

Article

Eco-Physiological Traits and Phenylpropanoid Profiling on Potted *Vitis vinifera* L. cv Pinot Noir Subjected to *Ascophyllum nodosum* Treatments under Post-Veraison Low Water Availability

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Abstract: In Mediterranean regions, extreme weather conditions during the growing season may alter grapevine physiology and metabolism, thus modifying the quality of wines. The objective of this study was to investigate the effects of *Ascophyllum nodosum* treatments on plant physiology and berry metabolism in *Vitis vinifera* exposed to water stress. The experiment was performed on potted vines subjected to two irrigation regimes (well-watered, WW, and water stressed, WS) both associated with *A. nodosum* treatments (SWE), compared with control plants (CTRL). Gas exchanges, chlorophyll fluorescence, and water relations were monitored on SWE and CTRL leaves, both in WW and WS vines at three times. Moreover, the quantification of secondary metabolites and their partitioning were performed in berry skins. Plants treated with *A. nodosum* extract showed higher photosynthesis and stomatal conductance than CTRL in both irrigation regimes and maintained a better plant hydraulic conductivity at the end of the sampling period. In addition, secondary metabolites in berry skins and their partitioning were significantly affected by the treatments in both irrigation regimes. Our results suggest that foliar application of *A. nodosum* extract may help the acclimation of grapevines to post-veraison water stress, likely improving plant physiological and biochemical performances under environmental constraints.

Keywords: biostimulants; abiotic stress; climate change; drought; water potential; gas exchanges; berry skin metabolites; enzymatic activity; phenylpropanoid pathway

1. Introduction

Soil water deficit is the main environmental constraint for viticulture in the Mediterranean basin [1,2]. Climate projections for this area predict changes in rainfall and seasonal temperature patterns in the next decades, with a higher frequency of extreme climate events [3,4]. These future trends due to global climate change are expected to negatively affect grapevine physiology and grape quality.

Stomatal closure is one of the first responses to drought. It is widely reported that the reduction in carbon fixation due to stomatal limitations is the crucial issue for grapevine performances under mild drought, as indicated by increased water use efficiency (WUE, i.e., the ratio of photosynthesis to transpiration) [5–10]. For more prolonged and/or more pronounced water deficits leading to

decrease of stomatal conductance (g_s) below 50 mmol H₂O m⁻² s⁻¹, photosynthetic limitations becomes more dependent on nonstomatal processes, especially decreased mesophyll conductance to CO₂ and impaired photochemistry [11,12]. Moreover, abiotic stresses, such as drought and high temperatures, influence the primary and secondary metabolism thus accelerating phenological stages (i.e., budburst, flowering, and veraison) and berry ripening [13]. In particular, the synthesis of phenolic compounds and aromatic ripeness in berry skin are delayed, while total sugars increase, thus resulting in wine with low acidity and high alcohol concentration [14–16]. In grape berries, the most abundant polyphenol subclasses are flavonols and anthocyanins [17]. These phenols are especially responsible for stability to oxidation, color, and flavor of wines [18–20]. The total amount of anthocyanins and the relative abundance of single anthocyanins are under genetic control [21]. This means that all cultivars express functional genes coding for enzymes that lead to the biosynthesis of di-hydroxylated (peonidin and cyanidin) or tri-hydroxylated (delphinidin, petunidin, and malvidin) anthocyanins as well as to the methylation of primary anthocyanins. Harsh environmental conditions, such as water shortage, high radiations, and hot temperatures, interacting with genotype, boost the shift of cyanidin and delphinidin (hydroxylated anthocyanins) into their methoxylated counterparts, namely peonidin, petunidin, and malvidin [22,23].

Hence, the cultivation of grapevines in a climate change scenario might require a selection of sustainable management practices to maintain grape quality under the future environmental constraints, like the foliar application of biostimulants [24–26]. Many studies have reported the ability of *Ascophyllum nodosum*-based biostimulants to promote plant growth, yield, and fruit quality [27–29] and to enhance the tolerance of grasses and crops to drought events [30–35]. This broad effectiveness may be ascribed to the high concentration of amino acids found in the *Ascophyllum*-based extracts, which may act as compatible solutes under osmotic stress [36,37], as well as to the presence of bioactive secondary metabolites such as vitamins and their precursors [38,39], that may protect plant tissues against stress-induced reactive oxygen species (ROS) [40]. If compared to the published research on the effects of *A. nodosum* applications on the yield of table grapes [41–44], only a few studies have focused on grapevines responses to abiotic stresses [45,46] and on berry composition [46–50] following seaweed extract treatments. In a previous two-year field experiment, Salvi et al. [46] demonstrated an improvement of gas exchanges and grape maturity balance, induced through the application of *A. nodosum* treatments on the phenylpropanoid pathway. Although these results supported the potential effect of *A. nodosum* in promoting the biosynthesis of anthocyanins and improving water stress tolerance in vines, further experimental evidences conducted under drought controlled conditions are needed to validate these hypotheses. In addition, although considerable evidences are available [46,50], the effect on *A. nodosum*-treated plants phenylpropanoid pathway is currently unclear and only hypothesized.

