



An increase in xylem embolism resistance of grapevine leaves during the growing season is coordinated with stomatal regulation, turgor loss point and intervessel pit membranes

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Received: *3 August 2020* Accepted: *2 October 2020*

New Phytologist (2020) **doi**: 10.1111/nph.17025

Key words: grapevine, hydraulic plasticity, hydraulic vulnerability, pit membrane, seasonality, stomatal regulation, turgor loss point, *Vitis vinifera*, xylem.

Summary

• Although xylem embolism resistance is traditionally considered as static, we hypothesized that in grapevine (*Vitis vinifera*) leaf xylem becomes more embolism-resistant over the growing season.

• We evaluated xylem architecture, turgor loss point (Ψ_{TLP}) and water potentials leading to 25% of maximal stomatal conductance (g_{s25}) or 50% embolism in the leaf xylem (P_{50}) in three irrigation treatments and at three time points during the growing season, while separating the effects of leaf age and time of season.

• Hydraulic traits acclimated over the growing season in a coordinated manner. Without irrigation, Ψ_{TLP} , g_{s25} , and P_{50} decreased between late May and late August by 0.95, 0.77 and 0.71 MPa, respectively. A seasonal shift in P_{50} occurred even in mature leaves, while irrigation had only a mild effect (< 0.2 MPa) on P_{50} . Vessel size and pit membrane thickness were also seasonally dynamic, providing a plausible explanation for the shift in P_{50} .

• Our findings provide clear evidence that grapevines can modify their hydraulic traits along a growing season to allow lower xylem water potential, without compromising gas exchange, leaf turgor or xylem integrity. Seasonal changes should be considered when modeling ecosystem vulnerability to drought or comparing datasets acquired at different phenological stages.

Introduction

Plants evolved a water transport system that relies on water sustaining a tensile force. From a thermodynamic perspective, water under tension should be vaporized (Or & Tuller, 2002). Still, the steric organization of water molecules and the limited nucleation points inside the xylem are assumed to enable the xylem sap to maintain a liquid metastable state (Dixon & Joly, 1895). These system conditions imply the following problem: when the tension grows beyond a certain threshold, as a result of high water demand and/or low water availability, the xylem sap vaporizes (a process known as xylem cavitation). Cavitation results in the formation of a gas bubble (embolism) inside a xylem conduit, thus breaking its hydraulic continuity. However, the structure of bordered pits restricts the embolism to a single xylem conduit, meaning that a single cavitation event may not spread and therefore is not necessarily catastrophic (Delzon et al., 2010; Johnson et al., 2020). However, if the tension intensifies, the gas could spread into the xylem network, reducing the capacity of the plant to

deliver water to its leaves and can lead to leaf shedding and even death (Tyree *et al.*, 1993; Choat *et al.*, 2018).

To maintain the integrity of their hydraulic system, plants maintain a safety margin, usually defined as values of water potential (Ψ) between complete stomatal closure and 12%, 50% or 88% cavitation (P12, P50, P88; Pammenter & Van der Willigen, 1998; Martin-StPaul et al., 2017). In most - if not all species, the safety margin is positive, meaning that complete stomatal closure precedes any sign of cavitation, as illustrated in Fig. 1 (solid gray and dashed gray lines; Martin-StPaul et al., 2017; Creek et al., 2020). For example, in potted grapevines, a common model plant for hydraulic studies (Brodersen et al., 2013), complete stomatal closure occurred before leaf wilting and 0.3 MPa before the first cavitation event (Hochberg et al., 2017b; Dayer et al., 2020). Differences between these processes were even more remarkable when the timing of stomatal closure and cavitation is considered. Full stomatal closure occurred 3 d before cavitation. Once cavitation had occurred, substantial leaf wilting was observed (Hochberg et al., 2017b). Accordingly,

these authors assumed that stomatal regulation would prevent cavitation in the vineyard under most conditions (including moderate drought stress) and that vines that do not exhibit wilting or leaf shedding did not get to their P_{12} .

In apparent contradiction, the xylem water potentials (Ψ_x) that are frequently measured in vineyards during late summer in the Mediterranean or semiarid regions (Martorell et al., 2015; Munitz et al., 2018; Netzer et al., 2019) are near the reported P₅₀ of grapevine leaves $(-1.9 \le P_{50} \le -1.2 \text{ MPa};$ Charrier *et al.*, 2016; Hochberg et al., 2016a, 2017b; Dayer et al., 2020). Because such vineyards maintain their stomata open $(30 < g_s < 150 \text{ mmol m}^{-2} \text{ s}^{-1})$ and do not show any visible signs of wilting or leaf shedding, we should consider one of two possibilities. The first option is that late in the season grapevines are prone to cavitation (Munitz et al., 2018) and when approaching harvest, grape leaves maintain their stomata open and drive their xylem sap into cavitating water potentials (Ψ_x ; Fig. 1, solid black and dashed gray lines). This could be viewed as a profitable strategy for a plant that desires to maximize its productivity before winter dormancy. The second option, and the one which we advocate for, is that P₅₀ is a plastic trait, shifting in coordination with other hydraulic traits to more negative values as the season progresses (Fig. 1, solid black and dashed black lines).

Evidence for a seasonal shift of hydraulic traits can be found in the turgor loss point (Ψ_{TLP}), known for its seasonal plasticity (Bartlett *et al.*, 2014; Maréchaux *et al.*, 2017; Degu *et al.*, 2019). Owing to the high correlation between stomatal regulation and Ψ_{TLP} (Brodribb *et al.*, 2003; Farrell *et al.*, 2017; Creek *et al.*, 2020), it is probable that the Ψ of stomatal closure will also adjust to lower values during the season. There are even a few studies that supported a seasonal shift in stem (Kolb & Sperry, 1999) or leaf P₅₀ (Martorell *et al.*, 2015), but this possibility has been given little attention. In fact, many studies tried to estimate the amount of xylem embolism by following the seasonal dynamics of Ψ and combining it with a constant xylem vulnerability curve (VC; e.g. Hochberg *et al.*, 2016b). Establishing the seasonal plasticity of plants could resolve this contradiction and open up a new perspective on how we comprehend plants' drought response.

