



Multi-hormonal analysis and aquaporins regulation reveal new insights on drought tolerance in grapevine

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ABSTRACT

Disentangling the factors that foster the tolerance to water stress in plants could provide great benefits to crop productions. In a two-year experiment, two new PIWI (fungus resistant) grapevine varieties, namely Merlot Kanthus and Sauvignon Kretos (*Vitis* hybrids), grown in the field, were subjected to two different water regimes: weekly irrigated (IR) or not irrigated (NIR) for two months during the summer. The two varieties exhibited large differences in terms of performance under water-limiting conditions. In particular, Merlot Kanthus strongly decreased stem water potential (Ψ_s) under water shortage and Sauvignon Kretos maintained higher Ψ_s values accompanied by generally high stomatal conductance and net carbon assimilation, regardless of the treatment. We hypothesized differences in the hormonal profile that mediate most of the plant responses to stresses or in the regulation of the aquaporins that control the water transport in the leaves. In general, substantial differences were found in the abundance of different hormonal classes, with Merlot Kanthus reporting higher concentrations of cytokinins while Sauvignon Kretos higher concentrations of auxins, jasmonate and salicylic acid. Interestingly, under water stress conditions ABA modulation appeared similar between the two cultivars, while other hormones were differently modulated between the two varieties. Regarding the expression of aquaporin encoding genes, Merlot Kanthus showed a significant downregulation of *VvPIP2;1* and *VvTIP2;1* in leaves exposed to water stress. Both genes have probably a role in influencing leaf conductance, and *VvTIP2;1* has been correlated with stomatal conductance values. This evidence suggests that the two PIWI varieties are characterized by different behaviour in response to drought. Furthermore, the findings of the study may be generalized, suggesting the involvement of a complex hormonal cross-talk and aquaporins in effectively influencing plant performance under water shortage.

1. Introduction

In agriculture, duelling drought is both a longstanding and an actual problem. Every year drought affects many areas worldwide causing yield losses and plants' death. The future scenario, due to climate change, would suggest an increase in the severity of this phenomenon (IPCC, 2018). In particular, higher temperatures will intensify the water evaporative demand from the atmosphere, thereby increasing the risk of water stress risk (van Leeuwen and Darriet, 2016). Consequently, the future will face a decreasing water availability, leading current research

efforts to focus on creating favourable conditions to protect crop production while limiting water use. A better understanding of the mechanisms adopted by plants to improve their drought tolerance is crucial to achieve this objective. Plant processes are mainly regulated by phytohormones (Vanstraelen and Benkov, 2012; Waadt et al., 2022). In several plant species, abscisic acid (ABA) stands out as the most important hormone associated to water stress responses, triggering stomatal closure (Sussmilch et al., 2017) and driving the responses mechanism through the regulation of many transcription factors (Nakashima et al., 2014; Cochotel et al., 2020; Leng and Zhao, 2020).

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Nevertheless, other phytohormones have been shown to influence plant processes under drought. Extensive reviews, demonstrated that auxins positively impact drought tolerance in several manners, including interacting with ABA to maintain root growth under drought stress, as reported for several plant species (Jogawat et al., 2021; Salvi et al., 2021). In addition, the crosstalk between ABA and jasmonate was also found to be essential to reduce oxidative damage in response to water stress, with the involvement of salicylic acid accumulation (Jogawat et al., 2021). On the other hand, cytokinins are negatively affected by drought and are also connected to ABA in the modulation of water stress responses (Waadt et al., 2022; Singh and Roychoudhury, 2023). Therefore, the role of hormones and their interaction should not be overlooked in order to comprehend the mechanisms underpinning drought tolerance in plants. While hormonal control plays a predominant role in coordinating plant responses, the cause-effect relationship between hormones and physiological modulations or the plant behaviour under stress still remains to be fully elucidated.

When plant water relations are discussed, another topic of high interest is represented by aquaporins (AQPs) modulation. AQPs are small channels that facilitate the transport of water and other small molecules (CO₂, urea, etc.) across the cellular membranes and are divided in different families (Afzal et al., 2016). During stress conditions AQPs expression could be modulated in different ways, with reports of both upregulation and downregulation in different plant species under water stress (Afzal et al., 2016; Singh et al., 2021). Galmés et al. (2007) suggested that AQPs modulation serves to maintain the water status homeostasis in grapevine. Shelden et al. (2009) identified at least 23 genes encoding full-length proteins classified as membrane intrinsic proteins (MIPs) in the grapevine genome, several of which were assigned to the families of plasma-membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs), considered the most important AQPs for water transport. Subsequent studies investigated the possible roles of AQPs in grapevine ascribing to the expression of some AQPs an effect on functional hydraulic traits, such as, hydraulic leaf conductance (K_{leaf}) and stomatal conductance (Pou et al., 2013; Dayer et al., 2020), hydraulic root conductance (Vandeleur et al., 2014), embolism recovery in petioles (Chitarra et al., 2014), leaf capacitance (Vitali et al., 2016), and grapevine hydraulic behaviours (Vandeleur et al., 2009; Shelden et al., 2017; Dayer et al., 2020). For these reasons AQPs are considered as potential targets capable to improve drought tolerance in plants.

Combined studies analysing the physiological, the molecular and the biochemical status of different grapevine cultivars are important to understand the complexity of drought response reactions. Moreover, grapevine varieties resistant to fungal diseases are gaining interest in viticulture, although their characterization is still limited. In this context, two new PIWI grapevine varieties, undergoing two different irrigation levels, were studied for two consecutive seasons, with specific regard to their water status, photosynthetic performance, hormonal balance and AQPs regulation.

2. Material and methods

2.1. Plant material, site and experimental design

In 2018 and 2019 seasons, a two-year field trial was carried out in an experimental vineyard, planted in 2013 at the experimental farm "A. Servadei" of the University of Udine (Italy). Two new PIWI (international acronym for fungus resistant grapevines) grapevine genotypes were considered for the experiment: the white grape Sauvignon Kretos® (Sauvignon blanc x 20-3) and the red Merlot Kanthus® (Merlot x 20-3). Both varieties originated from a breeding program for the introgression of resistance genes to powdery and downy mildew (*Erysiphe necator* and *Plasmopara viticola*). The plants, grafted on SO4 rootstock (*V. berlandieri* x *V. riparia*), were arranged in two contiguous rows (N-S oriented) and pruned to a single Guyot with a vertical shoot positioned (VSP) trellis system retaining 8–9 buds/vine. For each genotype, 24 plants were

considered and divided in two plots, irrigated (IR) and non-irrigated (NIR) treatments, respectively. Within each treatment, 4 plots of three vines were considered for both physiological measurements and sampling. The experimental trial started from approx. 10 days after flowering (on June 3rd in 2018, and on June 10th in 2019). The experimental vineyard was equipped with a drip irrigation system. In detail, two irrigation lines were installed for each vine row, the former was used for weekly irrigation of IR plots (blind tube on NIR plots), while the second line was used for irrigation of both treatments only when Ψ_s values in NIR plants fell below -1.4 MPa. Since in Friuli Venezia Giulia region the summer season is characterized by scattered rains, in NIR plots, the ground was covered with ethylene-vinyl-acetate film to avoid the penetration of the rain in the soil. The film was further covered by 3–4 cm of soil to prevent microclimate alteration due to soil heating and radiation reflection.