For these reasons, the aims of this study were (1) to test if *A. nodosum* extract treatments were able to positively affect grapevine physiology promoting higher tolerance to drought in controlled conditions; and (2) to validate the effect on berry phenylpropanoid metabolism, investigating the activity of the enzymes involved in the pathway.

In order to achieve these goals and validate the previous results reported in Salvi et al. [46], the comparison between vines treated with *A. nodosum* extract and nontreated ones was set up on Pinot noir cv. (*Vitis vinifera* L.) potted vines subjected to two irrigation regimes, integrating physiological and metabolic measurements.

2. Materials and Methods

2.1. Experiment Location and Plot Settings

This study was conducted for one growing season (2017) on 11-year-old homogeneous potted vines (average number of leaves/vine 103.5; average shoot length after topping 73.2 cm; average canopy leaf area/vine 1.13 m²) (*Vitis vinifera*) of cv. Pinot noir (clone Entav 115), grafted on 1103 Paulsen rootstock and grown outdoor at CREA-VE, Arezzo, Italy (Lat. 43.476° N, Long. 11.824° E; 260 m a.s.l.). Pots (70 L) were filled with a clay-loam soil with the following average characteristics:

clay 40%; silt 35%; and sand 25%, with a volumetric soil water content of ~34% at field capacity, collected from a vineyard of the Chianti Classico D.O.C.G. district in Tuscany, Italy [51]. The vines were trained on vertical shoot positioned trellis, with spur cordon pruning and an average of 10 buds per vine. Every year, at the end of February, each container was fertilized with 40 g of controlled-release fertilizer (Nitrophoska, 12N–12P–17K, Eurochem Agro, Cesano Maderno (MB), Italy). To avoid excessive soil over warming, which can negatively affect roots, and maintain a stable temperature, pots were protected by wrapping their lateral surface with a shade net.

At the beginning of the experiment (veraison, modified Eichorn and Lorenz (E-L) stage 35; 13 July 2017), 20 plants were maintained at 90% of maximum water availability as controls (WW, well-watered vines) until harvest (E-L stage 38), while the other 20 plants were subjected to a water deficit at 40% of maximum water availability (WS, water-stressed) [52]. The water regime was imposed later in the season, because post-veraison water deficit tends to have greater effects on berry ripening as compared to pre-veraison stress, which, conversely, affects primarily berry size [53]. Until the beginning of treatment, all plants were maintained at field capacity. During water limitation, the surface of the plant containers was covered with aluminum foils to avoid rainfall interference and to minimize soil water evaporation. The water supply per pot was determined monitoring the soil moisture, as volumetric content, by time domain reflectometry using Trase System 1 (Soil Moisture Equipment Corporation, Goleta, CA, USA). Readings were taken every day in the early morning with 30 cm long electrodes located in the pots. In each pot, water was supplied at 2 day intervals with drip irrigation emitters.

During the differential irrigation period, ten of the WW vines and ten of the WS ones were treated twice with 3 g/L of a noncommercial *A. nodosum* extract (SWE) [35,46,49]. This extract is mainly rich in amino acids, among which the most abundant were alanine, phenylalanine, proline, and methionine. A full extract characterization was provided in Salvi et al. [46]. The first application was performed approximately 20 days before the expected harvest (E-L stage 36; 27 July 2017) and another one was repeated after 14 days (E-L stage 37; 10 August 2017) on the same plants. On the same days, the other ten vines of WW and ten vines of WS were sprayed with the same amount of water (CTRL).

At three stages, on 10 vines/treatment, berries samplings and eco-physiological measurements were conducted: t_0 (before *A. nodosum* treatments and one week after irrigation regimes differentiation; E-L stage 36; 20 July 2017), t_1 (7 days after the first *A. nodosum* treatment; E-L stage 37; 3 August 2017), and t_2 (7 days after the second *A. nodosum* treatment; E-L stage 38; 17 August 2017).

2.2. Climate Parameters

Daily values of mean, minimum, and maximum air temperatures ($^{\circ}\text{C}$) and global radiation (Wm^{-2}) values were recorded using a meteorological station (Ecotech, Bonn, Germany) nearby.

2.3. Leaf Gas Exchange, Chlorophyll Fluorescence, Leaf Water Potential, and Content

At t_0 , t_1 , and t_2 , net photosynthesis (P_n), stomatal conductance (g_s), and transpiration rate (E) were measured on ten fully developed and intact leaves per treatment (one each vines, 10 replicates) using a portable infrared gas analyzer (model Ciras 3, PP Systems, Amesbury, MA, USA).