In the current study, we monitored three hydraulic traits (stomatal closure, Ψ_{TLP} and leaf vulnerability to cavitation) throughout a single growing season. We hypothesized that these three hydraulic traits shift in a coordinated way to support turgor maintenance and gas exchange at lower Ψ as the season progresses, but also to maintain a constant safety margin. We applied three irrigation treatments to determine if the plasticity in the traits measured would depend on the water availability conditions. Additionally, to comprehend if the seasonal plasticity in P_{50} is a function of leaf age or the time of season, we compared the young (but fully mature) leaves in late May with both young leaves and old leaves (taken from the same nodal position) at the end of August. Finally, to understand the anatomical differences that are associated with the plasticity of P_{50} , we monitored petiole vessel diameter and pit membrane thickness throughout the growing seasons, which are both important characteristics associated with hydraulic efficiency and drought-induced embolism resistance (Kaack et al., 2019).

Materials and Methods

Experimental setup

The study was conducted in the 2019 growing season in an experimental vineyard (*Vitis vinifera* cv Cabernet Sauvignon) that had several irrigation treatments (as described later) during seven successive years, from 2012 to 2019 (Ohana-Levi *et al.*, 2020).



Fig. 1 Potential seasonality trend of leaf hydraulic traits: stomatal regulation (g_s ; solid line), turgor loss point (TLP; arrow), and xylem vulnerability to cavitation (cavitation; dashed line). The gray color represents the commonly reported coordination between the three parameters (e.g. Creek *et al.*, 2020). The solid black line and black arrow, in combination with the gray dashed line, demonstrate the possibility that osmotic adjustment modifies stomatal regulation to drive plants into cavitating xylem water potential (Ψ_x) at the end of the growing season. The black color for all parameters demonstrates the hypothesis that seasonal shift of all three traits drives them into lower Ψ_x while maintaining a similar safety margin.

New Phytologist (2020) www.newphytologist.com

The vineyard was located in the central mountain region of Israel (32.2°N, 35.1°E), 759 m above sea level. The precipitation was 650 mm in the 2018-2019 winter, and the last rain was on 20 April 2019. The daily minimum and maximum temperatures, the vapor pressure deficit (VPD) and the potential evapotranspiration for this season are presented in Supporting Information Fig. S1. Budbreak occurred on 1 April and leaf fall commenced at the end of October. The irrigation treatments were as follows: no irrigation (NI), deficit irrigation (DI), and super irrigation (SI). The DI treatment received 127 mm during the season: 40% from maximal vine water consumption (ET_c) until veraison, and 10% from veraison until harvest. Irrigation was applied once a week. The SI treatment was composed of six vines planted in six lysimeters filled with local Terra Rossa soil, with a total volume of 1.47 m³ (Munitz et al., 2019). Irrigation was applied on an hourly basis, that is, 24 irrigation pulses d⁻¹ during the entire growing season, to ensure 'optimum soil water conditions' (Pereira et al., 2015). The daily irrigation amount exceeded the vines' estimated daily ET_c by 10–20%.

The SI treatment was applied to six vines, grown side by side in lysimeters, and considered as six replicates. The NI and DI treatments were applied to 48 vines, organized in four random blocks (12 vines in a block), considered as four replicates. Row orientation was east–west, and the vines were trained to a vertical shoot positioning training system with two foliage wires. The vines were designed as a bilateral cordon and pruned during the winter to 16 spurs per vine, each comprising two buds. The vines were fertilized and sprayed for pests following the local commercial growing practices. Because the SI treatment suffered from downy mildew from the middle of July, it was terminated, and data for that treatment was available only for the first half of the season.

In the 2019 season, physiological parameters such as xylem water potential, gas exchange, leaf area index and leaf osmotic content were evaluated periodically. Leaf responses to dehydration were evaluated at three time points during the experiment (late May, early July and late August). Shoots were brought to the laboratory and dehydrated while their VCs and stomatal closure dynamics were recorded. In parallel, leaves were sampled to analyze their anatomical properties.

Field measurements

he field measurements were taken weekly on the same plants, three plants from each of the four plots for NI and DI treatments, and six plants from the SI treatment. Leaf area index (LAI) was measured weekly during canopy development and every fortnight for the remainder of the season using a canopy analysis system (SunScan model SS1-R3-BF3; Delta-T Devices, Cambridge, UK) as described in Munitz *et al.* (2018)

During the season, we marked the newly formed leaves (< 3 cm²). The length of the midrib and the petiole of six of the newly formed leaves from each treatment were measured weekly until reaching their final size. This procedure allowed us to know when we sampled the leaves in relation to leaf growth.

Midday xylem water potential (Ψ_x) was measured weekly with a pressure chamber (model Arimad 3000; MRC, Hulon, Israel;

or model 600D; PMS, Albany, OR, USA) at midday (from 12:00 to 14:30 h) on the day before the DI treatment was irrigated. Measurements were conducted following the protocol described by Boyer (1995). Sunlit, fully expanded leaves were bagged for at least 30 min before the measurement with plastic bags covered with aluminum foil. The time elapsing between leaf excision and chamber pressurization was < 15 s.

Every 2 wk, gas exchange measurements were made at midday using a commercial gas exchange system (Li-6400; Li-Cor Inc., Lincoln, NE, USA) on the youngest fully expanded mature leaf. The conditions were set to photosynthetic active radiation (PAR) of 1000 μ mol m⁻² s⁻¹, a flow rate of 500 μ mol s⁻¹, 400 μ mol CO₂ mol⁻¹ with ambient humidity and temperature.

Generating VCs

Embolism spread was evaluated using the optical technique (Brodribb *et al.*, 2016) in dehydrating shoots three times during the season: late May (20 May–2 June), July (2–23 July) and August (19–25 August). On each of these dates, the youngest fully mature leaf was monitored for embolism spread. Additionally, to evaluate the seasonal and environmental (available water) effects on the VC of a leaf, we compared the embolism resistance of four mature leaves that were measured in May with that of August leaves taken from the same nodal positions as the ones measured in May.

On those dates, we collected four shoots (1.5 m long) from each treatment before sunrise and immediately recut them underwater. During the trip back to the laboratory (1 h), the cut shoots were kept submerged underwater and covered with a dark plastic bag. In the laboratory, we verified that the Ψ_x was close to zero, and then started to dehvdrate them for 3 d while imaging the youngest fully mature leaf. Leaves were imaged every 5 min with a scanner (9000F mark II; Canon) or with a custom-built imaging clamp (http://www.opensourceov.org). The imaged area was at least 300 mm² in the center of the leaf and encompassed all vein orders, including the midrib. $\Psi_{\rm x}$ was measured on other bagged leaves from the same shoot every 1 to 8 h using a pressure chamber (model 600D; PMS) to capture the dehydration dynamics at a 0.2 MPa interval. A best-fit regression model of the Ψ_x vs time was used to determine the Ψ_x of all the images. Image sequences were then analyzed as described in http://www.open sourceov.org/process/ to determine the embolized area for each image and generate the VC, expressed as a percentage of embolized pixels vs Ψ_x .