At harvest time of each season, three representative shoots (from the basal, the central and the terminal position on the cane) were selected on three vines within each experimental group. Thereafter, the leaves were collected and leaf area was assessed using a leaf area meter (LI-3100, LICOR, Lincoln, NE). For each plant, shoot number was determined, and total leaf area estimated by multiplying the average shoot leaf area by the number of shoots (Bubola et al., 2017).

2.2. Meteorological data and soil moisture monitoring

The meteorological data were collected from a weather station of Udine - Sant'Osvaldo (ARPA FVG–OSMER, <http://www.meteo.fvg.it/>), located within the experimental farm, thereby providing an accurate reflection of the vineyard's climate conditions. Temperature and humidity data were used to calculate the daily maximum vapour pressure deficit (VPD_{max}) values. Moreover, 4 EC-5 sensors (Decagon Devices, Inc., Pullman, WA, USA) per plot were buried at -30 cm in the soil, along the vine rows and near the plant roots, in order to monitor the soil water content (SWC). In each plot, the sensors were connected to an EM50 datalogger (Decagon Devices, Inc., Pullman, WA, USA) that collected data, once per hour, throughout the experimental period.

2.3. Physiological measures

During the imposition of the two treatments, the plant water status was weekly monitored around midday in sunny days. For each variety, four fully expanded leaves per treatment (one for each subplot) were bagged and covered with aluminium foil 1 h before the measurement, and then excised with a razor blade. Thereafter, a Scholander pressure bomb (Soil Moisture Co., Santa Barbara, USA) was used to assess the midday stem water potential (Ψ_s). Moreover, gas exchanges were measured with an infra-red gas analyser (LI-6400 XT, LI-COR, Inc., NE, USA), using a constant light intensity ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$), CO₂ concentration ($400 \mu\text{mol mol}^{-1}$), and ambient temperature and humidity.

2.4. Hormonal profiling

During both years, 3 leaves (the youngest fully developed leaf from the first or the second cane) from each plot were collected, wrapped in aluminium foil, flash-frozen in liquid nitrogen directly in field and stored in dry-ice until reaching the laboratory. Samples were collected in two time points in each of the seasons from the beginning of water stress imposition (at 32 and 65 DOE in 2018, and at 16 and 49 DOE in 2019). Leaf samples were ground to a fine powder and stored at -80 °C for subsequent analysis. Frozen samples (ca 10–20 mg FW) were extracted by 100 μl cold extraction solvent (1 M formic acid). Isotope labelled standards (10 pmol/sample) were added to samples: ¹³C₆-IAA, ²H₄-OxIAA, ²H₄-OxIAA-GE (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA), ²H₄-SA, ²H₂-GA₁₉ (Sigma-Aldrich, St. Louis, MO, USA), ²H₃-PA, ²H₃-DPA (NRC-PBI), ²H₆-ABA, ²H₅-JA, ²H₅-tZ, ²H₅-tZR, ²H₅-tZRMP, ²H₅-tZ7G, ²H₅-tZ9G, ²H₅-tZOG, ²H₅-tZROG, ¹⁵N₄-cZ, ²H₃-DZ,

$^2\text{H}_3\text{-DZR}$, $^2\text{H}_3\text{-DZ9G}$, $^2\text{H}_3\text{-DZRMP}$, $^2\text{H}_7\text{-DZOG}$, $^2\text{H}_6\text{-iP}$, $^2\text{H}_6\text{-iPR}$, $^2\text{H}_6\text{-iP7G}$, $^2\text{H}_6\text{-iP9G}$, $^2\text{H}_6\text{-iPRMP}$ (Olchemim s.r.o., Olomouc, Czech Republic). Plate SPE Oasis HLB 96 (10 mg/well, Waters Corporation, Milford, MA, USA) was activated by 100 μl 100 % acetonitrile, followed by 100 μl H_2O and 100 μl 1 M HCOOH . Supernatant was applied to SPE-wells. Pellet was reextracted by 100 μl 1 M HCOOH , shaken, centrifuged and supernatant was applied to the same SPE-well. Wells were washed with 100 μl H_2O and samples were eluted from wells twice by 50 μl 50 % acetonitrile using a Pressure+ 96 manifold (Biotage, Uppsala, Sweden). Phytohormones were separated on a Kinetex EVO C_{18} column (2.6 μm , 150 \times 2.1 mm, Phenomenex, Torrance, CA, USA). Mobile phases consisted of A - 5 mM ammonium acetate and 2 μM medronic acid in water, and B - 95:5 acetonitrile:water (v/v). The following gradient program was applied: 5% B in 0 min, 7% B in 0.1 min–5 min, 10%–35% B in 5.1 min–12 min, 100% B in 13 min–14 min, and 5% B in 14.1 min. Hormone analysis was performed with a LC/MS system consisting of UHPLC 1290 Infinity II (Agilent Technologies, Inc., Santa Clara, CA, USA) coupled to a 6495 Triple Quadrupole Mass Spectrometer (Agilent Technologies). MS analysis was done in MRM mode, using the isotope dilution method.

Data processing was performed with Mass Hunter software B.08 (Agilent Technologies).

2.5. Gene expression

In order to analyse the gene expression, 100 mg of fine leaf powder from sets of 4 samples spanning the stress phase (32, 47, 59, 65 DOE in 2018 and 16, 28, 49, 59 DOE in 2019) were used to extract total RNA using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA), following manufacturer's instructions. The quantity of the extracted RNA was checked with a NanoDrop 1000® spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA, USA). 1 μg of RNA was used to synthesize cDNA with QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany). qRT-PCR was performed using CFX96 Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA) and SsoFast™ EvaGreen® Supermix (Bio-Rad). The qRT-PCR cycle profile was set as follows: one cycle of 2' at 95 C°; 40 cycles of 5" at 98 C° and 5" at 56 C°. Primers used to detect gene expression are listed in Table S1. All quantifications were normalized to Ubiquitin1 housekeeping gene (Bogs