Measurements were carried out at ambient light intensity ($1300 \mu\text{mol m}^{-2} \text{s}^{-1}$) and ambient CO_2 concentration (400 ppm). Water use efficiency (WUE) was calculated as the ratio of photosynthesis to transpiration. The maximum quantum yield of photosystem II (PSII) photochemistry (F_v/F_m) was recorded with a portable fluorometer (Handy-PEA[®], Hansatech Instruments, King's Lynn, UK) on the same leaves used for gas exchanges at t_0 , t_1 , and t_2 . Then, F_v/F_m was calculated as Maxwell and Johnson [54]. At the same stages, leaf predawn (Ψ_{pd} , MPa) and leaf (Ψ_{leaf} , MPa) and stem midday (Ψ_{stem} , MPa) water potentials were determined following Scholander et al. [55] with a pressure chamber (model 600, PMS Instrument Co., Albany, OR, USA). Ψ_{pd} was measured between 04.00 and 05.00 h on ten fully expanded leaves per treatment, whereas Ψ_{leaf} and Ψ_{stem} were measured at 12 p.m. on the same leaves used for leaf gas exchange measurements (leaves over 60-min dark-adapted for Ψ_{stem}). The whole-plant hydraulic conductance (K_{plant}), expressed as the relationship between the

plant water loss by transpiration and the water potential drop from roots to leaves was calculated as $K_{\text{plant}} = E/(\Psi_{\text{pd}} - \Psi_{\text{leaf}})$ [56,57]. Other 10 leaves per-treatment were taken to calculate relative water content (RWC, as $\text{RWC} = ((\text{FM} - \text{DM})/(\text{TM} - \text{DM})) \times 100$ where FM, TM, and DM denote fresh, turgid, and dry masses, respectively).

2.4. Grape Composition and Yield

At t_0 , t_1 , and t_2 , a sample of 50 berries/vine was picked from the clusters of 10 vines (10 berry samples per treatment), weighed (PCE Italia s.r.l digital scale, Capannori (LU), Italy), and juiced. Total sugars ($^{\circ}\text{Brix}$) were determined with a refractometer (ATAGO, Bellevue, WA, USA). Titratable acidity (TA, g L^{-1} tartaric acid) was measured on a 10 mL sample by manual glass burette, titrating with 0.1 M NaOH to an end point of pH 7.0. Juice pH was measured using a portable pH meter (Hanna instrument, Woonsocket, RI, USA). At harvest (t_2), clusters from 10 vines/treatment were picked, computed, and weighed with a portable electronic scale (Bonso Advanced Technology Ltd., Hong Kong, China) to obtain cluster weight (g) and yield per vine (kg).

2.5. Berry Skin Phenylpropanoids

At t_0 , t_1 , and t_2 , flavonol, hydroxycinnamic acid, and anthocyanin contents were determined in berry skins (5 samples/treatment, each composed by a pool of 30 berries collected from 2 of the 10 vines/treatment). The samples were collected randomly within the clusters of the same vine at each sampling time, also assuming that all the vines of the same treatment and irrigation regime were at the same developmental stage. After freezing berry samples in liquid nitrogen, skins were peeled and lyophilized (Lio-5P, Cik solution, Karlsruhe, Germany), then, phenylpropanoid contents were detected with the same protocol of extraction, calibration, and quantification reported in Salvi et al. [46] and expressed as $\mu\text{mol g}^{-1}$ of dry weight (DW). Briefly, 0.7 g/sample of lyophilized berry skin were ground in a mortar and extracted with 75% of aqueous ethanol acidified to pH 2 by HCOOH (3×5 mL). The supernatant was partitioned with 3×5 mL of n-hexane, the extracts were added together and reduced to dryness under vacuum, then rinsed with MeOH/H₂O (50/50, pH 2). The analytical determinations were conducted using a liquid chromatograph equipped with a quaternary 200Q/410 pump and an LC 200 diode array detector (DAD) (all from Perkin Elmer, Waltham, MA, USA). Individual anthocyanins and flavonols were quantified at 530 and 330 nm, respectively, using calibration curves of authentic standards (Extrasynthese, Genay, France).

In order to investigate how SWE treatments affected anthocyanin composition, the percentage of methoxylated anthocyanins on total amount of 3-glucoside anthocyanins and the ratio of methoxylated to nonmethoxylated derivatives were estimated by the individual anthocyanin content.

Moreover, in order to compare the global balance of the biosynthesis generating anthocyanins in SWE and CTRL berries in both irrigation regimes, three key enzymatic activities, flavonoid 3',5'-hydroxylase (F3'5'OH), 3'-O-methyltransferase (3'-OMT), and 5'-O-methyltransferase (5'-OMT), linked to known structural genes involved in the synthesis of anthocyanins, were investigated following Mattivi et al. [19].

2.6. Statistical Analysis

To compare *A. nodosum* treatments effects in different irrigation regimes and factors interactions, all data were subjected to a two-way analysis of variance ($p \leq 0.05$) using SPSS Statistic 25 (IBM, Endicott, NY, USA).

The two irrigation regimes (WW and WS) were combined with *A. nodosum* treatments (SWE and CTRL) and supposed as fixed factors.

After running preliminary Shapiro–Wilk's ($p \leq 0.05$) and Levene's ($p \leq 0.05$) tests to verify the normal distribution and the homogeneity of variance of each dataset, since the purpose of this study was to elucidate the effects of *A. nodosum* treatments on grapevine physiology, berry phenylpropanoid metabolism and drought stress tolerance, factor significant interactions were

checked also with one-way ANOVA ($p \leq 0.05$) and reported in figures and tables. Mean values were separated by Fisher's least significant difference (LSD) post-hoc test ($p \leq 0.05$). Linear regression analysis was performed to assess possible relationships between Ψ_{pd} and Ψ_{leaf} using Sigmaplot (Systat Software Inc., San Jose, CA, USA).