Evaluation of the embolism degree

The optical vulnerability method requires that no embolism is present in the leaf before the dehydration starts. This was evident by comparing Ψ_x measured in the vineyard with the generated VC. Additionally, to directly evaluate the degree of embolism, we used the reversed light transmission technique (Hochberg *et al.*, 2017b). At three different dates, we collected five branches from the NI treatment at the time it exhibited its lowest Ψ_x throughout the season (middle of August to the middle of September). The

shoots were recut underwater and transported to the laboratory while their cut ends were dipped in water (to avoid dehydration) and their canopy was still transpiring to avoid refilling (Knipfer et al., 2016). In the laboratory, the youngest fully mature leaf was tightly taped to a custom-built imaging clamp and imaged once. To refill potentially embolized conduits, we pressurized the branch with water at 0.2 MPa for 10 min before the leaf was imaged again. From each image, we measured the gray value of 10 random polygons, containing 200-400 pixels, taken from the first and second vein orders. Then, the gray value ratio of after/ before pressurization was calculated for each leaf. No difference in gray values (a ratio of 1) suggests that no embolism was present in the leaf. As a positive control (leaf with embolism), we repeated this protocol with four branches that were bench-dehydrated to an average Ψ_x of -2.15 MPa. As a negative control (leaf without embolism), we measured leaves from the SI treatment for which Ψ_x was never < -0.5 MPa.

Stomatal regulation throughout dehydration

In the same weeks of shoot collection for the VC, we also sampled four branches from the NI treatment (in the same manner as described earlier for VC) to measure the stomatal conductance dynamics during dehydration. It was previously shown that measuring the relation between g_s and Ψ_x for a bench-dehydrating grapevine shoot resulted in a similar curve to that acquired when drving the whole plant (Tombesi et al., 2014). The measurements took place in an environmentally controlled room with steady light (100 μ mol m⁻² s⁻¹), humidity (40%), and temperature (30°C). Apart from the youngest fully mature leaf, all other leaves were bagged to increase the dehydration time of the branch. The youngest fully mature leaf was attached to a gas exchange system (Li-6400 or Li-6800; Li-Cor) set to PAR = 1000 μ mol m⁻² s⁻¹ and 400 μ mol CO₂ mol⁻¹ in the cuvette with ambient temperatures and humidity. Gas exchange measurements were logged every 2 min. When gs was stable (after c. 1 h), the branches were disconnected from the water reservoir. When the stomatal conductance decreased to at least 20% of its initial value (typically 4–6 h from the beginning of dehydration), we stopped the measurement. Throughout dehydration, Ψ_x was monitored every 30-60 min (in the same manner as described for the VC generation). A best-fit regression model of Ψ_x vs time was used to determine the Ψ_x of every g_s measurement. Then, to generate the stomatal response curve, gs, expressed as a percentage of the initial value, was plotted against $\Psi_{\rm x}$.

Osmolality measurements

Every 2 wk, osmolality was measured on the same leaves used for Ψ_x measurements. From each leaf, two pieces from both sides of the midrib were placed in two 1.5 ml plastic tubes that were immediately frozen in liquid nitrogen. Leaves were frozen *c*. 3 min after excision. In the laboratory, the tubes were thawed and centrifuged at 20,200 *g* (Eppendorf 5417R, Hamburg, Germany) for 1 min. The extracted sap was measured in a vapor pressure osmometer (Vapro 5600; Wescor Inc., Logan, UT, USA)

and the mean of the two halves of the leaf was taken as leaf osmolality (mmol $\rm kg^{-1}).$

Turgor loss point calculation

Turgor loss point was calculated from leaf osmolality (π), leaf water potential (Ψ_1 ; assuming it is lower than the Ψ_x by 0.2 MPa, as shown by Williams & Araujo (2002) for many grape cultivars), symplastic water content fraction (s_f) and cell wall elasticity (ϵ). A detailed explanation of the turgor loss point calculation appears in Methods S1.

Anatomy

Petioles were collected in May, July and August. We sampled the six uppermost mature leaves per treatment. The petioles were sectioned by sliding microtome, stained in phloroglucinol-HCL and analyzed as described in Shtein *et al.* (2017).

Transmission electron microscopy (TEM) was used for leaf samples collected in May, July and September. The fresh samples were shipped to Ulm University and prepared for TEM following a standard protocol (Li *et al.*, 2016; Schenk *et al.*, 2018). Only a single sample was prepared per month for each treatment. For both light microscopy and TEM, in the last sampling date we also collected basal leaves that were already mature during the sampling in May. A detailed protocol for the preparation, image acquisition and image analysis can be found in Methods S2.

Statistics

All analyses were computed in R v.3.5.2. The differences between treatments and dates were tested using a one-way ANOVA with Tukey honestly significant difference *post hoc* test using the AGRI-COLAE package. To create the curves of the vulnerability to cavitation and stomatal response to dehydration (Fig. 3) that can be averaged with their corresponding repetitions and be compared with other treatments, for each measurement, we round the Ψ_x by 0.05 MPa, and then averaged the values and their corresponding percentage cavitation, or % g_s .

Results

Vineyard measurements

Physiological parameters were measured periodically to follow the treatment status (Fig. 2). Canopy development was mostly in the first month after budbreak. By June, the SI treatment had the highest LAI with 1.6 m² m⁻², while the DI and the NI had 1.2 and 0.9 m² m⁻², respectively (Fig. 2a). During the first 2 months of the experiment, soil water content was adequate to give high predawn water potentials (Ψ_{PD}) in the three treatments (> -0.2 MPa). While the SI vines maintained a stable midday Ψ_x of *c*. -0.4 MPa during the entire season, Ψ_x of the two other treatments decreased from -0.4 to -1.3 MPa at harvest (Fig. 2b). The DI treatment had higher Ψ_x in the first 8 wk after budbreak as compared with the NI. Later in the season, both treatments had similar Ψ_x , probably because the irrigation of the DI was reduced from 40% to 10% ET_c in accordance with the irrigation protocol. The seasonal patterns of net photosynthetic assimilation, A_n , and g_s were similar in all treatments.

