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# Anthocyanin and proanthocyanidin evolution during ripening of the Refošk grapevine is modulated by deficit irrigation

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## Summary

The effects of two deficit irrigation regimes (50% $ET_c$ and 20% $ET_c$) on skin and seed phenolic compounds of the red *Vitis vinifera* L. cultivar 'Refošk' were studied during the veraison – maturation period. The study was undertaken for two successive seasons in the Karst area of North-East Italy, where rainfall is scarce and subterranean drainage limits surface water. To trace the effect of water deficit on both phenol biosynthesis and berry size reduction, phenols were extracted from lyophilised, cryo-milled skins and seeds using organic solvents and evaluated in skin and seeds, per units of both dry and fresh weight. The anthocyanin concentration was higher in 20% $ET_c$ early after veraison, whereas at harvest the difference was not significant. A decreasing trend with some fluctuations in proanthocyanidin (PA) concentration in skins and seeds was found during maturation with both water regimes. 20% $ET_c$ resulted in a decrease in skin PA concentration and increase in seed PA concentration during ripening. As regards PA structural characteristics, there was a trend for higher galloylation and polymerisation of seeds and skins during ripening with the 20% $ET_c$ irrigation regime, whereas the effect on prodelphinidins in skins was inconsistent. In 2018, when most of the differences in vine water status between irrigation regimes occurred during veraison – maturation, the concentrations of PAs were affected more significantly than the mean degree of polymerisation and percentage of galloylation. In contrast, in 2019, when differences in vine water status occurred in the pre-veraison-maturation period, the structural characteristics of PAs were affected more significantly than concentrations, especially galloylation of skins and polymerisation of seeds. The results of the study showed that the biosynthesis of PAs, their polymerization and galloylation can be significantly modulated by water deficit regimes applied in the vineyard, in combination with the meteorological course of the season.

## Keywords

**Vine water stress, polyphenol biosynthesis, proanthocyanidin polymerization, galloylation**

## Introduction

By the end of this century, climate scenarios predict an intensification of extreme meteorological conditions, with a reduction in rain mainly in the central months of the growing season in Italy (Faggian, 2021) and elsewhere in Europe (Rajczak and Schär, 2017). The lack of water during the summer months (longer periods without rain at different phenological stages, also coupled with heat waves and high VPD), will create water stress conditions in plants, particularly for grapevines, reducing yield and affecting grape quality.

Among grape metabolites, phenolics are key compounds affecting red wine quality. At berry level, water stress alters skin and seed phenolic compounds because of both berry size reduction and changes in biosynthesis and extractability. Several studies have shown that moderate water stress conditions are useful for improving the biosynthesis of secondary metabolites in both red and white varieties (Acevedo-Opazo *et al.*, 2010; Castellarin *et al.*, 2007; Savoi *et al.*, 2017, 2016; Sivilotti *et al.*, 2005; Van Leeuwen *et al.* 2009). Starting at berry-set, several classes of phenols accumulate in berry tissues until harvest, namely anthocyanins, flavan-3-ols and proanthocyanidins (PAs) the main flavonoids represented in grapes. Different experiments have been carried out during the last few decades, highlighting the effect of water stress on yield (Acevedo-Opazo *et al.*, 2010; van Leeuwen *et al.* 2009; Yang *et al.*, 2022) and also on secondary metabolism. In detail, water stress increases the concentration of anthocyanins (Càceres-Mella *et al.*, 2017; Calderan *et al.*, 2021; Ojeda *et al.*, 2002; Petruzzellis *et al.*, 2022; Zarrouk *et al.*, 2012), but also promotes a shift towards tri-substituted and methoxylated monomers, related to an upregulation of *VvF3'5'Hs*, *VvAOMTs* and *VvMYBAs* genes (Castellarin *et al.*, 2007; Savoi *et al.*, 2017). As regards PAs, Genebra *et al.* (2014) and Càceres-Mella *et al.* (2017) highlighted that water stress increased the concentration of flavan-3-ols, and also upregulated *VvANR*, *VvLARs* and *VvMYBPAs* genes. As compared to anthocyanins, Bucchetti *et al.* (2011) showed that the impact of water stress on PAs was much lower, and also not always sig-

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nificant, depending on the season. During maturation several structural and compositional changes in PAs make evaluation of their total concentration much more difficult to determine (Garrido-Bañuelos *et al.*, 2022). Bindon *et al.* (2012, 2010) and Gagné *et al.* (2006) reported higher cell-wall adsorption of PAs with a higher degree of polymerisation at the end of maturation. Bindon *et al.* (2014) examined modifications to skin cell-walls using the scanning electron microscopy technique, revealing that the surface was relatively smooth at veraison, but there was an increase in cell wall porosity during ripening which might promote the sequestration of more polymerised PAs within pores. More polymerised PAs might bind to cell-walls thanks to a higher number of hydrogen bondings, thus becoming unextractable even in strong organic solvents. Concentration, degree of polymerisation, and cell wall porosity were reported as factors that affect skin PAs' extractability in both wine-like medium and strong organic solvents (Bindon *et al.*, 2014). So-called total extraction of phenols is usually performed with lyophilised ground skins and seeds using two-step extraction, first with aqueous acetone and then aqueous methanol (Chira *et al.*, 2009). Both biological factors and analytical limitations impact the contrasting trends in skin and seed proanthocyanidin evolution during grape ripening. Moreover, there is no evidence of the enzymes involved in the polymerisation of flavan-3-ols, while Bontpart *et al.* (2015) discovered a couple of putative genes responsible for the galloylation of PAs. Evaluating the effects of two levels of water deficit, generating moderate to severe water stress during two seasons, Calderan *et al.* (2021) reported that at harvest time the concentration of PAs in both skins and seeds extracted in wine-like and organic solvents was not significantly modified by the degree of water deficit. On the other hand, the most evident differences were shown in the galloylation of PAs; skin and seed PA galloylation significantly increased in both tissues with 20% $ET_c$ water deficit.