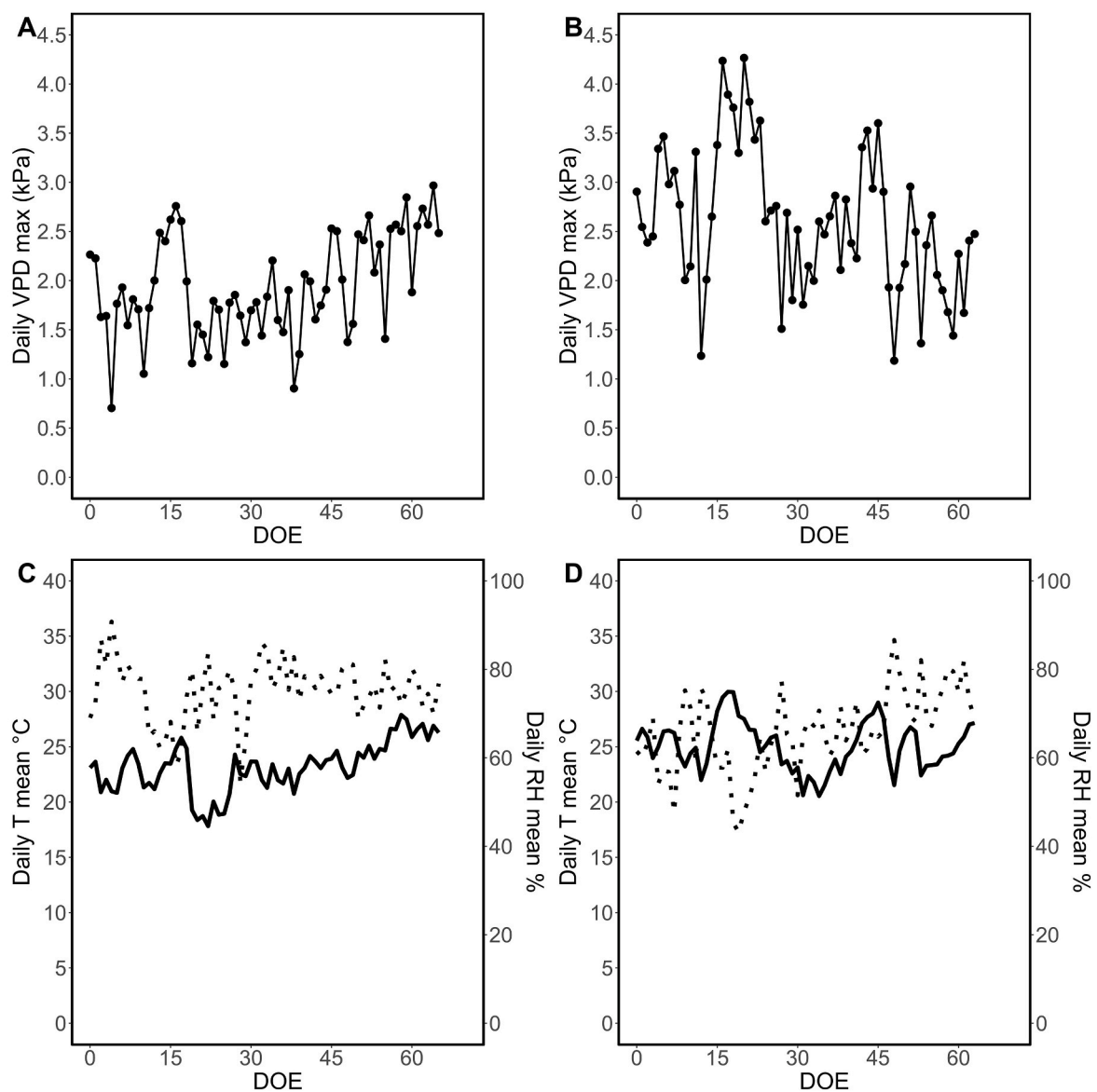


Fig. 1. On the top, daily maximum vapour pressure deficit (VPD_{max}) values calculated in the two years, 2018 (A) and 2019 (B). On the bottom, daily mean temperature (solid line) and daily mean relative humidity (dotted line) recorded in the same periods in 2018 (C) and 2019 (D). Time on x-axis is expressed as days after the beginning of the experiment (DOE).

et al., 2005) with the $2^{-\Delta\Delta Ct}$ method. In addition, the expression of Actin1 (Tashiro et al., 2016) was tested as a second housekeeping gene, highlighting a high reliability of the former.

2.6. Statistical analysis

Data of physiological parameters and gene expression were analysed using one-way ANOVA (*ns* = not significant, while *, **, ***, represent significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). Hormonal profile data were instead processed through two-way ANOVA, with irrigation treatment and variety as factors; when the interaction between factors was significant, means were separated using Tukey's HSD test ($p < 0,05$). All data were analysed separately for each date. Principal Component Analysis (PCA) was performed combining active compounds of the hormones of the five considered classes, separately for the two seasons. All statistical analysis and graphs were carried out using the R software (R Core Team, 2022; version 4.2.1) and the packages agricolae (1.3.5), rstatix (0.7.0), scales (1.2.1), cowplot (1.1.1), factoextra (1.0.7), FactoMineR (2.6), corrplot (0.92), ggpubr (0.5.0) and ggplot2 (3.3.6).

3. Results

3.1. Meteorological conditions and soil water content

In both years, the experimental period spanned from the first half of June and the first half of August. During these two seasons, we observed substantial differences in terms of temperature (Fig. 1 C, D), thus affecting the maximum vapour pressure deficit (VPD_{max}) values (Fig. 1 A, B). In detail, in 2018 the highest VPD was recorded in August, at the end of the experimental period. Conversely, in 2019, exceptionally high temperatures coupled with lower values of daily mean relative humidity were recorded in June and July. In summary, the VPD_{max} was generally lower in 2018 (consistently below 3 kPa) than in 2019, when occasional peaks of VPD_{max} exceeded 4 kPa.

On the other hand, soil water content analysis highlighted a progressive dehydration trend in the soil under the NIR treatment, compared to IR treatment (Fig. S1). Moreover, soil dehydration appeared to occur more rapidly in 2019, probably due to the higher values of VPD_{max} (Fig. S1 B, D). Rainfall data were not reported or discussed as they had a relevance only in case of the IR treatment