3. Results

The Shapiro–Wilk's test output values and the visual inspection of histograms and box plots showed that each dataset was approximately normally distributed. Skewness and kurtosis values of each parameter were reported in Supplementary Table S3. Moreover, the Levene's test verified the equality of variances in the samples.

3.1. Climate Parameters

The 2017 microclimate conditions of the experimental area are reported in Figure 1.

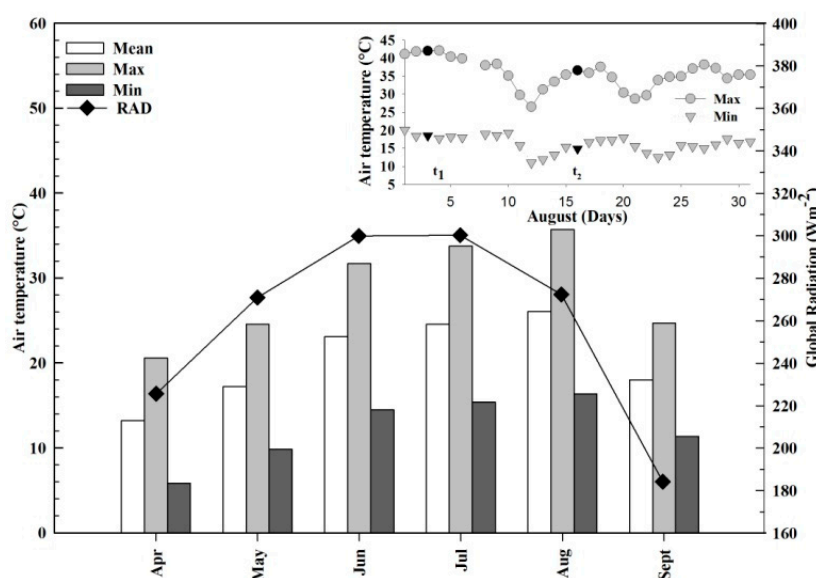


Figure 1. Climate parameters of the experiment location. Monthly averages of mean, maximum, and minimum air temperature (°C) and global radiation (RAD, $W m^{-2}$) measured from April to September; in the inset the daily averages of maximum and minimum air temperature (°C) measured in August are shown.

Average mean, maximum, and minimum air temperatures, measured from bud break to leaf fall (April–September), were 19.5°, 27.5°, and 11.4 °C, respectively. The most torrid month of this period was August (maximum temperatures were always above 40 °C in the first five days of this month). The hottest day was the 3rd of August (corresponding to t_1 for eco-physiological measurements and berries samplings), when maximum and minimum air temperatures were +2° and +6° higher, respectively, than the corresponding monthly averages.

3.2. Leaf Gas Exchange, Chlorophyll Fluorescence, and Leaf and Stem Water Potential

All eco-physiological parameters of SWE and CTRL plants in both irrigation regimes resulted similar at t_0 , because at this stage treatments with *A. nodosum* were not performed yet and only the differential irrigation regimes were applied (Table 1).

Gas exchanges, water potentials, K_{plant} , and RWC showed significant differences after *A. nodosum* treatments and irrigation regime differentiation, whereas a statistical difference was not

detected in F_v/F_m . Lower values of P_n , g_s , WUE, Ψ_{pd} , Ψ_{stem} , and K_{plant} were observed in WS than in WW vines (Table 2).

Table 1. Leaf gas exchanges and chlorophyll fluorescence in *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL), under two irrigation regimes (WW, well-watered; WS, water stressed).

Irrig. Regime	Samp. Time	P_n ($\mu\text{mol}/\text{m}^2 \text{ s}$)		g_s ($\text{mmol}/\text{m}^2 \text{ s}$)		F_v/F_m		WUE ($\text{mmol}/\text{m}^2 \text{ s}$)	
		CTRL	SWE	CTRL	SWE	CTRL	SWE	CTRL	SWE
WW	t ₀	13.9 ± 0.7 a	15.0 ± 1.5 a	162.2 ± 19.6 a	146.1 ± 9.3 a	0.77 ± 0.01 a	0.77 ± 0.01 a	3.4 ± 0.4 a	3.5 ± 0.4 a
	t ₁	6.6 ± 0.6 a	8.6 ± 0.5 a	94.4 ± 3.4 a	92.8 ± 5.4 a	0.76 ± 0.02 a	0.78 ± 0.01 a	4.9 ± 0.2 a	3.7 ± 0.2 b
	t ₂	6.6 ± 1.9 b	11.6 ± 0.7 a	86.0 ± 14.7 b	161.5 ± 12.6 a	0.80 ± 0.01 a	0.77 ± 0.03 a	2.6 ± 0.7 a	2.6 ± 0.1 a
WS	t ₀	4.8 ± 1.1 a	5.3 ± 2.3 a	92.4 ± 14.8 a	82.2 ± 12.4 a	0.77 ± 0.01 a	0.77 ± 0.01 a	1.8 ± 0.5 a	1.0 ± 0.5 a
	t ₁	8.1 ± 0.4 a	7.0 ± 0.6 a	62.3 ± 3.4 a	62.2 ± 6.4 a	0.73 ± 0.03 a	0.76 ± 0.02 a	4.6 ± 0.4 a	3.4 ± 0.1 b
	t ₂	1.0 ± 0.2 b	4.3 ± 1.1 a	18.2 ± 5.0 b	49.5 ± 11.4 a	0.80 ± 0.01 a	0.80 ± 0.01 a	1.1 ± 0.4 b	2.1 ± 0.3 a

Means (\pm SE, $n = 10$) followed by distinct letters within the same parameter and row are significantly different according to the LSD test ($p \leq 0.05$). Measurements were conducted at t₀, t₁, and t₂.