Vine water status is a key factor in grape ripening and vintage quality for red wines. Therefore, the objectives of this research were to investigate the effects of two irrigation regimes, 50% $ET_c$ and 20% $ET_c$, on the evolution of berry weight, total soluble solids, acidity, and polyphenols during berry ripening of the red *V. vinifera* 'Refošk'. The effects of two water deficit regimes on both the concentration and structural characteristics of proanthocyanidins from seeds and skins extracted in organic solvents were studied over two consecutive growing seasons.

## Material and Methods

### Location, plant material and experimental design

An experiment was conducted during the 2018 and 2019 growing seasons in a *Vitis vinifera* L. 'Refošk' vineyard located in Sagrado (Karst, North-East Italy), in the Friuli Venezia Giulia region. The vineyard, planted in 2007 with 'Refošk' clone ISV F2 grafted on 110 Richter, has a plant density of 4273 plants/ha (2.6 m between rows and 0.9 m between plants) and is managed using the single Guyot training system. An irrigation system was installed along six vineyard rows to create two different irrigation regimes: 50% $ET_c$ (supplying 50% of crop evapotranspiration, $ET_c$) and 20% $ET_c$ (supplying 20% of $ET_c$). Water was supplied by a IRRITEC MULTIBAR drip irrigation system (Irritec S.p.A., Rocca di Caprileone, Messina, Italy), with 0.6 m emitter distance and a water volume of 2.1 $L \cdot h^{-1}$. Irrigation was applied 1–2 times per week, depending on rainfall and on the calculated $ET_c$. Three replicates were randomly selected along the central row of each treatment, and for each replicate 20 homogeneous vines were labelled to collect physiological measurements, yield parameters, and berry samples. $ET_c$ was computed using Penman–Monteith, applying a $K_c$ as proposed by Lopez-Urrea *et al.* (2012), and meteorological data were obtained from the Gradisca d'Isonzo weather station (ARPA FVG–OSMER, http://www.meteo.fvg.it/). Irrigation started on 26 June in both the 2018 and 2019 seasons, and the water applied during the summer accounted for 45.6 and 18.1 mm in 2018 and 76.1 and 34.6 mm in 2019, for the 50% $ET_c$ and 20% $ET_c$ treatments, respectively (Fig. S1). In 2018 irrigation was carried out only twice before veraison, with 5.0 and 1.8 mm of water for 50% and 20% $ET_c$ respectively, while after veraison, there were four interventions, with a total of 40.6 and 16.3 mm for the same treatments. A different situation can be observed in the following season in 2019, when irrigation was carried out 5 times before and 2 times after veraison (Fig. 1).

### Stem water potential

Plant water status was monitored during the summer by measuring the stem water potential ($\Psi_{stem}$). Four fully expanded (mature) leaves were bagged and covered with aluminium foil between 12:00 and 14:00 (solar time). After 1 h, the leaves were excised with a razor blade, and immediately wrapped in cling film. The application of cling film has been shown to allow more reliable measurement of $\Psi_{stem}$ (Tomasella *et al.*, 2023). $\Psi_{stem}$ was measured by placing the leaves in a Scholander-type pressure chamber (Soil Moisture Corp., Santa Barbara, USA) with the petiole protruding from the chamber. After pressurising the chamber using compressed nitrogen, $\Psi_{stem}$ was recorded within a few seconds after the initial xylem sap emerged from the cut end of the petiole. For each season, the values of $\Psi_{stem}$ were computed to also calculate the integral of the accumulated water deficit using the model proposed by Myers (1988).

### Berry sampling and basic analysis of grape juice

During the 2018 and 2019 seasons, samples of 80 berries per plot were collected from veraison until harvest. A set of 40 berries for each replicate was weighed and immediately stored at −80 °C for the analysis of phenolic compounds. The remaining berries were manually pressed at room temperature. Total soluble solids (°Brix) and pH were measured using a manual refractometer (ATC-1, Atago, Tokyo, Japan) and a pH meter (HI2211, Hanna Instruments, Woonsocket, RI), respectively. Titratable acidity ($g \cdot L^{-1}$ tartaric acid equivalents) was determined by titrating the juice with 0.1 M NaOH until pH 7.

### Extraction of seed and skin polyphenols

Anthocyanins and PAs were extracted from ground skins and seeds with organic solvents, as described in Calderan *et al.*

| **flowering-veraison** | **veraison-harvest** |
|---|---|
| Rain = 178 mm | Rain = 168 mm |
| Irrigation<br>50% $ET_c$ = 5.0 mm<br>20% $ET_c$ = 1.8 mm | Irrigation<br>50% $ET_c$ = 40.6 mm<br>20% $ET_c$ = 16.3 mm |

| **flowering-veraison** | **veraison-harvest** |
|---|---|
| Rain = 126 mm | Rain = 178 mm |
| Irrigation<br>50% $ET_c$ = 58.0 mm<br>20% $ET_c$ = 25.7 mm | Irrigation<br>50% $ET_c$ = 18.1 mm<br>20% $ET_c$ = 8.9 mm |