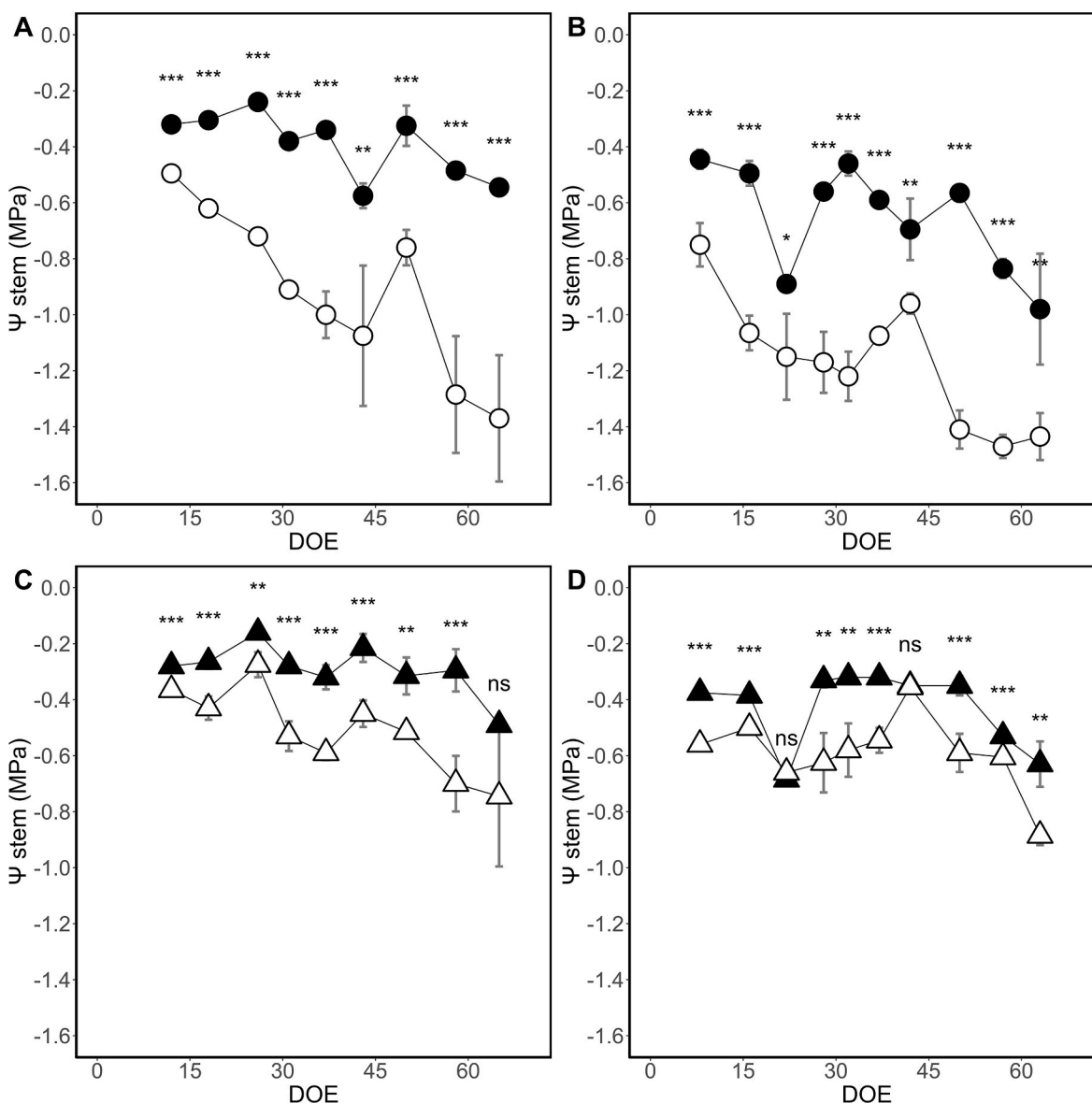


Fig. 2. Evolution of Ψ_s during the experiment in Merlot Kanthus (A, B) and Sauvignon Kretos (C, D) in the seasons 2018 (A, C) and 2019 (B, D). Full symbols represent irrigated treatment (IR) while empty symbols represent non-irrigated treatment (NIR). Vertical bars indicate standard deviation.

(weekly irrigated), while, for the NIR treatment, the plastic covering the ground, prevented rain infiltration.

3.2. Plant water status and leaf stomatal conductance

The stem water potential was separated according to years, treatments and varieties (Fig. 2). In both years, we observed the significant negative effect on Ψ_s due to the NIR treatment, in both varieties. While in 2018 (Fig. 2 A, C), the decrease occurred more gradually throughout the experimental period, water stress was more severe and manifested in a shorter time in NIR plants in 2019 (Fig. 2 B, D). The stressful meteorological conditions in 2019 also affected the Ψ_s values in the IR treatment, resulting in a reduction of Ψ_s at 22 DOE and in the latter part of the season. In 2018, IR treatments constantly maintained favourable Ψ_s values, with Merlot Kanthus vines never dropping below -0.6 MPa. Conversely, Sauvignon Kretos Ψ_s remained always above -0.5 MPa. Interestingly, despite the similar and expected effect of water shortage on water status, in both years, the NIR treatment exhibited stronger effects on Merlot Kanthus, resulting in lower Ψ_s values attained more rapidly, as compared to Sauvignon Kretos for which even values not

significantly different from the IR treatment have been observed.

Overall, extreme and challenging water stress conditions were avoided in both seasons, and the lowest recorded values of Ψ_s measured were -1.4 MPa in Merlot Kanthus vines at the end of the experimental period, across both seasons. For Sauvignon Kretos, the minimum Ψ_s values recorded were -0.7 MPa and -0.9 MPa in 2018 and 2019, respectively.

Regarding the measurements of leaf stomatal conductance (g_s), both cultivars showed lower values under NIR treatment conditions (Fig. 3 A, B, C, D) as compared to IR plants. Nonetheless, Merlot Kanthus consistently displayed much lower g_s values under NIR conditions than Sauvignon Kretos, which could maintain g_s values comparable to IR-treated plants for a long time period during the experimental season, especially in 2018 (Fig. 3 C). Interestingly, absolute g_s values were higher in Sauvignon Kretos, regardless treatment, with the minimum of $170 \text{ mmol H}_2\text{O m}^{-2}\text{s}^{-1}$ reached at 42 DOE in 2019, while in the first year of observation g_s values never dropped below $200 \text{ mmol H}_2\text{O m}^{-2}\text{s}^{-1}$. Conversely, in Merlot Kanthus the g_s decreased during NIR treatment to values typical of severe drought at 65 and 22 DOE, in 2018 and 2019, respectively. The net photosynthesis (A) (Fig. S2) and leaf transpiration