At t₁, similar values of P_n and g_s were observed in SWE than CTRL in both WW and WS vines. At t₂, in well-watered vines, SWE plants showed significant higher values of P_n and g_s than CTRL plants, whereas WUE was largely unaffected by *A. nodosum* treatments. At the same sampling time, water stressed SWE vines were able to maintain significant higher levels of P_n , g_s , and WUE than corresponding CTRL vines (Table 1).

At t₁, Ψ_{pd} and Ψ_{stem} were largely unaffected by *A. nodosum* treatments in WW plants (Figure 2A,B). On the contrary, under water stress conditions, both at t₁ and t₂, SWE vines displayed higher Ψ_{pd} and Ψ_{stem} values with respect to CTRL. In addition, RWC was significantly higher in SWE compared to CTRL vines at t₁ (first week of August) only in WS vines (Figure 2C). At t₂, RWC and K_{plant} values were significantly higher in SWE than CTRL in both water regimes (Figure 2C,D). F_v/F_m did not show significant changes after *A. nodosum* treatments.

A strong linear relationship was found between Ψ_{pd} and Ψ_{leaf} , considering *A. nodosum* treated and untreated vines under both irrigation regimes ($R^2 = 0.97$ and $p < 0.001$) (Supplementary Figure S2).

3.3. Grape Composition and Yield

Irrigation regime induced significant differences ($p \leq 0.05$) in berry weight, pH, and titratable acidity (Table 2). Berry weight, pH, and titratable acidity were +29%, +6%, and +33% higher in WW than in WS vines, respectively (Table 2). Consequently, significant differences induced by irrigation regimes were also observed in cluster weight and yield/vine (Table 2). *A. nodosum* treatments did not induce significant effects in berry composition and productivity parameters in both water regimes (Supplementary Table S1).

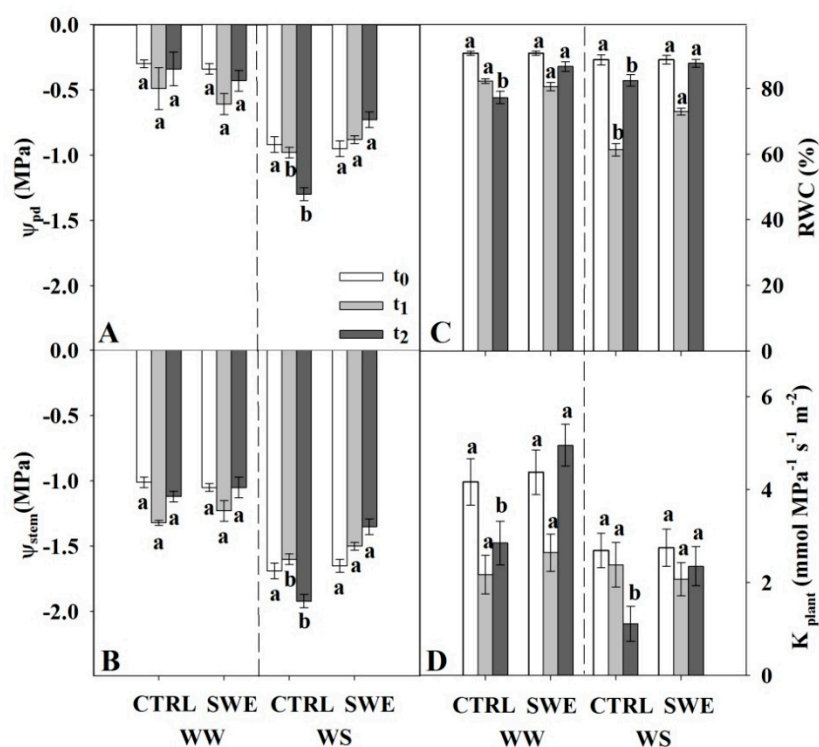


Figure 2. (A) Predawn (Ψ_{pd}) and (B) midday stem (Ψ_{stem}) water potentials, (C) relative water content (RWC), and (D) plant hydraulic conductivity (K_{plant}) in *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL), under two irrigation regimes (WW, well-watered; WS, water stressed). Means (\pm SE, $n = 10$) followed by distinct letters within the same water regime and sampling time are significantly different according to the LSD test ($p \leq 0.05$). Measurements were conducted at t_0 (white bar), t_1 (grey bar), and t_2 (dark grey bar)

Table 2. Two-way ANOVA ($p < 0.05$) for eco-physiology, chemical features of the berry and productivity parameters in *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL), under two irrigation regimes (WW, well-watered; WS, water stressed).