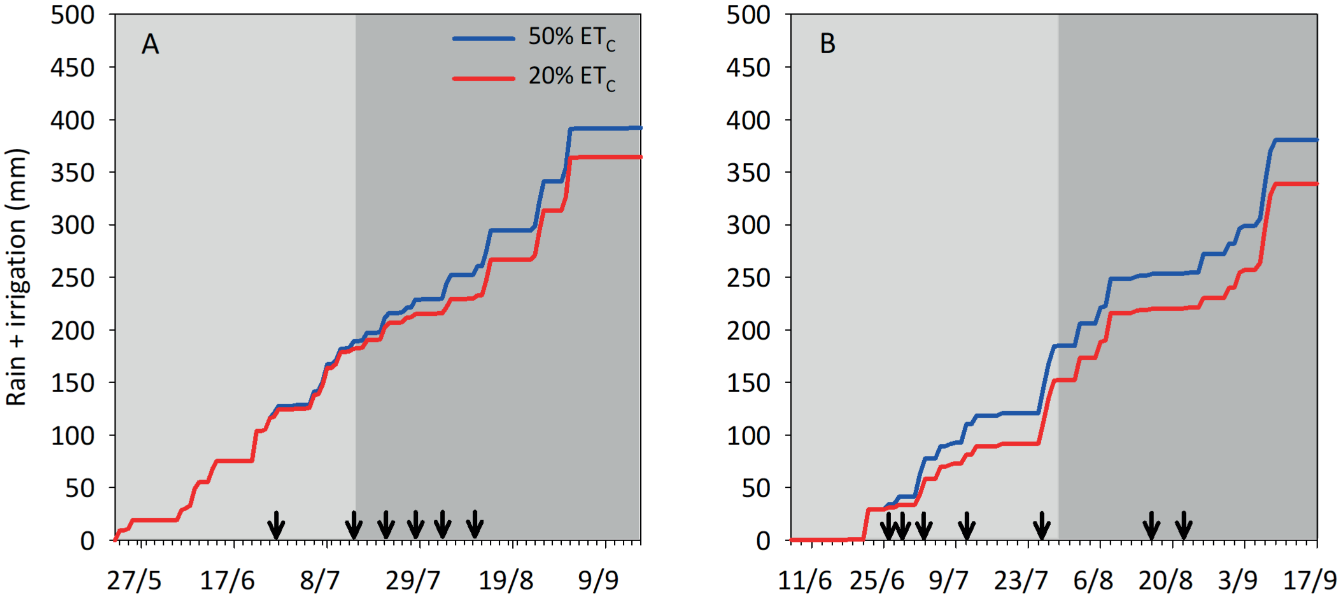

Fig. 1: Trends for mean, min and max temperatures, and rain during the 2018 (A) and 2019 (B) seasons. Pale grey shading shows the period between flowering (EL 23) and veraison (EL 35), while the dark grey shading relates to the period between veraison and harvest (EL 38). Data recorded at the Gradisca d'Isonzo weather station (ARPA FVG–OSMER, http://www.meteo.fvg.it/).

(2021). Briefly, the skins and seeds of frozen berries were separated, then immediately dropped into liquid nitrogen, cryomilled and lyophilised. Lyophilised skin or seed powder was extracted in the ratio 1:9 (w/v) with two-step extraction, first with 80% aqueous acetone, followed by 60% aqueous methanol. Supernatants were pooled, evaporated, and the residue was re-dissolved in water and freeze-dried to obtain a crude extract.

## *Spectrophotometric analysis*

Analysis of phenol content in lyophilised crude extracts of seeds and skins was performed with an Agilent 8453 spectrophotometer (Agilent Technologies Inc., Palo Alto, USA). The method reported in Rigo *et al.* (2000) was used without the solid phase extraction step, as described by Sivilotti *et al.* (2020).

Total anthocyanins (TAs) were determined based on maximal absorbance in the visible range (536 to 542 nm). The results were expressed against the corresponding blank in mg·g$^{-1}$ of skin DW.

The Vanillin Index (VAN) provides a good estimation of flavanols and low molecular weight proanthocyanidins corresponding to two to four units. VAN was evaluated as (+)-catechin in mg·g$^{-1}$ of skin or seed DW and mg·g$^{-1}$ of berry FW.

High molecular weight proanthocyanidins (PAs) were evaluated by transformation into cyanidin. The method is a highly specific assay, which provides a good evaluation of total PAs, and is linked mainly to variations in PAs corresponding to at least five units (Vrhovsek *et al.*, 2001).

## *UHPLC-DAD-MS/MS analysis of seed and skin proanthocyanidin structural characteristics*

The structural characteristics of PAs, comprising mean degree of polymerisation (mDP), percentage of galloylation (%G), and percentage of prodelphynidines (%P), were determined after acid-catalysed degradation with phloroglucinol (Drinkine *et al.*, 2007; Kennedy and Jones, 2001), as described in detail by Calderan *et al.* (2021). Crude seed or skin tannin extracts were diluted in methanol, and an aliquot was mixed with phloroglucinol reagent (Sigma Aldrich, Steinheim, Germany) and ascorbic acid (Merck, Darmstadt, Germany). The mixture was kept at 50 °C for 20 min for fractionation and the reaction was stopped with aqueous sodium acetate. Samples were then filtered through a 0.22 µm PVDF filter from Millipore (Billerica, MA, USA) and analysed using UHPLC-DAD-MS/MS (Agilent Technologies, Santa Clara, USA) as described by Lisjak *et al.* (2020) and Sivilotti *et al.* (2020). mDP, %P, and %G were estimated using the response factors of PA cleavage products at 280 nm and calculated as described in Kennedy and Jones (2001). The mDP value represented the molar ratio between the sum of all flavan-3-ol units produced by phloroglucinolysis and the sum of terminal units.

### *Statistical analysis*

Separate data for each sampling date were analysed using the *t*-test (*p* indicated). SigmaPlot 13 (Systat Software GmbH, Erkrath, Germany) was used to test for significant differences between treatments and to draw the figures.

## Results

### *Weather conditions, phenology and plant water status*

The trends for temperatures and rain distribution were different in the two seasons, thus affecting phenology, plant water status and grape maturation. In 2018, temperatures were consistently high throughout the season, only decreasing when periods of rain occurred (Fig. 2). In detail, in April most of the rain was concentrated in the first half of the month, while in May, significant rainfall was registered from 15 May onwards. Several episodes of rain were also recorded during the summer months. From 4 to 22 June, there was heavy rainfall on two occasions, with over 70 mm and 90 mm respectively, and 3 further significant episodes of rainfall occurred between the middle of August and the beginning of September, accounting for 37, 48 and 53 mm, respectively. After 25 August, temperatures decreased by ca 5 °C, and showed a reduction after 22 September. In this season, the most critical phenological stages were advanced, thus flowering and veraison occurred on 21 May and 13 July respectively.

The meteorological trend in 2019 was very different. At the beginning of April, several episodes accounted for a total of 50 mm of water. However, from 23 April to 12 May, rainfall totalled over 150 mm, and temperatures remained as low as at the beginning of April. The average temperature in May 2019 was 12.7 °C, namely 7 °C lower than in the previous season, and was responsible for the delay in flowering and veraison, recorded on 7 June and 29 July, respectively. After this period, June was characterised by a lack of rain, with only one significant event on 22 June. Compared to May, temperatures rose by around 9 °C; the average temperature in June was 2.5 °C higher than in 2018. There was scattered rain in the first half of July, with a significant three-day event from 26 to 28 July, and temperatures were comparable to June. In August, there was major rainfall at the beginning of the month (with about 80 mm in three days), with little precipitation recorded afterwards. The mean temperatures remained similar to July, while a reduction in maximum temperatures was observed. From the beginning of September there was intense rainfall, mainly from 7-9 September, and a drop in mean temperature of ca 5.5 °C was recorded, determining the need to harvest early.