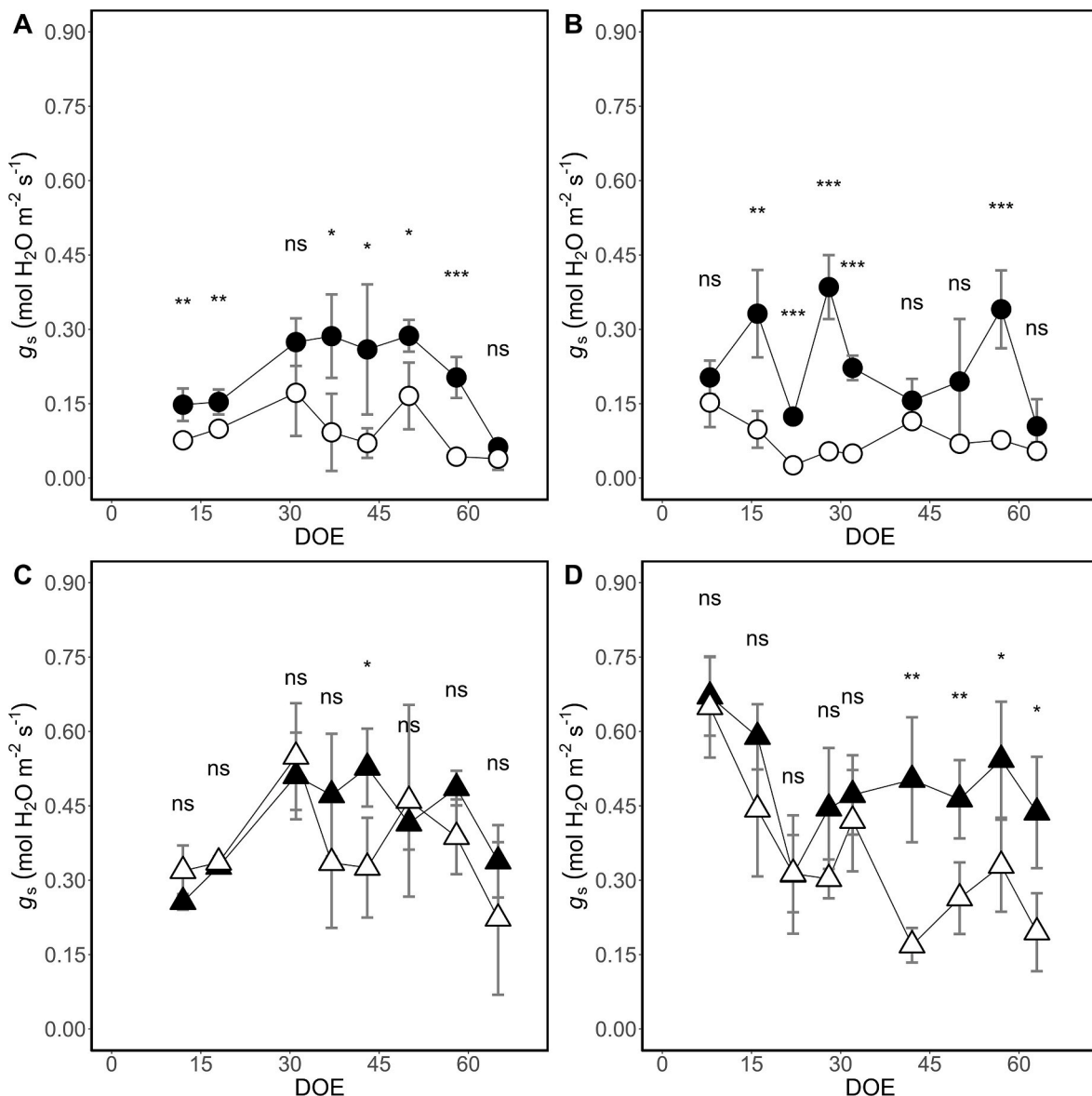


Fig. 3. Dynamics of stomatal conductance (g_s) in Merlot Kanthus (A, B) and Sauvignon Kretos (C, D) in 2018 (A, C) and 2019 (B, D). Full symbols represent irrigated treatments (IR) while empty symbols present non-irrigated treatments (NIR). Vertical bars indicate standard deviation.

(E) (Fig. S3), mirrored the trend of g_s . Thus, Sauvignon Kretos constantly displayed higher A and E than Merlot Kanthus, when comparing the same treatment. Finally, the intrinsic water use efficiency (*iWUE*) (Fig. S4), calculated as the ratio between A and g_s , demonstrated that, mainly in Merlot Kanthus, water-stressed plants were more efficient than irrigated ones. In Sauvignon Kretos, the *iWUE* was consistently low throughout the season, and the difference between treatments was evident only in 2019, in the final stages of the experiment.

3.3. Leaf hormonal profile

The phytohormonal profile was determined from leaves collected at two time points per year: at the onset of stress (at 32 DOE in 2018 and 16 DOE in 2019) and at the maximum stress level (at 65 DOE in 2018 and 49 DOE in 2019). Samples in 2019 were collected earlier than in 2018 due to the faster stress evolution caused by meteorological conditions. In the two earlier sampling points, we observed a limited influence of the irrigation treatments, whereas the main differences were related to the variety. Conversely, significant differences for both irrigation treatment and variety were found at the second sampling point of both seasons (Table 1). Moreover, two-ways ANOVA highlighted several interactions between the two factors that need to be taken into account as shown by the means separation by Tukey's HSD (Table S2). Examining the effects of main factors, and focusing on the irrigation treatments, NIR samples collected during the first stage of water stress, showed increased levels of ABA as an active compound (statistically significant in 2019) and also as relative metabolites (ABA met) (statistically significant in both years). At the subsequent sampling, NIR showed significant higher concentrations of both ABA and ABA met in both seasons. As regard to the interaction between factors, in the season 2019 a significant higher concentration of ABA was ascertained for the Merlot Kanthus NIR treatment as compared to IR. More significant interactions were

observed for ABA met, with NIR treatment reporting significantly higher values in case of Merlot Kanthus (except for 65 DOE in 2018), and only a slight increase for Sauvignon Kretos (Table S2).

Regarding auxins (IAA), inconsistent differences between varieties in both seasons and at two sampling times were observed, even if a tendential increase of both IAA and IAA metabolites (IAA met) was measured in late samplings for Sauvignon Kretos (Table 1).

Sauvignon Kretos reported significant lower concentrations of active cytokinins (act CKs) in both years and for both sampling times, and the same result (although not significant at 65 DOE in 2018) was ascertained for the CK metabolites (CK met). As regard to irrigation treatments, the act CKs significantly increased in both years at the later sampling time as a consequence of water stress, while, as for the CK met, only a negligible change was observed. When examining the interaction effects, the aforementioned increase of act CKs was significant only for Merlot Kanthus, while no effect was observed for Sauvignon Kretos.

As far as Jasmonic acid (JA) and the JA metabolites (JA met) are concerned, higher concentration were measured in case of Sauvignon Kretos, at the last sampling time in both seasons, and both compounds were enhanced in NIR treatment, although significant differences were only obtained for JA met mainly at the later samplings.

At last, salicylic acid (SA) showed significantly higher concentrations in Sauvignon Kretos as compared to Merlot Kanthus, while no differences caused by irrigation treatment were observed (Table 1).

In summary, the results obtained highlighted strong variety and treatment effects on the phytohormonal profiles. ABA, JA and their metabolites were strongly enhanced by water stress, while no change in SA content was detected. However, variety-dependent responses were also observed, with higher concentrations of ABA and CK in Merlot Kanthus and higher levels of IAA, JA and SA in Sauvignon Kretos (Table 1).

To better understand the overall effect of the hormones data,

Table 1

Phytohormonal profiles in leaves of cultivar Merlot Kanthus (MK) and Sauvignon Kretos (SK) under irrigation (IR) and non-irrigation (NIR) treatment. Data are presented as mean values expressed as pmol g^{-1} DW (leaf dry weight). Significant differences between treatments, varieties and both factors (T. x V.) were calculated with two-ways ANOVA and are indicated by asterisks.