Gas exchange rapidly increased at the beginning of the season and gradually decreased, starting in July. By the beginning of September, g_s decreased to 0.05 ± 0.011 and 0.04 ± 0.005 mol m⁻² s⁻¹, and A_n decreased to 4.4 ± 0.6 and $3.5 \pm 0.6 \ \mu\text{mol m}^{-2} \ \text{s}^{-1}$ for the DI and NI treatments, respectively. The highest values for the SI, DI and NI treatments were 0.42 ± 0.02 , 0.3 ± 0.03 , and $0.2 \pm 0.04 \ \text{mol m}^{-2} \ \text{s}^{-1}$ for g_s , respectively, and 16.1 ± 0.3 , 15.3 ± 0.2 , and $13.8 \pm 0.4 \ \mu\text{mol m}^{-2} \ \text{s}^{-1}$ for A_n (Fig. 2c,d). The leaves accumulated osmolytes in all treatments during the season. DI and NI accumulated osmolytes gradually from 332 ± 18 and $302 \pm 40 \ \text{mmol kg}^{-1}$ at the beginning of May, to 700 ± 30 and



Fig. 2 Physiological and vegetative parameters measured in the three irrigation treatments of grapevines: super irrigation (SI, black circles), deficit irrigation (DI, gray triangles), and no irrigation (NI, white squares). (a) leaf area index (LAI); (b) midday (symbols) and predawn (bar graph) xylem water potential (Ψ_x) ; (c) net photosynthetic assimilation (A_n); (d) stomatal conductance (g_s); (e) leaf midday osmolality; (f) turgor loss point (Ψ_{TLP}). Because the SI treatment suffered from downy mildew from the middle of July, it was terminated, and data for that treatment are available only for the first half of the season. Each value is the mean of four replicates (three leaves per replicate, 12 leaves in total) except for the SI treatment, which consists of six repetitions. The gray area represents the SE.

 $693\pm46~\text{mmol}~\text{kg}^{-1}$ at the beginning of September, respectively. Osmotic concentration was lower in the SI treatment and increased from $255\pm15~\text{mmol}~\text{kg}^{-1}$ at the beginning of May to $430\pm35~\text{mmol}~\text{kg}^{-1}$ in mid-June, but then maintained a similar osmotic content for the remainder of the experiment (Fig. 2e). Based on these osmotic values, the calculated Ψ_{TLP} of the SI treatments decreased from $-0.69\pm0.04~\text{MPa}$ in May to $-1.2\pm0.05~\text{MPa}$ in July, and the Ψ_{TLP} of DI and NI decreased from $-0.83\pm0.04~\text{MPa}$ in May to $-1.88\pm0.04~\text{and}~-1.86\pm0.05~\text{MPa}$ at the beginning of September, respectively (Fig. 2f). Statistical comparison of these physiological parameters is presented in Table S1.

Bench dehydration in May, July and August showed high seasonal plasticity and high coordination between the hydraulic traits measured. In the NI treatment, for which we also evaluated the stomatal regulation in bench-dehydrated shoots, it was coordinated with Ψ_{TLP} and xylem embolism resistance (Figs 3, 4), maintaining a safety margin between 0.36 and 0.83 MPa, with Ψ_x values corresponding to 25% stomatal conductance (g_{s25}) and P12. Absolute gs values were higher in May and July $(g_{s100} = 0.156 \pm 0.028 \text{ and } 0.16 \pm 0.028 \text{ mol m}^{-2} \text{ s}^{-1}, \text{ respec-}$ tively) than in August $(g_{s100} = 0.104 \pm 0.007 \text{ mol m}^{-2} \text{ s}^{-1})$. Remarkably, the coordination of these three parameters (g_{s25} , Ψ_{TLP} , P₁₂) was maintained despite their seasonal plasticity. Namely, they all showed a similar decline as the season progressed. The g_{s25} changed significantly (P < 0.001) from -1.01 ± 0.05 MPa in May to -1.25 ± 0.04 in July and to -1.78 ± 0.1 MPa in August, while at the same time the P₁₂ changed significantly (P < 0.001) from -1.55 ± 0.07 MPa in May to -1.83 ± 0.03 in July and to -2.15 ± 0.11 MPa in August. Ψ_{TLP} changed from -0.9 ± 0.06 in May to -1.44 ± 0.05 in July and -1.85 ± 0.05 in August. The raw data of Fig. 3 are available in Fig. S2. The high coordination between the different hydraulic traits is further manifested by the high correlation ($R^2 = 0.96$) between Ψ_{TLP} and P_{12} for all of the irrigation treatments throughout the growing season (Fig. 4).

Contrary to the large coordinated hydraulic shift through the growing season, we found only a minor change in hydraulic vulnerability in response to the irrigation treatment. In fact, over the growing season, P_{12} , P_{50} and P_{88} were not significantly different between the treatments. Only on a few occasions were significant differences measured showing a 0.1-0.2 MPa drought acclimation: P_{12} in May was significantly higher in the SI vines $(-1.33 \pm 0.03 \text{ MPa})$ than in the DI vines $(-1.5 \pm 0.06 \text{ MPa})$, and in July the P_{12} of SI plants (-1.68 ± 0.07) was significantly higher than those from the DI treatment $(-1.76 \pm 0.03 \text{ MPa})$ (Fig. S3; Table 1).

As we always sampled the 'youngest fully mature leaf' as the growing season progressed, leaves from further up the shoot were collected. To understand if the seasonal plasticity is limited to newly formed leaves, or if leaves formed earlier in the season could also change their hydraulic traits, we measured again at the end of the season (August) leaves from the same nodal position as those that were measured at the beginning of the season (the basal leaf that was formed on 6 May). We found that in the NI treatment leaves formed early in the season also changed, and became

less vulnerable to cavitation with time (Fig. 5; Table 1). The P₁₂ and P₅₀ of the leaves formed on 6 May were significantly more vulnerable at 3 wk after leaf formation (-1.55 and -1.89 MPa, respectively) than at 16 wk after formation (-1.82 and -2.5 MPa). Still, the P₁₂ was not as low as that of leaves formed on 1 July (P₁₂= -2.15 ± 0.11 MPa), although the P₅₀ values were similar (-2.6 MPa).

As noted, the optical vulnerability technique assumes that no embolism was present before measurement. This was confirmed based on the Ψ_x values that never approached the P₁₂ of even the most vulnerable leaves. To show that the reduction in the whole plant hydraulic conductivity (Fig. S4) was not related to xylem embolism, we used the reverse optical technique (Hochberg *et al.*, 2017b). The measurement confirmed that no embolism was present in the vineyard even in the NI treatment in late August or early September (lowest Ψ_x measured throughout the experiment). The gray value ratio of the most stressed leaf was close to 1, implying that no cavitation had occurred in the vineyard. The value was similar to the negative control (SI treatment; 0.99 ± 0.03) and significantly lower than the positive control (1.07 ± 0.01) shoots that were bench-dried to an average Ψ_x of -2.15 MPa (Fig. 6).