As already described in Calderan *et al.* (2021), in both seasons, meteorological conditions were responsible for moderate water stress during the summer period. On 26 June 2018 the $\Psi_{stem}$ values were -0.71 and -0.75 MPa for 50% and 20% $ET_C$, respectively; in 2019, on the same date the same treatments reported $\Psi_{stem}$ values of -0.86 and -0.90 MPa, respectively. In 2018, weak-to-moderate water stress conditions occurred with both irrigation levels, while in the following period $\Psi_{stem}$ dropped down below -1.2 MPa in the case of 20% $ET_C$ (Fig. S1A), with a recovery in the following period approaching harvest. In 2019, $\Psi_{stem}$ was also low in June, but during the season never reached the lowest values registered in the previous season (Fig. S1B). Examining the trend for the water stress integral in the 2018 season (Fig. S1C), this parameter reached 10 MPa days approximately between the last ten days of July and the first few days of August, while only the 20% $ET_C$ treatment exceeded 20 MPa days by the middle of August. In contrast, in 2019 the water deficit integral reached higher values, exceeding 10 MPa days at the beginning of July, 20 MPa days between the end of July and

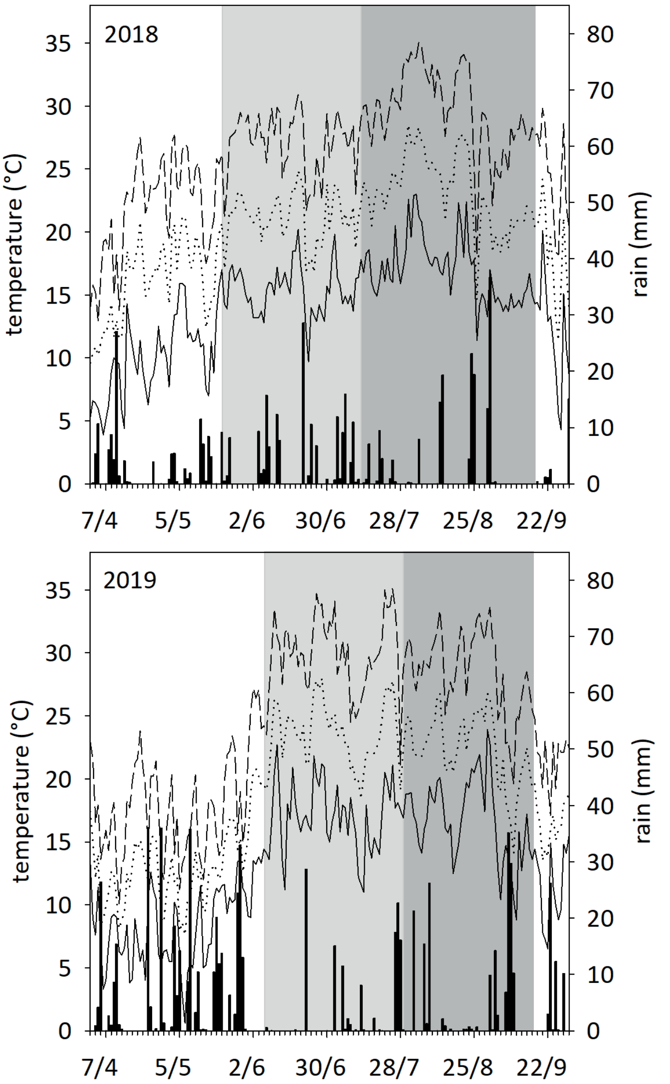

Fig. 2: Cumulative trend for rain + irrigation with the two deficit irrigation strategies during the 2018 and 2019 seasons. Rainfall data recorded at the Gradisca d'Isonzo weather station (ARPA FVG–OSMER, http://www.meteo.fvg.it/). The arrows at the bottom show the irrigation timings. Pale grey shading shows the period between flowering (EL 23) and veraison (EL 35), while the dark grey shading relates to the period between veraison and harvest (EL 38). The tables at the top show separately the amount of rain in the two different periods and the water in the two periods with the two deficit irrigation strategies.

the beginning of August, and even 30 MPa days by the middle of August (Fig. S1D).

### *Basic weight, basic maturation parameters and anthocyanins*

Berry weight was significantly affected by water deficit during both seasons, with lower values for the 20% $ET_c$ treatment, and the effect was much greater in 2019 (Figs. 3A, 3B). The trends for total soluble solids (Figs. 3C, 3D) and titratable acidity (Figs. 3E, 3F) were instead slightly affected by water deficit.

The anthocyanin concentration (mg·g$^{-1}$of skin DW) increased in the first stages after veraison (Figs. 3G, 3H). Thereafter, there was a reduction until harvest in both seasons. Moreover, there was significantly higher biosynthesis of anthocyanins in the skins of the 20% $ET_c$ treatment (first two timepoints) compared to the 50% $ET_c$ treatment. By the second sampling date, the skin concentration was still 32% and 27% higher in the case of 20% $ET_c$ in 2018 and 2019, respectively. At harvest, the concentrations of anthocyanins in mg·g$^{-1}$of skin DW did not significantly differ between the treatments. However, when these data were calculated in mg·g$^{-1}$ of berry FW the relative difference in the skin-to-berry ratio for the two deficit irrigation treatments also showed a significantly higher anthocyanin concentration in 20% $ET_c$ at harvest (as reported in Calderan *et al.*, 2021).

