. First, t0 spectra were collected on raw tissues. Then, the slides were used for the starch-iodine complexation: 3 min reaction with a drop of Lugol solution [10 $g \cdot L^{-1}$ potassium iodide and 2.5 $g \cdot L^{-1}$ iodine in water] according to Rustioni *et al.* (2017). A total of 540 spectra were recorded. The index was calculated following Rustioni *et al.* (2017) by the formula:

$$INDEX_{\text{Starch-Iodine Complex}} = \frac{R_{IC(900)}}{R_{IC(555)}} - \frac{R_{t0(900)}}{R_{t0(555)}}$$

where $R_{x(n)}$ corresponds to the reflectance value of the x spectrum ($t0$ = raw tissue; IC = iodine complexed) at the n wavelength. These formulas are representative of the absorption intensities (estimated by the reflectance decrease) of the specific on-solid reactions considered. In the $INDEX_{\text{Starch-Iodine Complex}}$, the Iodine complexation with starch produces a characteristic purple colour.

Statistical analysis: The obtained values were elaborated and analysed by using Microsoft Office Excel and SPSS statistical software (version PASW Statistics 24, SPSS, Inc. Chicago, IL). Data were analysed by three-way ANOVA with cultivar, treatment, and internode position as fixed factors. All interaction between factors were computed (Tab. 1), and when significant, means were separated with Tukey's HSD test ($p < 0.05$). Physiological data of stem water potential (Ψ_S), stomatal conductance (g_s), and net assimilation (A_N) in WW and early/late WS were compared using t-test ($p < 0.05$). The Figure was constructed using SigmaPlot 13 (Systat Software GmbH, Erkrath, Germany).

Table 1

The calculated $INDEX_{Starch\text{-}Iodine\ Complex}$ in 'Cabernet Sauvignon' and 'Syrah' grapevines under well-watered (WW), early and late water stress (WS) conditions. The analysis was performed on dormant canes at the 4th, 9th and 14th node position

Factors of variability			INDEX-Starch-Iodine Complex
Cultivar (C)	Cabernet Sauvignon		2.06
	Syrah		1.99
		sign. F	n.s.
Treatment (T)	control WW		2,08 a
	early WS		2,13 a
	late WS		1,88 b
		sign. F	**
Internode (I)	4th		2.07
	9th		2.04
	14th		1.97
		sign. F	n.s.
Interactions	C x T	sign. F	*
	C x I	sign. F	n.s.
	T x I	sign. F	n.s.
	C x T x I	sign. F	n.s.

The difference between cultivars, treatments and interaction between factors was assessed with a two-way ANOVA. The level of significance is reported within the columns: *, **, or n.s., significant at $P < 0.05$, 0.01 or not significant, respectively.

Results and Discussion

A full factorial analysis was carried out concerning the calculated $INDEX_{Starch\text{-}Iodine\ Complex}$ considering the two cultivars, the irrigation treatments, the internode positions, and the interactions among these factors (Tab. 1). The availability of a rapid and low-cost method allowed to easily obtain a high number of replications and to describe a biological variability otherwise difficult to highlight. It is important to note that each data (540 analysed samples) were calculated as the average of 15 records (analytical replications) and all the starch analyses were carried out within few days of measurements. Furthermore, the use of a starch-specific stain (Lugol's iodine solution) allowed to quantify the stored carbon reserves by a direct and specific measurement, differently from other optical methods commonly based on the identification of wavelengths of interest by statistical and indirect correlations (not necessarily representative of the target compounds).

Only the irrigation treatment and the interaction between the cultivar and the irrigation treatment were significant (Tab. 1). Furthermore, only the late WS resulted in a significant reduction in the wood starch index (Tab. 1). The results are in general not surprising and in line with expected results and hypothesis. The water stress limited the vine carbon assimilation by restricting leaf photoassimilation in accordance with previous research (Escalona *et al.* 1999, Hochberg *et al.* 2016, Lavoie-Lamoureux *et al.* 2017) impacting the carbon storage in woody organs (Dayer *et al.* 2013, Herrera *et al.* 2015, Bianchi *et al.* 2018). In particular, during the early water stress period, stomatal conductance (g_s) was severely reduced by WS to 0.012 and 0.016 $mol \cdot m^{-2} \cdot s^{-1}$ in 'Cabernet Sauvignon' and 'Syrah', respectively, while WW vines maintained g_s values above 0.250 $mol \cdot m^{-2} \cdot s^{-1}$ in both cultivars (Tab. 2); accordingly, net carbon assimilation (A_N) reached values close to zero in WS, while was maintained above 11 $mol \cdot m^{-2} \cdot s^{-1}$ in WW. Moreover, it has been shown that embolism (most probably a certain degree occurred here given the low water potential reached for several days) recovery involves the carbohydrate consumption (Savi *et al.* 2016, Klein *et al.* 2018). However, the early water stress imposition was followed by full irrigation, which resulted in fully restored gas exchange and photosynthesis activity similar to WW vines (data not shown) that, given the long period of recovery before leaf fall (ca. 90 d after rehydration), most probably allowed the plants to replenish wood carbon reserves. On the other hand, late water stress imposition determined a strong reduction in leaf photoassimilation (g_s values of 0.097 and 0.051 $mol \cdot m^{-2} \cdot s^{-1}$ in 'Cabernet Sauvignon' and 'Syrah', respectively; A_N values of 5.86 and 3.07 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in 'Cabernet Sauvignon' and 'Syrah', respectively; Tab. 2) that probably forced the vines to use part of the carbon storage pool to sustain metabolism (Rossouw *et al.* 2017), embolism recovery (Trifilò *et al.* 2017), while the time after rehydration (30 d) was not enough to reach starch reserves values similar to fully irrigated plants. Another parameter that should be considered is leaf area; at the beginning of early WS, leaf area was quite low (0.15 and 0.17 $m^2 \cdot vine^{-1}$ in 'Cabernet Sauvignon' and 'Syrah', respectively), while at the beginning of late WS was much higher (0.49 and 0.34 $m^2 \cdot$vine in 'Cabernet