Growth rate, leaf morphology and anatomy were similar in the DI and NI treatments. They did, however, show significant changes during the season, exhibiting significant decreases in leaves that were formed later in the season. Maximal growth rate and final size of newly formed leaves gradually decreased as the season progressed (Figs 7, S5). Maximal growth rate, single leaf area and petiole diameter of NI plants decreased from 7.9 mm d^{-1} , $83.8 \pm 5.06 \text{ cm}^2$ and $2.3 \pm 0.06 \text{ mm}$ in May to 4.7 mm d⁻¹, 53.53 ± 3.19 cm² and 1.71 ± 0.05 mm in July, and to 2.2 mm d $^{-1}$, 24.1 \pm 1.92 cm 2 and 1.28 \pm 0.03 mm in August, respectively (Table 2). These changes were also reflected in the xylem anatomical parameters measured. Mean vessel diameter decreased significantly during the season, but no significant differences between treatments were observed. For example, in the NI treatment, mean vessel diameter in the center of the midrib decreased from $23.69 \pm 0.34 \,\mu\text{m}$ in May to $21.42 \pm 0.31 \,\mu\text{m}$ in July, and to $16.92 \pm 0.24 \,\mu\text{m}$ in August (Table 2). In addition, the vessel structural strength $(t/b)^2$ was not significantly different between the treatments or between May and July, but had a significant increase in August in plants from both the DI and NI treatments. For example, in the NI treatment, $(t/b)^2$ increased from 0.0024 ± 0.00022 in July to 0.0043 ± 0.00051 .

Surprisingly, pit membrane thickness ($T_{\rm PM}$) showed considerable seasonal shrinkage (Figs 8, 9b). $T_{\rm PM}$ was significantly reduced between May and July, from 643.9 ± 23.9 to 296.5 ± 59.5 nm, for all treatments (Table 2). The reduction in $T_{\rm PM}$ over the growing season was also noticeable when sampling leaves from the same nodal position. Leaves that were formed early in the season (fifth nodal position) in the NI treatment had a relatively high $T_{\rm PM}$ in May (474.1 ± 39.5 nm), but when sampled again in August, $T_{\rm PM}$ (303.3 ± 72.6 nm) had reduced considerably, showing values that were similar to the apical leaves in August (389.5 ± 13.2 nm). Leaves of the NI treatment in May showed considerably thinner pit membranes (474.1 ± 39.5) than





Fig. 3 Coordinated hydraulic plasticity of grapevines from the no irrigation treatment. Stomatal conductance (g_{s} ; solid lines), turgor loss point (Ψ_{TLP} ; arrows) and xylem embolism resistance (dashed lines) of the leaves during the growing season (May, blue; July, green; August, red). The gray area represents the SE (n = 4). The raw data for the figure are presented in Supporting Information Fig. S2.



Fig. 4 Relationship between turgor loss point (Ψ_{TLP}) and 12% cavitation (P_{12}) during the growing season (May, blue; July, green; August, red) in grapevines from three irrigation regimes: super irrigation (SI, circles), deficit irrigation (DI, triangles) and no irrigation (NI, squares). For Ψ_{TLP} , each value is the mean of four replicates (three leaves per replicate) except for the SI treatment which had six replicates (one leaf per replicate). P_{12} is the average of four leaves, and the bars represent the SE.

leaves collected at the same time from SI (725.2 \pm 42.3) and DI treatments (690.8 \pm 26.7). Minor differences in T_{PM} were found for the DI and NI treatments between July and August, with T_{PM} values of c. 300-400 nm. In July and August, the leaves from treatments that exhibited lower P12 were found to have higher $T_{\rm PM}$ (Fig. 9b). For example, the increased xylem vulnerability in August compared with July was accompanied by higher $T_{\rm PM}$ in August than July for both the DI (340.3 ± 14.4 nm vs. 287.4 ± 8.3) and NI treatments (389.5 ± 13.2) vs 309.6 ± 15.3 nm). Additionally, the most resistant leaf in terms of xylem vulnerability (NI in August) also had the highest T_{PM} $(389.51 \pm 13.2 \text{ nm})$. Besides T_{PM} , there was also a considerable change in electron density of pit membranes between the leaves

collected in May and later in the growing season. Pit membranes in May were highly transparent and had a low contrast under TEM (Fig. 8a–d), while darker pit membranes with a granular appearance were more pronounced in July and September (Fig. 8e–h).

Discussion

Seasonal plasticity of plant hydraulics

Our results show that a seasonal shift in xylem embolism resistance is well coordinated with Ψ_{TLP} and stomatal regulation dynamics (Fig. 3). This leads to the affirmation of the hypothesis:

Table 1 Xylem embolism resistance of grapevine leaf	veins obtained by the optic vulnerabilit	y method in June, July and Au	gust in the three treatments
(super irrigation, SI; deficit irrigation, DI; no irrigation,	, NI).		

Leaf formation time	Treatment	Sampling time	Ν	P ₁₂ (MPa)	P ₅₀ (MPa)	P ₈₈ (MPa)
6 May	SI	May	3	-1.33 ± 0.03^{Aa}	-1.92 ± 0.09^{Aa}	$-2.44\pm0.54^{\text{Ab}}$
,	DI	,	4	-1.5 ± 0.06^{Aab}	-1.79 ± 0.05^{Aa}	-2.16 ± 0.71^{Aa}
	NI		5	$-1.55\pm0.07^{\text{Ab}}$	-1.89 ± 0.02^{Aa}	-2.09 ± 0.3^{Aa}
17 June	SI	July	4	-1.68 ± 0.07^{Ba}	-2.03 ± 0.1^{Aa}	$-2.35\pm0.26^{\text{Aa}}$
	DI		4	-1.76 ± 0.03^{Bb}	-2.07 ± 0.06^{Ba}	-2.56 ± 1.77^{ABa}
	NI		4	-1.83 ± 0.03^{Bab}	-2.15 ± 0.04^{Ba}	-2.65 ± 1.1^{Ba}
1 July	SI	August	_	_	_	_
,	DI	0	4	-2.1 ± 0.05^{Ca}	-2.53 ± 0.1^{Ca}	$-2.99\pm1.5^{\rm Ca}$
	NI		4	-2.15 ± 0.11^{Ca}	-2.6 ± 0.07^{Ca}	-3.11 ± 0.96^{Ca}
6 May	NI	August	4	-1.82 ± 0.08^{B}	$-2.6\pm0.16^{\text{C}}$	$-3.27\pm0.26^{\rm C}$

Values are averages \pm SE. Capital letters indicate statistically significant differences ($\alpha = 0.05$) between dates within the same treatment based on one-way ANOVA followed by *post hoc* Tukey HSD test, and small letters indicate differences between treatments on the same date.