### *Skin proanthocyanidins*

The trend in skin PA concentration showed a reduction from veraison to harvest as expressed by both mg·g$^{-1}$ of berry FW and mg·g$^{-1}$ of skin DW. In 2018, monomers and low molecular weight PAs (VAN; Fig. S2) and PA concentrations (Figs. 4C, 4D) were lower in case of the 20% $ET_c$ deficit irrigation treatment. Significant differences during maturation were ascertained for PAs near veraison but not at harvest. A similar trend was observed in 2019, with a steep decrease in monomers and low molecular weight PAs (VAN). The differences between the two deficit irrigation treatments during ripening in 2019 were never statistically significant.

The mDP of skin PAs was slightly increased during maturation and variable when ripening progressed, with non-significantly higher values in 20% $ET_c$ in both seasons (Figs. 4E, 4F). The same trend was also observed for %G; on a few sampling dates between veraison and harvest, significantly higher %G was found in 20% $ET_c$ (Figs. 4G, 4H). As regards %P, no significant differences between treatments were ascertained during maturation in both seasons (Fig. S3).

### *Seed proanthocyanidins*

The monomeric and oligomeric fraction of PAs (VAN) in seeds decreased during maturation in both seasons, with higher values for 20% $ET_c$ in 2018, whereas in 2019 the differences between treatments in the seed VAN index were not significant (Fig. S4). Significantly higher VAN was ascertained on the first and third dates in 2018 for the 20% $ET_c$ treatment. In 2019, VAN was higher than in 2018. In contrast, the concentration of polymeric PAs was relatively stable during maturation in 2018, and significantly higher concentrations were measured in the 20% $ET_c$ treatment on all dates, but not at harvest. in 2019, the differences in seed PA concentrations between treatments were not significant (Fig. 3B).

As regards PA structure, in 2018 only the first date showed significantly higher values for mDP and %G for the 20% $ET_c$ treatment (Figs. 3E, 3G). In 2019 the mDP and %G were higher in the case of the 20% $ET_c$ treatment on all sampling dates, although the difference was not always significant (Figs. 3F, 3H). In both years, galloylation and the mean degree of polymerisation of seed PAs increased during ripening and dropped near maturation.

## Discussion

Deficit irrigation represents an optimal strategy to enhance grapevine resilience to water shortage while maintaining sustained yield and a good grape quality. In our experiment, we investigated the autochthonous variety 'Refošk' in Karst, an area characterised by shallow clay soil called "Terrarossa". Due to water scarcity in this area, it is not possible to sustain a full irrigation regime. Thus, we compared two levels of water deficit, by examining plant water status during summer as well grape maturation trends in two consecutive seasons characterised by differences in the meteorological conditions, in particular in terms of rainfall distribution.

Nowadays, plant water status can be measured using different techniques, but a considerable number of papers have reported leaf water potential to be a reliable parameter (Deloire *et al.*, 2004; Tomasella *et al.*, 2023). Despite some controversy among researchers regarding the thresholds for this parameter, it is generally accepted that $\Psi_{stem}$ values below -1.4 MPa are critical and can compromise yield, fruit quality and the basic physiological functions of grapevines. As reported in the results section, the experiment carried out in the Karst area never led to critical $\Psi_{stem}$ values, thus excessive water stress was avoided in both seasons. However, the $\Psi_{stem}$ and water stress integral (Fig. S1) demonstrated that water stress differed between the seasons and was longer and more challenging in 2019. It is well-known that the lack of water during the herbaceous phase of berry growth has a significant and irreversible effect on berry weight (Caruso *et al.*, 2022; Ollé *et al.*, 2011; Zsófi *et al.*, 2011). In this experiment, berry weight was lower in 2019 at harvest and significantly reduced in 20% $ET_c$ while the differences between water deficits were not significant in 2018 (Calderan *et al.* 2021) . We could speculate that the distribution of rainfall after veraison in 2018 nearly compensated for the irrigation applied with 20% $ET_c$, thus eliminating the differences between the two treatments at harvest. Conversely, the earlier occurrence of water stress in 2019 promoted a significant irreversible reduction in berry weight that was maintained until harvest.

In both seasons, the deficit irrigation treatments accounted for similar trends in terms of main maturation parameters (Fig. 3). In 2019, the first sample collected revealed delayed maturation compared to the same point in 2018, resulting in lower soluble solids and higher titratable acidity. This crucial