Parameter	P_n	g_s	E	WUE	Ψ_{pd}	Ψ_{stem}	RWC	K_{plant}	TA	Berry Weight	Cluster Weight	Yield/Vine
Unit	$\frac{mol}{m^2 \cdot s^{-1}}$	$\frac{mmol}{m^2 \cdot s^{-1}}$	$\frac{mmol}{m^2 \cdot s^{-2}}$	$\frac{Mmol}{m^2 \cdot s^{-2}}$	MPa	MPa	%	$\frac{mmol}{MPa^{-1} \cdot s^{-1} \cdot m^{-2}}$	gL^{-1}	g	g	g
Treatments												
Treated	8.915 2	111.0 000	3.270 7	2.755 2	-0.6176	-1.2872	73.5383	3.24	10.4737	0.6873	52.4	312.1
Nontreated	8.157 8	95.20 00	2.869 6	2.889 1	-0.7547	-1.4238	68.6305	2.50	10.6412	0.6852	46.1	276.7
Irrig. Regime												
WW	11.73	133.3 2	3.73	3.33	-0.43	-1.10	70.43	3.52	12.46	0.75	51.72	312.00
WS	5.13	71.00	2.37	2.28	-0.95	-1.63	71.68	2.22	9.34	0.58	46.81	289.79
Significance												
Treatments	0.512	0.039	0.019	0.387	0.017	0.035	0.020	0.042	0.874	0.001	0.606	0.459
Irrig. Regime	0.000	0.000	0.000	0.000	0.000	0.000	0.506	0.025	0.005	0.000	0.031	0.018
Treat. \times Irrig. Regime	0.500	0.634	0.646	0.002	0.005	0.003	0.967	0.011	0.571	0.041	0.694	0.412

Values are the mean of each parameter, considering treatments and irrigation regime (Irrig. Regime) as factors. In the last 3 rows is indicated the significance. Other abbreviations: net assimilation rate (P_n), stomatal conductance (g_s), transpiration (E), water use efficiency (WUE), predawn water

potential (Ψ_{pd}), midday stem water potential (Ψ_{stem}), relative water content (RWC), and total acidity (TA).

3.4. Berry Skin Phenylpropanoids

The irrigation regime affect both anthocyanin and flavonol derivatives and enzymatic activity. In detail, malvidin, total anthocyanin content and methoxylated to nonmethoxylated anthocyanin ratio were +43%, +35%, and +16% higher in WS than in WW grapes. Among flavonols in berry skin, kaempferol derivatives were significantly higher (+93%) in WS compared to WW vines. The activity of F3'5'OH, 3'-OMT, and 5'-OMT were similarly enhanced (+24%, +23%, and +19%, respectively) by water stress regime irrespective of *A. nodosum* treatments (Supplementary Table S2).

The anthocyanins detected in berry skin extracts were delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, and malvidin-3-O-glucoside (Supplementary Figure S1A). At t_0 , there were no significant differences in the anthocyanins content between *A. nodosum* treated and untreated plants in irrigation regimes (Figure 3). On the contrary, at t_1 and t_2 , both in WW and WS vines, delphinidin-3-O-, petunidin-3-O-, cyanidin-3-O-, and peonidin-3-O-glucoside contents were significantly more abundant in SWE than CTRL berry skins (Figure 3A,B,D,E). Moreover, at t_1 , berry skin contents of malvidin and total anthocyanin were higher in SWE than in CTRL plants (Figure 3F), irrespective of the irrigation regime. The opposite trend was observed at t_2 , when malvidin and total anthocyanin did not significantly differ in treated and untreated berry skins, in both water regimes.

In WW vines, the ratio of methoxylated to nonmethoxylated anthocyanins and the percentage of methoxylated anthocyanins/tot were both higher in CTRL than in SWE at t_1 and the amplitude of these ratios was larger at t_2 (Figure 4A,B). In WS vines, the methoxylated to nonmethoxylated anthocyanins ratio was higher in CTRL respect to SWE both at t_1 and t_2 (Figure 4A), whereas the percentage of methoxylated anthocyanins was unaffected by *A. nodosum* treatments in WS vines (Figure 4B).

At both sampling times and irrigation regimes, SWE berry skins presented a relevant increment in hydroxycinnamic acids (Figure 5A) and total phenolics (Figure 5D) content compared to CTRL counterparts.

The content of quercetin derivatives was more than two-fold higher in treated respect to untreated berry skins in both WW and WS vines at t_2 . This difference was found also at t_1 , but only in WS vines (Figure 5B). The opposite was observed for kaempferol derivatives (Figure 5C), that did not show significant changes at every sampling time and were marginally affected by the *A. nodosum* treatments only in WS vines, with higher values in SWE than in CTRL at t_2 .

The enzymatic activities of F3'5'OH, 3'-OMT, and 5'-OMT are reported in Table 3.

Both in WW and WS vines, *A. nodosum* treatments did not affect F3'5'OH enzymatic activity at t_1 , while decreasing its activity at t_2 , leading to lower values in SWE than in CTRL vines.