Fig. 5 Comparison of the vulnerability to cavitation as a function of leaf age or time of season in grapevines at the no irrigation treatment. The mean vulnerability curve shown was measured on the apical youngest fully mature leaf in May (leaf formation on 6 May; blue squares) and August (leaf formation on 1 July; red squares) and on the basal leaf in August (formed on 6 May; black squares). The gray area beside the lines represents the SE (n = 4).

the safety margin is maintained throughout the season (*c*. 0.4 MPa between stomatal closure and the onset of cavitation). This safety margin is similar to those previously reported for potted grapevines (Hochberg *et al.*, 2017b), and their seasonal maintenance explains the species' ability to support gas exchange while avoiding embolism despite a seasonal decline in Ψ_x . Our results match those of Martorell *et al.* (2015), who measured the hydraulic vulnerability of grapevine leaves using the rehydration kinetics method, and found that P₈₈ is significantly more negative in August than in July or June, and bears a high correlation with Ψ_{TLP} . The seasonal decrease in Ψ_{TLP} is well documented in grapevines (Patakas *et al.*, 1997; Alsina *et al.*, 2007; Martorell *et al.*, 2015) as well as in many other species (Bowman & Roberts, 1985; Bartlett *et al.*, 2014), but a seasonal increase in embolism resistance of leaves is far less explored (Martorell *et al.*, *et al.*).

2015). The finding of a coordinated seasonal plasticity presents a new perspective for our understanding of plant hydraulics, indicating that hydraulic traits are more seasonally dynamic than previously thought.

In general, the ability of the xylem vulnerability to adjust in response to environmental pressure is well documented. Exposure to light/shade (Lemoine *et al.*, 2002; Schoonmaker *et al.*, 2010), fertilization (Harvey & Van Den Driessche, 1999; Beikircher *et al.*, 2019) and drought stress (Stiller, 2009; Awad *et al.*, 2010; Cardoso *et al.*, 2018) led to substantial xylem plasticity. Thus, the seasonal change of xylem vulnerability presented in this study should not come as a surprise, especially when considering the enormous environmental changes experienced by deciduous species grown in Mediterranean climates between budbreak and leaf fall. In fact, this coordinated hydraulic shift seems to be the





Fig. 6 Evaluation of the cavitation level in leaves from the vineyard based on the relative change in the gray values of the midrib (after/before pressurization). Measured leaves (gray) are those that experienced the lowest xylem water potential (Ψ_x) throughout the experiment (the no irrigation (NI) treatment during late August and early September). The positive control is composed of leaves that were taken from the same treatment at the same time and bench-dehydrated to an average Ψ_x of -2.15 MPa (orange). The negative control represents leaves from the SI treatment, which did not experience water stress (blue). The data are presented as a whisker plot. n = 5. ***, P < 0.001 (statistically significant difference).



Fig. 7 (a, b) Lengths of midribs (circle; a) and petioles (triangle; b) of grapevine leaves throughout the growing season. The different colors represent the time of leaf formation as the visible light spectrum from 21 April to 24 June. Data are averages for all treatments. Each line represents the mean of at least 10 leaves. The gray area represents the SE.

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Treatment	Date	Leaf area (cm ²)	Petiole diameter (mm)	Mean vessel diameter (μm)	(<i>t/b</i>) ²	Pit membrane thickness (nm)
SI	May	70.59 + 5.35 ^{Aa}	2.4+0.14	24 ± 0.38^{Aa}	0.0029 ± 0.00043^{Ba}	725.22 + 42.37 ^{Aa}
DI		83.76 ± 7.82 ^{Aa}	2.6 ± 0.08	25.89 ± 0.36^{Aa}	0.0024 ± 0.00053^{Ba}	690.78 ± 26.74 ^{Aa}
NI		$83.8\pm5.06^{\text{Aa}}$	2.3 ± 0.06	23.69 ± 0.34^{Aab}	0.0026 ± 0.00032^{Ba}	474.07 ± 39.48^{Ab}
SI	July	43.84 ± 2.66^{Ba}	1.71 ± 0.07	20.3 ± 0.25^{Bab}	0.0029 ± 0.0003^{Ba}	289.93 ± 18.01 ^{Ba}
DI	,	55.85 ± 3.69 ^{Ba}	1.73 ± 0.05	$\textbf{20.73} \pm \textbf{0.29}^{\text{Bab}}$	0.0026 ± 0.00031^{Ba}	287.42 ± 8.31^{Ba}
NI		$53.53 \pm 3.19^{\; Ba}$	1.71 ± 0.05	$21.42\pm0.31^{\text{ Ba}}$	0.0024 ± 0.00022^{Ba}	309.56 ± 15.33^{Ca}
SI	August	_	_	_	_	
DI	0	22.58 ± 1.52^{Ca}	1.23 ± 0.04	$16.83\pm0.38^{\text{Ca}}$	0.005 ± 0.00034^{Aa}	340.26 ± 14.39^{Bb}
NI		24.1 ± 1.92^{Ca}	1.28 ± 0.03	16.92 ± 0.24^{Ca}	0.0043 ± 0.00051^{Aa}	389.51 ± 13.24^{Ba} $303.3 \pm 72.6*$

Table 2 Leaf anatomy parameters of grapevine in May, July and August in the three treatments (super irrigation, SI; deficit irrigation, DI; no irrigation, NI).

Values are means of six samples \pm SE. Capital letters indicate statistically significant differences ($\alpha = 0.05$) between dates within a specific treatment based on one-way ANOVA followed by *post hoc* Tukey's honestly significant difference test, and small letters indicate differences between treatments on the same date. *The value represents the basal leaf of the NI treatment that was formed on 6 May, and leaves of the same age from the same node sampled in August.