Year 2018	DOE 32							DOE 65						
	Treatment			Variety			T. x V.	Treatment			Variety			T. x V.
	IR	NIR	Sign.	MK	SK	Sign.		IR	NIR	Sign.	MK	SK	Sign.	
ABA	749	1074	ns	928	896	ns	ns	1006	2025	***	1883	1147	**	ns
ABA met	21301	35688	***	37533	19456	***	***	28629	46367	**	53764	21233	***	ns
IAA	70	62	ns	43	88	**	ns	44	56	ns	48	52	ns	ns
IAA met	214	206	ns	237	183	**	ns	247	211	*	212	247	*	***
act CKs	14	16	ns	25	6	***	ns	13	20	***	23	10	***	*
CK met	230	250	ns	364	115	***	ns	388	325	ns	373	341	ns	ns
JA	369	570	ns	220	718	***	ns	415	965	***	284	1097	***	***
JA met	415	968	***	779	604	*	***	535	1507	***	603	1439	***	***
SA	1135	1152	ns	923	1364	***	ns	915	1107	ns	747	1276	***	ns
Year 2019	DOE 16							DOE 49						
	Treatment			Variety			T. x V.	Treatment			Variety			T. x V.
	IR	NIR	Sign.	MK	SK	Sign.		IR	NIR	Sign.	MK	SK	Sign.	
ABA	1775	3059	**	3210	1625	***	**	1365	2549	**	2444	1471	*	ns
ABA met	24635	35986	**	38924	21697	***	*	25096	45440	***	46351	24185	***	***
IAA	65	105	ns	95	75	ns	ns	62	93	*	51	104	**	ns
IAA met	200	206	ns	234	171	***	*	230	216	ns	220	226	ns	ns
act CKs	17	17	ns	26	8	***	ns	16	20	**	26	10	***	**
CK met	131	125	ns	172	83	***	*	217	215	ns	294	138	***	ns
JA	336	760	ns	487	609	ns	ns	261	438	*	161	537	***	ns
JA met	766	2305	ns	1363	1708	ns	ns	3084	3796	*	1737	5143	***	ns
SA	1094	1190	ns	967	1317	*	ns	1765	1718	ns	1020	2464	***	*

Notes: MK, Merlot Kanthus; SK, Sauvignon Kretos; ABA, abscisic acid; ABA met, sum of dihydrophaseic acid, phaseic acid, neophaseic acid, abscisic acid glucosyl ester, 9'-hydroxyabscisic acid and 7'-hydroxyabscisic acid; IAA, indole-3-acetic acid; IAA met, sum of indole-3-acetyl aspartate, 2-oxindole-3-acetic acid, 2-oxindole-3-acetic acid glucosyl ester, indole-3-acetyl-1-glucosyl ester; act CKs: *trans*-zeatin, isopentenyladenine; CK met, sum of *trans*-zeatin riboside, dihydrozeatin riboside, isopentenyl adenosine, *cis*-zeatin riboside, *trans*-zeatin-O-glucoside, *trans*-zeatin riboside-O-glucoside, dihydrozeatin riboside-O-glucoside, *cis*-zeatin-9-glucoside, *cis*-zeatin-O-glucoside, *cis*-zeatin riboside-O-glucoside, *trans*-zeatin riboside monophosphate, isopentenyl adenosine monophosphate and *cis*-zeatin riboside monophosphate; JA, jasmonic acid; JA met, sum of jasmonic acid methyl ester and jasmonic acid-isoleucine; SA: salicylic acid.

Principal Component Analysis (PCA) was employed using all the data of the active forms (except for JA) of the five considered hormonal classes (data of hormone metabolites were excluded to avoid redundancy). The sum of the two eigenvalues accounted for the 74.43% of the variability of the entire dataset (Fig. 4). The analysis well addressed that the variety is the main driver of the differences ascertained, with Sauvignon Kretos exhibiting the higher concentrations of IAA, JA and SA, while Merlot Kanthus showed higher values of act CKs and ABA. Although not distinctly delineated, the correlation with the hormones was much stronger when considering the NIR samples.

3.4. Expression of candidate genes in leaves

Candidate genes involved in ABA signalling and encoding aquaporins (AQPs) were selected for qRT-PCR analyses of samples collected at four time points, in both years for all treatment and variety combinations. The selected genes included one RCAR homolog (*VvRCAR6*) that is an ABA receptor well known to mediate the response to ABA-dependent signals, component of the PYR/PYL/RCAR receptor chain (*Pyrobactin Resistance 1/PYR1-like/Regulatory Components of ABA Receptors*) and one homolog of the PP2C-type protein phosphatases (*VvPP2C9*) that is an ABA regulator. Four aquaporins belonging to the PIPs family and two to the TIPs family (Shelden et al., 2009) were also considered for expression analysis. The logarithmic fold-change (Log_2 NIR/IR) between irrigation treatments, calculated for all four sampling dates, in 2018 and 2019, in both cultivars, is shown in Fig. 5.

VvRCAR6 exhibited downregulation in response to NIR treatment across all the considered dates and in both varieties. The downregulation was more pronounced in Merlot Kanthus. In contrast, *VvPP2C9* displayed an opposite trend, with both varieties showing upregulation of this gene due to water-limiting conditions. The upregulation with NIR treatment was stronger in Merlot Kanthus as compared to Sauvignon Kretos.

VvPIP1;1 was not significantly affected by the water regime except in the last date of 2018 in Sauvignon Kretos, where it was downregulated by water stress. *VvPIP2;1* was mainly downregulated in Merlot Kanthus, while in Sauvignon Kretos was more stable except for the last sampling point in 2018 when it showed a significant downregulation. *VvTIP2;1* reported a trend similar to *VvPIP2;1*. In fact, in case of Merlot Kanthus the gene was mainly downregulated, whereas in Sauvignon Kretos the

gene expression was quite stable and decreased only in the last date of 2018. *VvPIP2;2* was unaffected or slightly upregulated in both varieties, and reported a significant upregulation in Sauvignon Kretos only, at 28 DOE in 2019. *VvPIP2;3* showed only a significant downregulation in Sauvignon Kretos, in the last date of 2018.

VvTIP1;1 displayed a consistent trend of significant upregulation in Merlot Kanthus over the two years, which was also observed in Sauvignon Kretos in 2018. However, in 2019, the trend of the same gene was different in Sauvignon Kretos, starting with downregulation on the first date followed by an increasing trend throughout the experiment, although without statistical significance.

The basal expression levels in IR plants showed a much higher expression of *VvPIP2;2* and *VvTIP2;1* in Sauvignon Kretos as compared to Merlot Kanthus, whereas other tested genes exhibited a similar expression level (data not shown).

4. Discussion

In water stress experiments, Ψ_s value is considered a good indicator for the plant water status since it is related both to the soil water availability and to the evaporative demand of the atmosphere (Choné et al., 2001). PIWI varieties play a well-established role in viticulture, thanks to their resistance to fungal diseases, offering a hopeful alternative to conventional cultivars. Nevertheless, understanding of how these varieties respond to abiotic stressors, such as drought conditions, remains limited. In this field experiments, Merlot Kanthus and Sauvignon Kretos displayed a similar control over g_s , both upon water stress and in condition of sudden increase in transpiration demand (i.e. increase of VPD_{max}). For example, a noteworthy example is what we observed at 22 DOE in 2019, where VPD_{max} increased fourfold and g_s strongly decreased in both varieties regardless of the treatment. In fact, it is widely recognized that VPD increases plant transpiration and soil water evaporation, affecting plant water status (Williams and Baeza, 2007) and inducing stomatal closure by hormonal and hydraulic signals in leaves (Merilo et al., 2018). Thus, high VPD exacerbates the negative effects on plant physiology associated to drought (Grossiord et al., 2020).