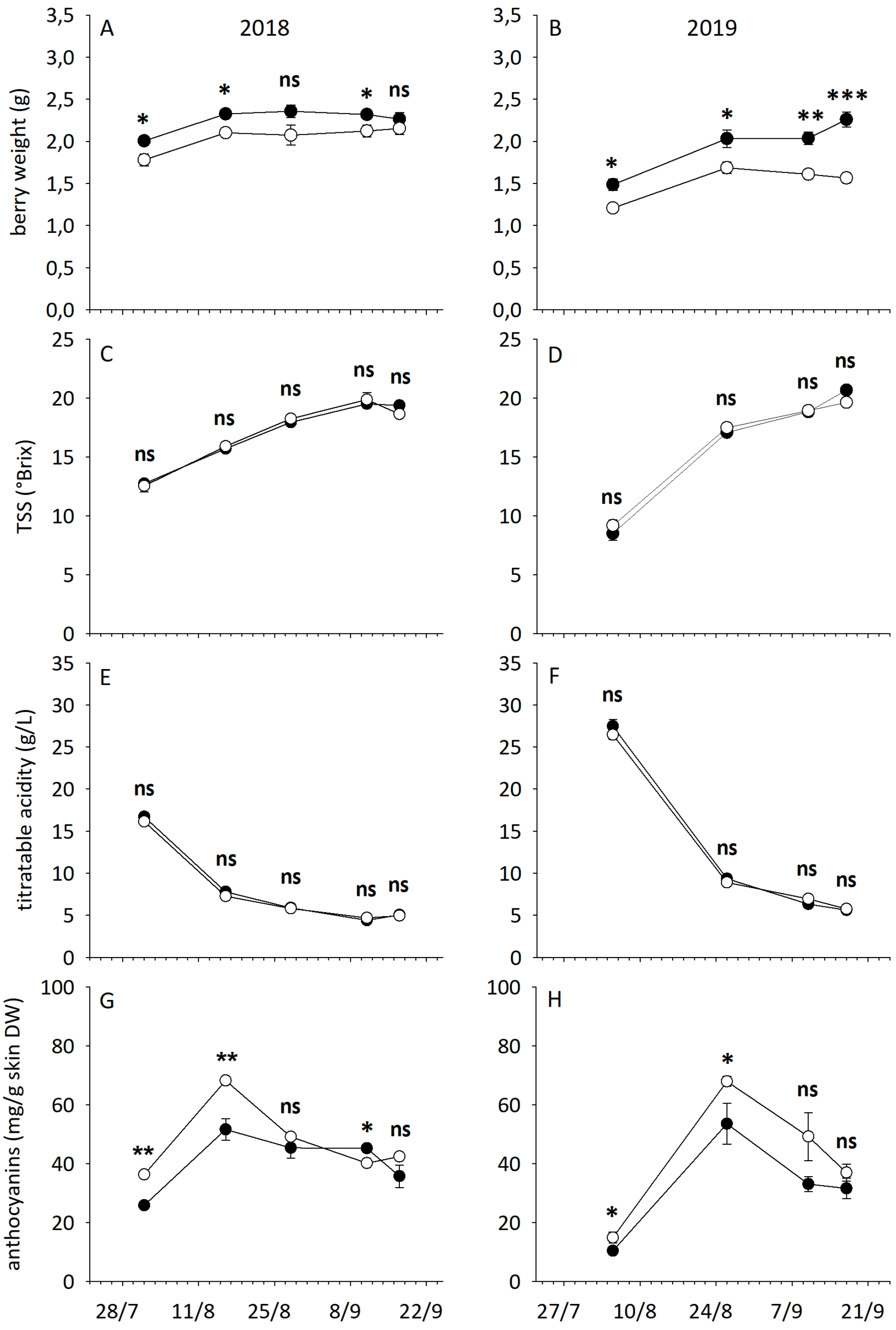

Fig. 3: Trends for berry weight (A,B), total soluble solids (TSS; C,D), titratable acidity (E,F) and anthocyanins (G,H) in 'Refošk' grapevines subjected to 50% ETC (●) and 20% EC (○) in the 2018 (left) and 2019 (right) seasons. Data for individual time-points were analysed with the *t*-test (ns, non-significant; *, **, ***, means significantly different for p<0.05, p<0.01 and p<0.001, respectively). Bars represent standard error (n=3).

aspect could help explain some of the results that have been described in the case of PAs. When comparing well-watered *vs* water-stressed grapevines, different studies have reported contrasting results on the effects of water regime on basic maturation parameters (Gambetta *et al.*, 2020; Mirás-Avalos and Intrigliolo, 2017). Moreover, in our experiment the comparison of two levels of water deficit instead of a fully irrigated *vs* a deficit treatment could be the reason for the negligible differences found between the treatments compared.

In our experiment, the accumulation of anthocyanins was very fast, and after the second sampling date, the concentration was reduced in both seasons (Figs. 3G, 3H). This result was not a surprise since Gómez-Plaza *et al.* (2008), Kyraleou *et al.* (2015), and Theodorou *et al.* (2019) have reported a similar evolution for anthocyanins in different grapevine varieties. Post-veraison water stress leads to an increase in berry sugar accumulation, but the concentration of abscisic acid (ABA) also increases. Both sugars and ABA are signalling molecules promoting gene expression and the protein synthesis involved in the phenylpropanoid pathway in berry skins, leading to the accumulation of flavonols and anthocyanins (Castellarin *et al.*, 2007; Deluc *et al.*, 2007; Pastenes *et al.*, 2014; Villalobos-González *et al.*, 2016). In 2018, after the first two sampling dates, the skin anthocyanin concentration was similar for the two treatments. Conversely, in 2019 the anthocyanin concentration was higher in 20% $ET_c$, but not at harvest. Many experiments conducted worldwide have ascertained that anthocyanin biosynthesis is promoted by limited water availability (Bucchetti *et al.*, 2011; Kyraleou *et al.*, 2017; Sivilotti *et al.*, 2005; van Leeuwen *et al.*, 2009). The concentration of anthocyanins in mg·g$^{-1}$ of berry FW at harvest was much higher for the 20% $ET_c$ treatment than for 50% $ET_c$, due to the higher skin-to-flesh ratio of the berries with the former treatment (as reported in Calderan *et al.* 2021).

Beside anthocyanins, PAs represent a huge class of compounds with specific characteristics in skins (higher mDP, lower %G, higher %P) and seeds (lower mDP, higher %G), and they are responsible for the mouthfeel and colour properties of wines. Modifications occurring during maturation thus have major implications for the quality of the resulting wines. In our experiment, a drop in the content of both VAN (Figs. S2, S4) and PAs (Figs. 4, 5) was ascertained during maturation until harvest in seeds and skins. As regards berry skins, slightly higher levels of both VAN and PAs were found with the 50% $ET_c$ treatment, with a few significant differences in terms of PAs between treatments during maturation in 2018, but not at harvest. These results are in agreement with the outcomes of studies by Kyraleou *et al.* (2017) and Allegro *et al.* (2016). Differences between grapevine varieties, environments and water stress conditions could significantly modify