Moreover, SWE vines showed lower 3'-OMT activity than CTRL only at t_1 , in both irrigation regimes. The 5'-OMT enzymatic activity was also affected by *A. nodosum* treatments, that lowered 5'-OMT in WW treated vines at t_1 and t_2 , and also in WS vines at t_1 (Table 3).

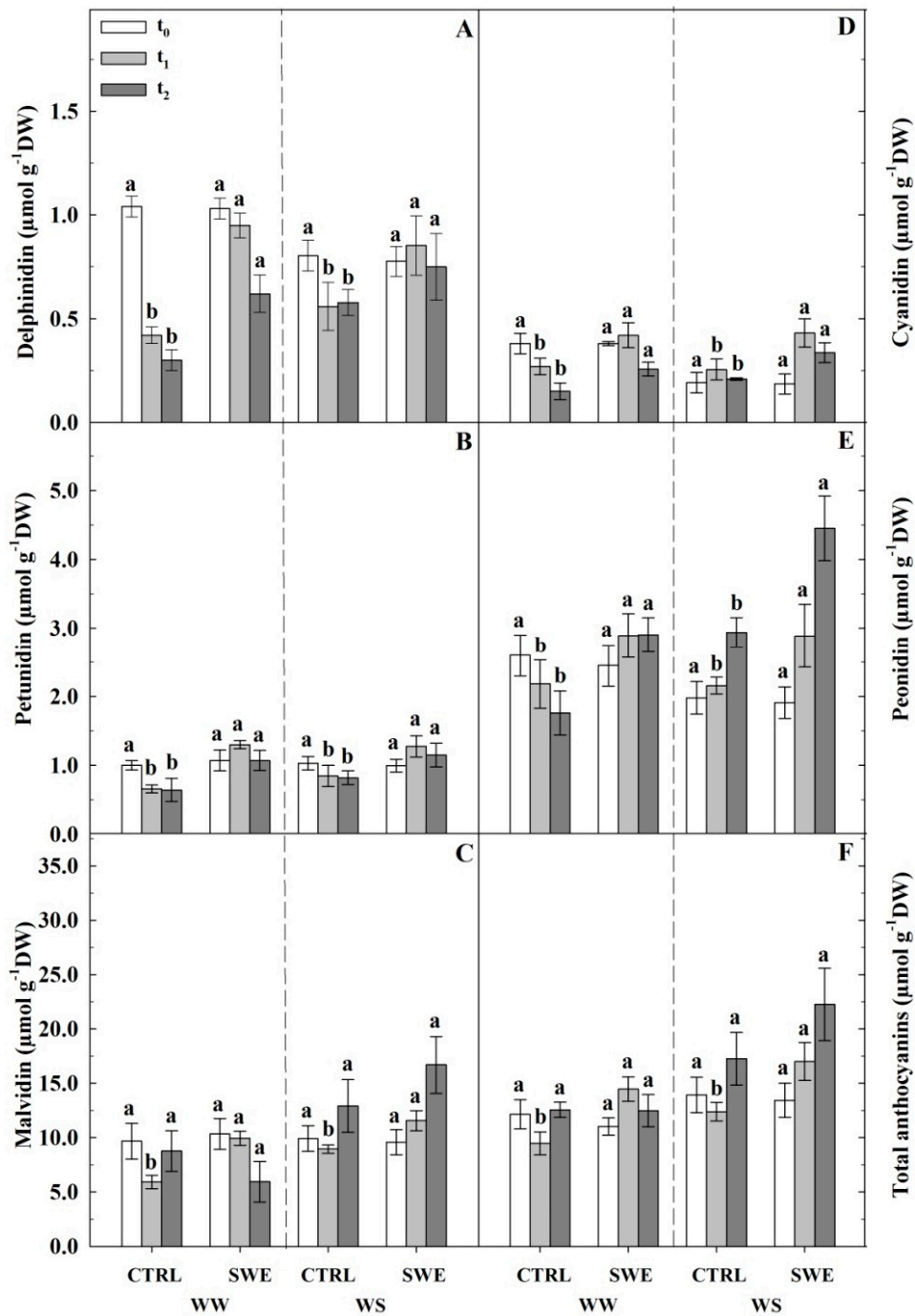


Figure 3. Berry skin individual anthocyanin amounts in *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL) under two irrigation regimes (WW, well-watered; WS, water stressed). (A) Delphinidin, (B) Petunidin, (C) Malvidin, (D) Cyanidin, (E) Peonidin, and (F) Total anthocyanin contents. Means (\pm SE, $n = 5$) followed by distinct letters within the same water regime and sampling time are significantly different according to the LSD test ($p \leq 0.05$). Measurements were conducted at t₀ (white bar), t₁ (grey bar), and t₂ (dark grey bar).