Although, as expected, water stress leads to a reduction in g_s in both the varieties, they exhibited markedly different absolute levels of gas exchange, seldom exceeding $0.30 \text{ mol H}_2\text{O m}^{-2}\text{s}^{-1}$ in Merlot Kanthus

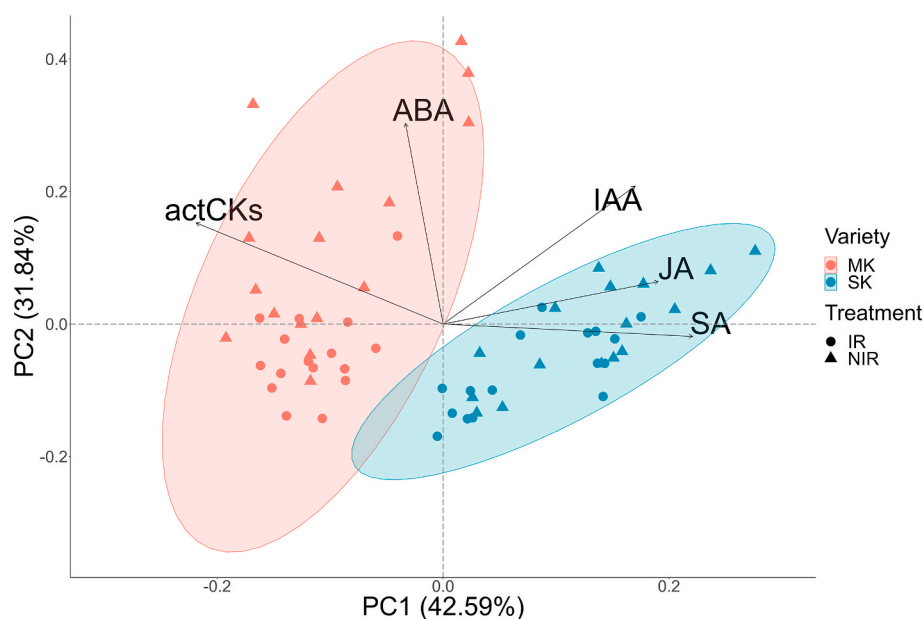


Fig. 4. Principal Component Analysis (PCA) projecting the single leaf replicate of each date analysed by the influence of the five hormones classes (ABA, IAA, act CKs, JA and SA) considered in this study. Abbreviations: MK, Merlot Kanthus; SK, Sauvignon Kretos.

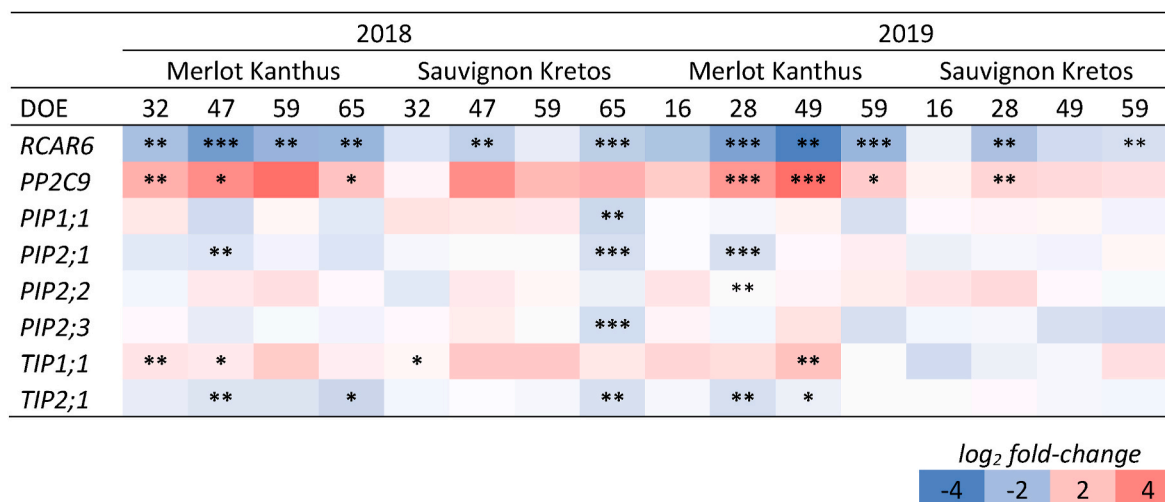


Fig. 5. Heatmap representing the logarithmic fold-change ($\text{Log}_2 \text{ NIR/IR}$) of the eight considered genes. Comparisons of the relative expression of the gene with treatment as factor were performed using one-way ANOVA. Statistically significant differences are reported.

and mostly above that level in Sauvignon Kretos.

In the two-year study period, very different meteorological conditions were observed. However, the two varieties investigated showed consistent behaviour and a clearly different aptitude to modulate Ψ_s under water shortage. The water stress conditions affected Merlot Kanthus more severely and at an earlier stage compared to Sauvignon Kretos.

Taken together, g_s and Ψ_s data in NIR treatments, give rise to a paradigmatic situation apparently conflicting with the well-established belief that plants experiencing water stress close their stomata in order to preserve hydration (i.e. higher Ψ_s values) for longer periods and, as opposite, plants that maintain active gas exchanges reduce faster the Ψ_s . These two distinctive ways to cope with water stress are identified as isohydric (pessimistic) and anisohydric (optimistic) behaviour, respectively (Tardieu and Simonneau, 1998). In the present study, in both years Sauvignon Kretos constantly exhibited higher values of g_s and of Ψ_s than Merlot Kanthus, under both irrigation regimes. In this context, we can consider Sauvignon Kretos as a variety showing higher resilience to water stress as compared to Merlot Kanthus. A behaviour similar to Sauvignon Kretos was observed by Bianchi et al. (2020) on the drought tolerant rootstocks 140Ru and 41B.

On the other hand, differences in stomata sensitivity to drought among grapevine cultivars has already been reported in the literature (Lavoie-Lamoureux et al., 2017) and seasonal osmotic adjustment in the leaves of grapevines can shift their sensitivity to drought towards lower (more negative) water potentials (Linhart et al., 2023).

One commonly proposed explanation for this behaviour could involve the increase of WUE and reduction in the total leaf area. Nonetheless, the increase of $iWUE$ in Sauvignon Kretos NIR was narrow as compared to what observed in Merlot Kanthus. In addition, the total leaf area was similar between varieties in IR plants and in NIR treatment Sauvignon Kretos recorded even tententially higher values than Merlot Kanthus in both years (Table S3).