Fig. 8 Transmission electron microscopy images of grapevine showing pit membranes (PM, arrows) in intervessel pits of xylem at the base of the midrib. Samples were prepared from the youngest fully mature leaves collected in May (a–d), July (e, g, h), and August (f), and followed three different watering regimes: super irrigation (d, h), no irrigation (a, b, e, f) and deficit irrigation (c, g). Pit membranes in spring showed a very low electron density, were sometimes invisible (a), and relatively thick (a–d), while more electron-dense and thinner pit membranes were observed in early and late summer (e–h).

desired strategy for any species that generally experiences high water availability early in the spring and gradually drier conditions as the summer progresses. This notion is supported by Kolb & Sperry (1999), who showed a more than 3 MPa decline in P_{50} of *Artemisia tridentata* stems as summer progressed and the soil dried out. In grapevines, Charrier *et al.* (2018) showed that stem

New Phytologist (2020) www.newphytologist.com





Fig. 9 (a, b) Relationship between mean vessel diameter (a) or pit membrane thickness (b) and P_{12} in the three treatments at the vineyard (super irrigation (SI), circles; deficit irrigation (DI), triangles; and no irrigation (NI), squares). Measurement time is represented by different colors (May, blue; July, green; apical leaf in August, red). The black squares represent the basal leaves measured in August, which were formed on the same date as those measured in May. Mean vessel diameter was measured on five petioles. For pit membrane thickness, the averages represent more than 15 pits that were measured in one leaf per treatment. P_{12} values are averages of four replicates. The error bars represent the SE. The dashed lines and R^2 in panel (b) were calculated without the May group data for the pit membrane thickness.

 P_{50} values shifted from -2.1MPa in July to -2.8MPa in September. As many studies did not consider the potential effect of seasonality on hydraulic traits, it could be speculated that many other species that experience significant seasonal changes in Ψ_{TLP} (Bartlett *et al.*, 2014) will also shift their embolism resistance.

Seasonal hydraulic plasticity seems to be, in part, a predetermined strategy rather than only a response to the seasonal change in the environment. Generally, in field conditions, it is hard to separate seasonal plasticity from drought acclimation because frequently, as the season progresses, drought frequencies, duration and intensity increase. Considering the atmospheric conditions, this was not the case in the current experiment where VPD was higher in May than in June, July and August (Fig. S1b). Therefore, the hydraulic plasticity of the SI treatment (Table 1; Fig. S2), despite its constant high water availability, implies that in grapevines, hydraulic plasticity is partly a function of leaf maturation. In support, the small – and mostly insignificant – modification of the VC or Ψ_{TLP} with respect to the water treatment (Table 1; Fig. S3) suggests that drought has a limited ability to shape grapevine hydraulics compared with the developmental changes. This is surprising in comparison to the large effect drought has on the VC of other species (Stiller, 2009; Awad et al., 2010; Cardoso et al., 2018), but in agreement with previous experiments that did not find improved xylem resistance in grapevine leaves (Hochberg et al., 2017a) or other species (Lamy et al., 2014) that were acclimated to drought. Moreover, the limited drought acclimation response of grapevines can also be inferred from the limited osmotic adjustment that drought treatments caused in several studies (c. 0.3 MPa) compared with the much larger seasonal osmotic adjustment (c. 1 MPa) (Fig. 2e; Alsina et al., 2007; Martorell et al., 2015). The magnitude of the seasonal change in osmotic adjustment seems reasonable when considering the challenges it presents to a deciduous species that has to regrow its entire canopy in a relatively short period. The osmotic content of leaves is composed of many inorganic ions

(Downton & Loveys, 1981; Patakas *et al.*, 2002; Degu *et al.*, 2019) and their transport from the soil and accumulation in tissues takes time. This is especially true for the early-season leaves that are formed when the growth rate is very fast compared with the limited supply of minerals (Fig. S4). In accordance with that, Patakas & Noitsakis (2001) showed that the immature grapevine leaf relies mostly on sugars (coming from previous year's storage) as osmolytes, replaced primarily by inorganic ions upon maturation. The factors that account for the major seasonal changes in the VC are more elusive, especially because we still lack a clear understanding of the drought-induced xylem cavitation mechanism (Jansen *et al.*, 2018).

Seasonal anatomical changes with respect to cavitation

Air-seeding from adjacent embolized conduits is currently the most probable explanation for drought-induced xylem cavitation (Cochard et al., 1992; Schenk et al., 2015; Hochberg et al., 2019). Based on the rare pit hypothesis, large vessels with a high number of intervessel pits would increase the likelihood of airseeding (Christman et al., 2012). Accordingly, if vessel dimensions would be positively related to the number of intervessel pits, the high correlation of xylem vulnerability with the vessel size can be linked to air-seeding (Fig. 9a; Saliendra et al., 1995). Having said that, this correlation (Fig. 9a) might be incidental because plants that sustain higher tensions require smaller vessels to prevent xylem collapse (Hacke et al., 2001). In support of an indirect relationship, the basal leaves that were formed early in the season and measured in August had large vessels despite low vulnerability to cavitation. Other factors, such as reduced lignification or partial cell wall thickening, could also contribute to the higher xylem vulnerability experienced by early-season leaves (Lens et al., 2016). Therefore, we should consider the possibility that more than one anatomical feature determines the likelihood of air-seeding. The surprising shift in P₅₀ of mature leaves could have resulted from changes in xylem sap composition, affecting surface tension, and also from dynamic structural changes. Even mature xylem elements are 'only mostly dead' (https://youtu.be/ xbE8E1ez97M) and some processes, such as cell wall thickening and lignification, can occur with the assistance of living neighboring cells (Barros et al., 2015; Blokhina et al., 2019).

The seasonal shift in xylem vulnerability to cavitation could result from the pit membrane structure, which is shown here for the first time to represent a dynamic trait by itself. Previously reported pit membrane thickness for grapevines (185 nm; Jansen *et al.*, 2009) is probably an underestimation as a result of irreversible shrinkage. The thickness measured in the current research (280–390 nm) seems to better represent the native pit membrane thickness of grapevines. When pit membranes undergo hydrolysis, slight swelling of the pit membrane has been observed (Schmid & Machado, 1968). It has also been shown that pit membranes become more electron-dense as the season progresses (Schmid & Machado, 1968; Wheeler, 1981). However, any major change in shrinkage has previously been attributed to aspiration and dehydration (Tixier *et al.*, 2014; Zhang *et al.*, 2020), which are unlikely to explain the large shrinkage between May and summer (Fig. 8). The correlation between pit membrane thickness and P12 in July and August matches previous findings (Jansen et al., 2009, 2012), which showed that thicker pit membranes lead to lower vulnerability to cavitation. As a 50% shrinkage of pit membranes is associated with a c. 23% reduction of the pore volume fraction (Zhang et al., 2020), smaller pore dimensions of shrunken pit membranes in summer are probably an explanation for the increased embolism resistance and do not contradict the overall relationship between embolism resistance and pit membrane thickness (Li et al., 2016; Kaack et al., 2019). Therefore, the thick pit membranes that were measured in May did not confer high resistance to cavitation of those leaves. How seasonal changes in electron density of pit membranes (Fig. 8) affect embolism resistance is currently unknown and would require further research on the functional role of xylem sap lipids (Schenk et al., 2015, 2018; Yang et al., 2020).