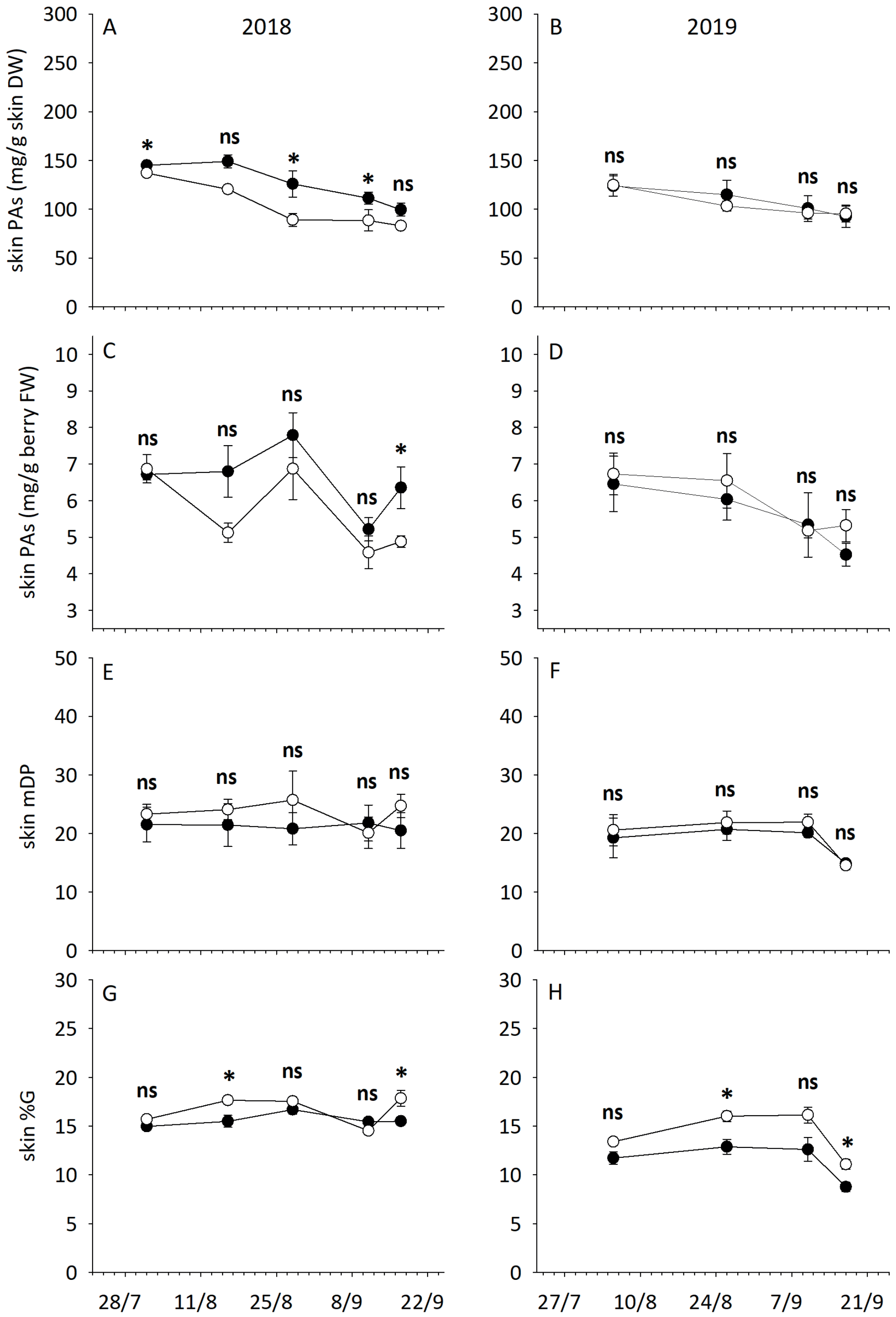

Fig. 4: Trends for low (LMWP; A,B) and high molecular weight PAs (HMWP; C,D), mDP (E,F) and %G (G,H) in 'Refošk' skins subjected to 50% ETC (●) and 20% EC (○) in the 2018 (left) and 2019 (right) seasons. Data for individual time-points were analysed with the *t*-test (ns, non-significant; *, **, ***, means significantly different for p<0.05, p<0.01 and p<0.001, respectively). Bars represent standard error (n=3).