We noticed that the primary factor influencing the leaf hormonal profile was the variety rather than the irrigation treatment. It is not surprising that the effect of ABA is predominant, and correlates with NIR replicates (mostly located in the upper part of the PCA graph). This finding is intriguing as it indicates that the two varieties, which vary in their tolerance to water stress, are not differentiated by ABA, the hormone commonly associated with drought response. This suggests that the different degree in tolerance of the two varieties is probably ABA-independent. In literature, the different modulation of ABA was demonstrated to be associated to the differences existing between isohydric and anisohydric behaviours (Tardieu and Simonneau, 1998; Soar

et al., 2006). This is consistent with the suppressor effect of ABA on stomatal conductance, ascertained in various plant species (Kollist et al., 2014). In the present study, it is difficult to assign to the two varieties a clear hydraulic behaviour in terms of isohydricity, while the different water stress tolerance degree is undeniable. In the second sampling point, when stress conditions were more severe, the two varieties reported a different modulation in CKs and JA. Interestingly, in literature, CKs, showing highest levels in Merlot Kanthus, have been reported to act as negative regulators of plant acclimation to drought (Li et al., 2016; Jogawat et al., 2021). Similarly, actCKs are normally reduced in leaves exposed to water stress (Havlová et al., 2008; Verslues, 2016), due to an antagonistic action of ABA on their biosynthesis (Huang et al., 2018). In addition, under oxidative stress, actCKs were associated to the promotion of the scavenging of reactive oxygen species (ROS) (Brenner et al., 2012). In the light of this observation, the increase of actCKs detected in Merlot Kanthus could reflect the reduced resilience or could be a response to the oxidative stress caused by the strong and prolonged inhibition of g_s and A in this variety.

Furthermore, it has been demonstrated that IAA, JA, and SA exhibit beneficial effects when plants are subjected to water stress. According to Shi et al. (2014), the application of exogenous IAA proved to be advantageous for *Arabidopsis* plants experiencing drought stress, and IAA concentration could be a proxy of the antioxidant activity (Cha et al., 2015).

JA and SA have been generally related to the response to biotic stresses, nonetheless recent studies on different plant species highlighted their role as important regulators also in abiotic stresses (Santisree et al., 2020; Haider et al., 2017; Abeer et al., 2021; Sadeghipour, 2018). In particular, SA application was shown to increase the carotenoids to cope with the oxidative stress, in grapevine leaves experiencing water stress (Abbaspour and Babae, 2017). Moreover, exogenous SA application on leaves increased tolerance to heat and cold stress in grapevine, promoting Ca^{2+} homeostasis and antioxidant activity (Wang and Li, 2006). Mitigation of negative effects of drought was also ascertained in case of SA application in *Eucalyptus globulus* Labill (Jesus et al., 2015) and annual crops such as maize (Saruhan et al., 2012) and barley (Habibi, 2012).

In summary, information from other plant species suggest that Sauvignon Kretos hormonal asset could be more suitable to cope with water limiting conditions as compared to Merlot Kanthus. Although the understanding of the physiological mechanisms involved may be challenging, we can hypothesize that these hormones play a role in Sauvignon Kretos' greater resilience to drought.

Consistently with ABA accumulation pattern, the two genes

VvRCAR6 and VvPP2C9 involved in the ABA perception and signalling, were not differently modulated in the two varieties when considering the contextual variation in stress intensity experienced by Merlot Kanthus and Sauvignon Kretos. In particular, the expression observed in NIR treatment, downregulated and upregulated respectively, is in agreement with what was previously reported in grapevine (Boneh et al., 2012; Rossedeutsch et al., 2016). These findings could partially support the speculation of a difference in the drought tolerance degree, between the two varieties, that is not ABA-based.

According to the functional characterization of grapevine AQPs (Shelden et al., 2009), VvPIP1 type AQPs have very low water permeability if compared to VvPIP2 type, while VvTIP1; 1 and VvTIP2; 1 have an intermedium water channel activity (between VvPIP1 and VvPIP2), that is higher in the second one. Based on our findings, it can be argued that the main differences between varieties are most probably related to VvPIP2;1, VvPIP2;2 and VvTIP2;1, which exhibit the highest expression levels and possess a great water channel activity. Anyway, the relative expression of VvPIP2;2 did not show a consistent pattern over the two years, making interpretation difficult. Pou et al. (2013) studied the expression of the same AQPs examined in our study in Chardonnay leaves subjected to a cycle of water stress and recovery. They found that the relative expression of VvPIP2;1 and VvTIP2;1 correlates with K_{leaf} . In our experimental conditions, both these AQPs were downregulated in NIR plants, particularly in Merlot Kanthus, indicating a decrease in K_{leaf} in this variety. The lower K_{leaf} in Merlot Kanthus may also be related to the higher ABA content observed in these plants. Indeed, ABA is known to reduce K_{leaf} by downregulating AQPs in bundle sheath, thereby reducing membrane water permeability and inhibiting water flow from the xylem to the apoplast (Shatil-Cohen et al., 2011; Pantin et al., 2013). In addition, Scoffoni et al. (2012) pointed out that an adequate K_{leaf} is necessary to sustain a good leaf water potential and therefore maintain stomata opened.

Moreover, Pou et al. (2013) observed that VvTIP2;1 expression was correlated also with g_s . Interestingly, Sauvignon Kretos exhibited both higher VvTIP2;1 expression and higher g_s than Merlot Kanthus and their correlation is statistically significant (p value < 0.001) (Fig. S5). Taken together, the differences in the relative expression of target AQPs could partially explain and support the greater ability of Sauvignon Kretos to maintain higher g_s and a better plant water status under water limiting conditions. Since stomatal conductance and photosynthesis are closely related processes in plants, the carbon net assimilation (A) mirrors the different physiological response of the two varieties, showing low susceptibility to water stress (particularly during the 2018 season with milder meteorological conditions) and generally higher absolute levels in Sauvignon Kretos.

5. Conclusions

Grapevine is an important perennial crop cultivated worldwide and a plant model for physiological studies. In this study, we examined two new PIWI varieties from various perspectives, revealing contrasting hydraulic behaviours in a two-year field experiment.

Despite our incomplete understanding of the physiological mechanisms involved, we have accumulated substantial data supporting the role of hormones and aquaporins in the distinct performances under water stress observed in two poorly studied new varieties.

Our results suggest that the observed differences in drought tolerance are not attributable to variations in the modulation of ABA during water stress. Conversely, the modulation of cytokinins (act CKs) and jasmonic acid (JA) following prolonged water deprivation may play a pivotal role in the performance disparities between Sauvignon Kretos and Merlot Kanthus. In addition, differences in the expression of target AQPs could support a most favourable hydraulic behaviour for Sauvignon Kretos in water limiting conditions.

CRedit authorship contribution statement

Riccardo Braidotti: Data curation, Investigation, Methodology, Writing – original draft. **Rachele Falchi:** Supervision, Conceptualization, Investigation, Writing – review & editing. **Alberto Calderan:** Investigation, Methodology. **Alessandro Pichierri:** Data curation, Formal analysis. **Radomira Vankova:** Data curation, Investigation, Methodology, Writing – review & editing. **Petre I. Dobrev:** Methodology. **Michaela Griesser:** Writing – review & editing. **Paolo Sivilotti:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jplph.2024.154243>.

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