What is the probability of cavitation in vineyards? Potential limitations for dataset extrapolations

The current dataset also highlights the problematic extrapolation of pot experiments into field-scale scenarios. Most of the available VC for grapevine leaves were acquired for potted vines (Hochberg et al., 2016b, 2017b; Charrier et al., 2018; Dayer et al., 2020). Those experiments suggested that cavitation starts between -1 and -1.5 MPa and that P_{50} is between -1.2 and -1.9 MPa, slightly more vulnerable than the VC we measured in May. Combining these values with Ψ_x measured under deficit irrigation conditions in the vineyard, $-2 < \Psi_x < -1.2$ MPa (e.g. Martorell et al., 2015; Netzer et al., 2019) was interpreted as substantial cavitation (Munitz et al., 2018). However, the VCs measured in July and August clearly demonstrate that P12 values (-1.8 MPa and -2.1 in July and August, respectively) are $\Psi_{\rm x}$ values that vines seldomly experience in the field. In support, Choat et al. (2010) also did not see an increase in cavitation in the stem, despite a reduction of Ψ_x from -0.7 to -1.3 MPa between fruit set and post-harvest. More important than the cavitation values themselves is the physiological sequence of events through dehydration. Stomatal closure and Ψ_{TLP} preceded cavitation throughout the season; accordingly, even in the rare event that Ψ_x would drop below -2 MPa in the vineyard, we suspect that the leaf VC should be modified accordingly and that cavitation is unlikely, unless the vines experience complete stomatal closure and wilting. Furthermore, cavitation should also lead to basal leaf shedding (Hochberg et al., 2017b) and its absence implies that no substantial degree of cavitation had taken place.

The difference between pots and vineyards probably originates from the significantly larger root volume accompanied by a more abundant soil water reservoir in plants growing in the field. As a result, dehydration of field-grown vines is much longer than pots (Essiamah & Eschrich, 1985; Bota *et al.*, 2001). In most vineyard experiments, a drop of 0.4 MPa (the safety margin) takes several weeks (Fig. 2), and it would be even slower if stomata had been closed. During the slow dehydration under field conditions, the vines have time to adapt and to shift their hydraulic properties, as a function of soil dehydration and/or maturation. Accordingly, by the time Ψ_x approaches the cavitation threshold of early-season leaves (at least 2 months after budbreak), the hydraulic characteristics of the vines have already been modified. These different values between pots and field experiments and the difference in hydraulic traits between the early season and the late season could explain why some meta-analysis (taking the VC and stomatal closure curves from different studies) results in a negative safety margin for some species, contrary to studies that simultaneously measured g_s and cavitation (Hochberg *et al.*, 2017b; Creek *et al.*, 2020).

The ability of plants to modify their hydraulic traits suggest that wilting and cavitation will only occur in one of two scenarios: when Ψ_x drops below the minimum value to which the vines can acclimate; or when the changes in Ψ_x are faster than the plant acclimation rate. Although we do not have any information on xylem vulnerability acclimation thresholds, we do know that Ψ_{TLP} in grapevines can be as low as -2.5MPa (Alsina *et al.*, 2007), lower than any reported measurement of grapevines' Ψ_x . Furthermore, the P₁₂ values measured in August in this experiment (-2.1 MPa) are not common in vineyards, implying that scenario 1 is not probable. On the other hand, fast changes in Ψ_x (scenario 2) are likely under heatwaves or when the soil waterholding capacity is low (karstic or sandy soils) compared with the canopy transpiration demand. The risk is even more significant in specific cultivars known for their high transpiration rates (e.g. Syrah) and towards the end of the season when plant hydraulic resistance is high (Fig. S4; Schultz, 2003; Choat et al., 2010). In support, late-season heatwaves frequently lead to leaf shedding in specific cultivars (Syrah, Malbec, Carignan and others), and vineyard managers are encouraged to take precautions (increased irrigation dosage and frequency) under such scenarios.

To conclude, our measurements provide clear evidence for significant and coordinated seasonal hydraulic plasticity in grapevines. This plasticity can occur even in mature leaves with mostly differentiated xylem. The plasticity of the VC is probably linked to the remarkable changes in pit membrane structure throughout the season and can explain the ability of grapevines to maintain photosynthesis despite growing xylem tension without compromising its hydraulic integrity.

Acknowledgements

We thank Dr Shabtai Cohen for reviewing the manuscript. SJ thanks Andrea Huppenberger and the Electron Microscopy Unit of Ulm University for preparation of the TEM samples. Financial support to SJ was provided by grants from the German Research Foundation (nos. 383393940 and 410768178). Financial support to YN for field study was provided by grants from the Ministry of Science and Technology (no. 6-6802), Israel, the Ministry of Agriculture and Rural Development (no. 31-01-0013), Israel, and the Israeli Wine Grape Council. The authors would like to thank the dedicated growers, Yoav David, Itamar Weis, and Shlomi Cohen. We thank Yossi Shteren for assisting in the field measurements.

Author contributions

YS performed the experiment with help from SG and YN. IS performed the petiole anatomical measurements. SJ performed the SEM measurements. UH conceived the experiment and wrote the main body of the text with a major contribution from YS, YN and SJ.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Meteorological data throughout the 2019 growing season.

Fig. S2 The raw data of the coordinated hydraulic plasticity for the no irrigation (NI) treatment.

Fig. S3 The leaf vulnerability curve based on the optical method during the 2019 growing season for the three irrigation treatments.

Fig. S4 Plant conductivity during the growing season for the irrigation treatments.

Fig. S5 The elongation rate of developing leaves across the growing season.

Methods S1 Detailed explanation on the calculation of turgor loss point.

Methods S2 Detailed protocols of the anatomical procedures.

Table S1 Statistical comparison of the physiological parameters

 between the different irrigation treatments.

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