Table 2

Average values of stomatal conductance (g_s), net assimilation (A_N), and stem water potential (Ψ_S), measured during the two water stress periods (early, *i.e.* end of July; late, *i.e.* September) in 'Cabernet Sauvignon' and 'Syrah' grapevines. All measurements were taken between 12.00 and 13.00 h on 9 vines per treatment. WW, well-watered; WS, water stress

		g_s (mol H_2O m^{-2} s^{-1})	A_N (µmol CO_2 m^{-2} s^{-1})	Ψ_S (MPa)
Cabernet Sauvignon	WW	0.256	11.7	-0.53
	early WS	0.016	0.10	-1.23
	sign	**	***	**
	WW	0.333	9.86	-0.37
	late WS	0.097	5.86	-1.24
	sign	***	*	*
Syrah	WW	0.287	11.2	-0.55
	early WS	0.012	0.42	-1.33
	sign	***	***	**
	WW	0.239	5.48	-0.6
	late WS	0.051	3.07	-1.43
	sign	***	*n.s.*	*

Data were analyzed by t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

Sauvignon' and 'Syrah', respectively). In case of early WS most of the leaf area developed later did not experience water stress, while at the time of late WS no further increase of leaf area was ascertained. Late WS produced a restriction in photoassimilation in a large amount of leaves and this could have affected the accumulation of starch in the shoots.The response of the two cultivars to the water stress treatment was different as expected (Hochberg *et al.* 2013). The WS treatment did not impact 'Cabernet Sauvignon' starch index, while 'Syrah' exhibited a significant decrease in wood starch content as a consequence of the late WS (Figure). 'Cabernet Sauvignon' is known to be more tolerant to water stress conditions than 'Syrah' (Hochberg *et al.* 2013), a behaviour that might be related to acclimation processes, a greater capacity of osmotic adjustment, and water-stress chemical signalling (Martorell *et al.* 2015, Hochberg *et al.* 2017). These characteristics allowed 'Cabernet Sauvignon' to maintain higher gas exchange than 'Syrah' during the late stress treatment (g_s values of 0.097 and 0.051 mol m^{-2} s^{-1} in 'Cabernet Sauvignon' and 'Syrah', respectively; A_N values of 5.86 and 3.07 µmol·m^{-2}·s^{-1} in 'Cabernet Sauvignon' and 'Syrah', respectively; Tab. 2) as well as slightly higher stem water potential (Ψ_S = -1.24 and -1.43 MPa in 'Cabernet Sauvignon' and 'Syrah', respectively; Tab. 2). Probably, while 'Syrah' was forced to use part of the starch reserve to guarantee carbon resources for metabolism and probably repair from embolism formation, 'Cabernet Sauvignon' managed to cope with the water stress situation without mobilizing much of the allocated starch or if so, the time of recovery was sufficient to replenish it to similar levels of the well-watered control. Moreover, 'Syrah' vines under WS suffered from greater leaf abscission (data not measured) than its 'Cabernet Sauvignon' counterpart, a fact that probably influenced strongly the total carbon assimilation capacity per plant and the final starch stored after re-watering.

Conclusions

In the present experiment, the starch iodine index allowed to highlight the differences between varieties and, in case of 'Syrah', also between treatments. Although the experiment was set up originally for a different purpose, and the parameters measured here don't allow a full explanation of the differences impaired by water stress on the starch measured during winter time, this study permitted to test the applicability of a fast method to determine a spectrophotometrical index related to the starch concentration in woody tissues.The on-solid reaction coupled with reflectance spectroscopy method used here could be potentially used for a rapid assessment of the starch in the canes, and so in more detailed studies regarding carbon partitioning and reserve accumulation, as well as for phenotyping and breeding programs.

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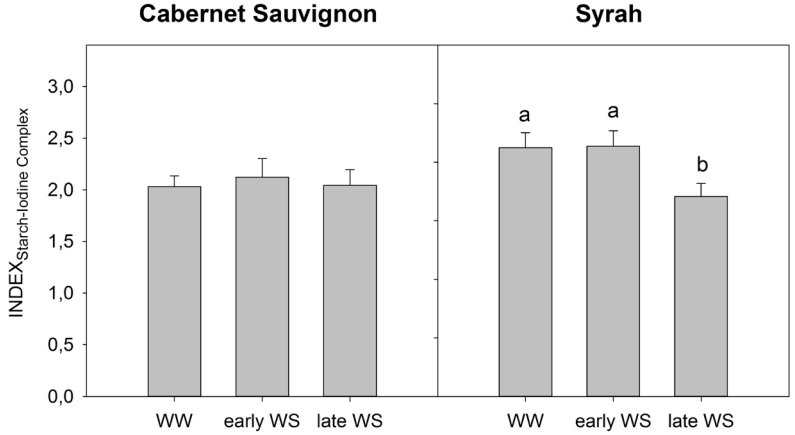

Figure: Water stress effect on the wood starch accumulation expressed as the $INDEX_{Starch\text{-}Iodine\ Complex}$. Average values are presented as the average ± standard error. Within each cultivar, data were processed through one-way ANOVA and when significant the means were separated based on the Tukey's HSD test. Different letters refer to significant differences ($p < 0.05$) between treatments

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