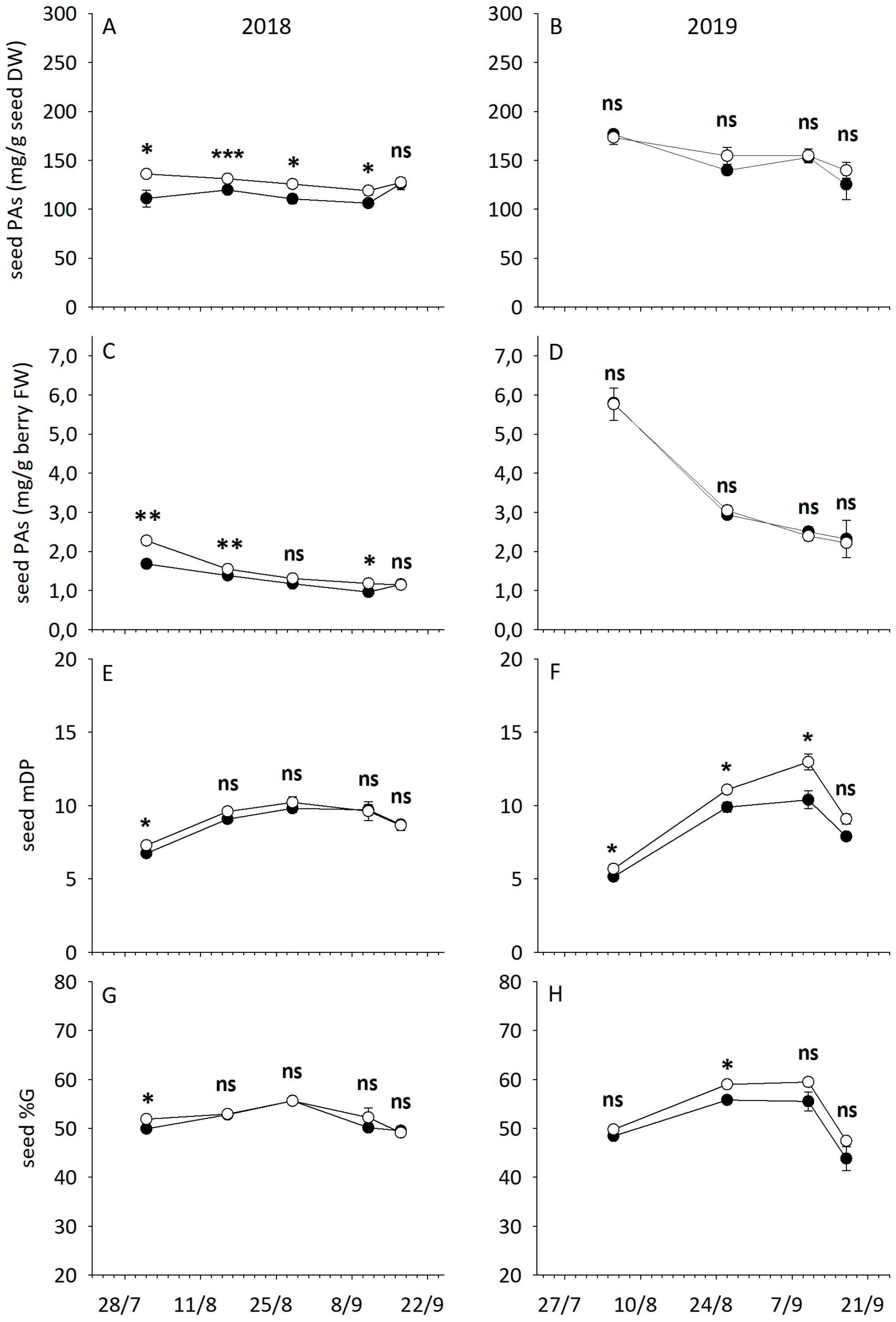

Fig. 5: Trends for low (LMWP; A,B) and high molecular weight PAs (HMWP; C,D), mDP (E,F) and %G (G,H) in 'Refošk' seeds subjected to 50% ETC (●) and 20% EC (○) in the 2018 (left) and 2019 (right) seasons. Data for individual time-points were analysed with the *t*-test (ns, non-significant; *, **, ***, means significantly different for p<0.05, p<0.01 and p<0.001, respectively). Bars represent standard error (n=3).

the biosynthesis, degradation and compartmentation of PAs in grape berry skins following different water stress conditions. The mDP (Figs. 4E-F) and the %G (Figs. 4G-H) of skin PAs was slightly higher in the case of 20% $ET_c$ at different sampling times in both years. Moreover, significantly higher %G was ascertained in both seasons for the 20% $ET_c$ treatment at harvest time. As already reported in relation to the concentration of skin tannins, the results reported in different experiments on both the concentration and structural composition of PAs are inconsistent (Bautista-Ortín *et al.*, 2012; Kennedy and Jones, 2001; Kyraleou *et al.*, 2017; Ollé *et al.*, 2011). At two different stages during maturation in both seasons, galloylation was significantly promoted in the 20% $ET_c$ treatment. Thanks to the studies of Bontpart *et al.* (2015) and Braidotti *et al.* (2024), we know that galloylation of flavan-3-ols occurs early during maturation. The differences between treatments in 2019 (Fig. 4H) could be related to the earlier water stress experienced by the vines. The mDP of skin PAs tends to increase during maturation, but with the progression of ripening a certain fraction of skin PAs is no longer extractable (Bindon *et al.*, 2010; Bindon *et al.*, 2014), as already explained in the introduction. Therefore, during the progression of maturation, sequestration of the more polymerised PAs could explain mDP variation around the similar value.

As regards the biosynthesis of seed PAs, this occurs between the beginning of berry-set and veraison, while thereafter there is a decline until harvest (Cadot *et al.*, 2006; Downey *et al.*, 2003; Genebra *et al.*, 2014). Irrigation experiments carried out worldwide have given inconsistent results on both the change in concentration and the structural characteristics of seed PAs (Chacon *et al.*, 2009; Kennedy *et al.*, 2000; Yu *et al.*, 2016). In the present experiment, the concentration of VAN (Fig. S3) and PAs (Fig. 5A-D) was higher in 20% $ET_c$, with a trend for reduction up to harvest in both seasons, in agreement with Yu *et al.* (2016). Interestingly, Chacon *et al.* (2009) highlighted that the change in seed PA concentration was related to the intensity of water limitation. Even if the water stress experienced by the vines was stronger in 2019 in this experiment, the irrigation treatments did not show significant differences in PA concentration during maturation and at harvest (Fig. 5B, 5D). On the other hand, greater differences were shown regarding the structural characteristics of PAs. Indeed, both mDP (Fig. 5F) and %G (Fig. 5H) were higher in 20% $ET_c$, and these last results agree with Kyraleou *et al.* (2017). Differences in both PA concentrations and structural characteristics might be connected to the advanced maturation promoted by higher water stress. Indeed, at harvest time, seed colour in 20% $ET_c$ regime was significantly darker than in 50% $ET_c$ (Calderan *et al.*, 2021) and similar findings were reported by Mihelčič *et al.* (2023). It should be noted that some PAs are subject to oxidation, and another fraction reacts with cell walls. Moreover, water deficit and seasonal conditions influence the understanding of PA dynamics, due to modulation of the reaction mentioned above.

## Conclusions

In this study, the effects of two deficit irrigation regimes (50% $ET_c$ and 20% $ET_c$) on the evolution of phenols during grape ripening were evaluated over two growing seasons in the Refošk grapevine. With both regimes, skin and seed proanthocyanidin concentrations decreased from veraison towards ripening. The decrease was more substantial in the case of monomers and low molecular weight proanthocyanidins, since this fraction is subjected to polymerisation during grape ripening. The 20% $ET_c$ deficit irrigation treatment between veraison and harvest had a strong impact on grape quality, by promoting anthocyanin and seed proanthocyanidin biosynthesis and promoting the polymerisation and galloylation of both skin and seed proanthocyanidins. The different meteorological trends during the two seasons resulted in a different impact of the deficit irrigation applied on proanthocyanidins; in 2018 the 20% $ET_c$ deficit irrigation treatment caused an increase in proanthocyanidin concentration, while in 2019 the same treatment did not show modifications in proanthocyanidin concentrations, but promoted changings in the their structural characteristics The results obtained suggest that relatively small differences in vine water status during grape ripening strongly affect the potential of grapes to produce high quality red wines.

## Supplementary Material

Supplementary Material can be found online: https://doi.org/10.5073/vitis.2024.63.12

## Conflicts of interest

The authors declare that they do not have any conflicts of interest.

## Author Contributions

Conceptualisation, PS, KL and AV; experimental set up, PS and ACa; data collection, PS, ACa, DB, EP, AF and MV; data analysis, PS and AV; statistical analysis, PS; writing-original draft preparation, PS and AV; writing-review and editing, all authors; supervision, PS. All authors have read and agreed to the published version of the manuscript.

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