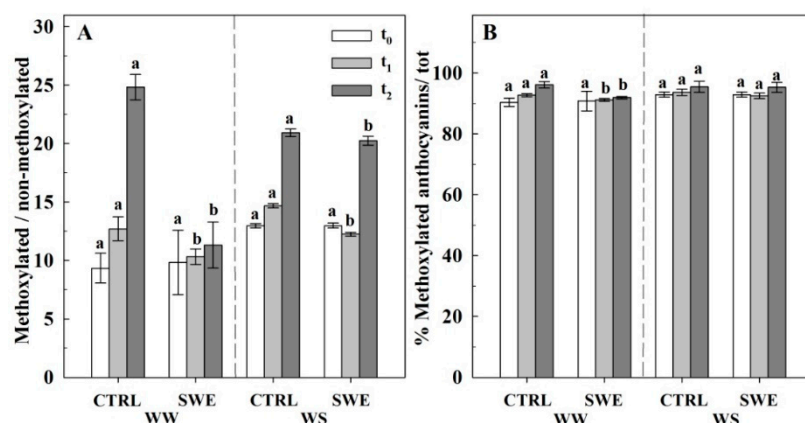


Figure 4. Berry skin anthocyanins methoxylation in *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL) under two irrigation regimes (WW, well-watered; WS, water stressed). (A) Methoxylated/non-methoxylated anthocyanins ratio; (B) % of methoxylated anthocyanins on total anthocyanin content. Means (\pm SE, $n = 5$) followed by distinct letters within the same water regime and sampling time are significantly different according to the LSD test ($p \leq 0.05$). Measurements were conducted at t₀ (white bar), t₁ (grey bar), and t₂ (dark grey bar)

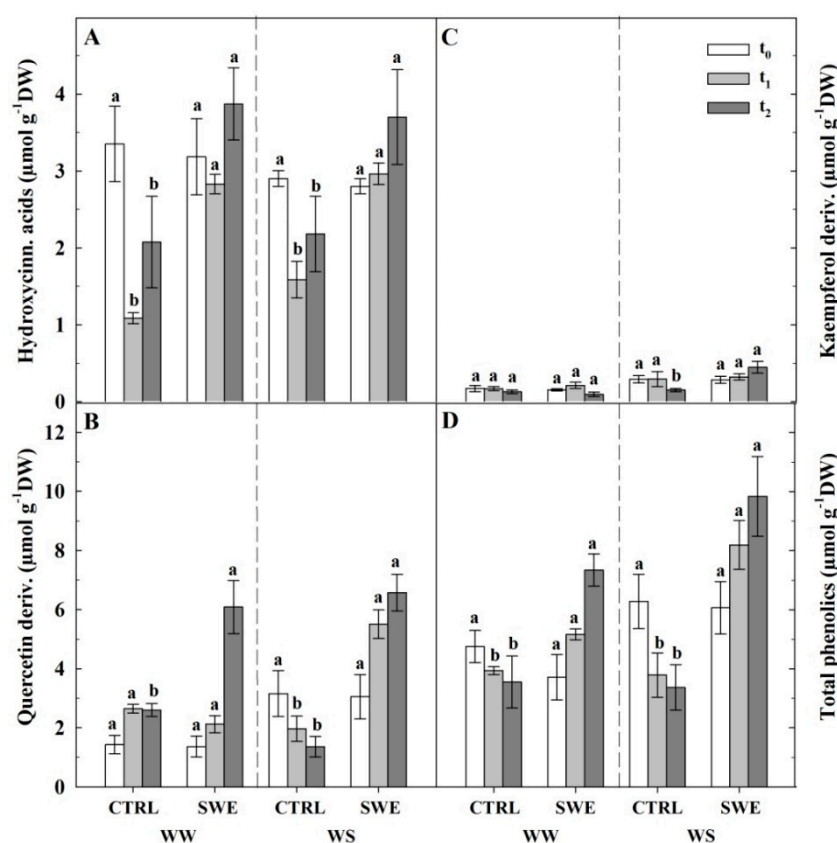


Figure 5. Berry skin hydroxycinnamic acids and flavonols in *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL) under two irrigation regimes (WW, well-watered; WS, water stressed). (A) Hydroxycinnamic acids, (B) Quercetin derivatives, (C) Kaempferol derivatives, and (D) Total phenolic contents. Means (\pm SE, $n = 5$) followed by distinct letters within the same water regime and sampling time are significantly different according to the LSD test ($p \leq 0.05$). Measurements were conducted at t₀ (white bar), t₁ (grey bar), and t₂ (dark grey bar).

Table 3. Enzymatic activities computed as ratios of anthocyanin amounts in berry skin of *V. vinifera* treated with *A. nodosum* extract (SWE) and untreated plants (CTRL), under two irrigation regimes (WW, well-watered; WS, water stressed).

Irrig. Regime	Samp. Time	F 3'5'OH Activity		3'-OMT Activity		5'-OMT Activity	
		CTRL	SWE	CTRL	SWE	CTRL	SWE
WW	t ₀	3.93 ± 0.05 a	4.40 ± 0.05 a	6.84 ± 0.7 a	6.45 ± 0.7 a	9.30 ± 0.6 a	10.04 ± 0.7 a
	t ₁	2.86 ± 0.40 a	3.69 ± 0.56 a	8.08 ± 0.6 a	6.88 ± 1.4 b	14.12 ± 1.4 a	10.48 ± 0.4 b
	t ₂	5.09 ± 0.15 a	2.42 ± 0.09 b	11.73 ± 0.9 a	11.31 ± 2.2 a	29.23 ± 3.9 a	9.60 ± 3.2 b
WS	t ₀	5.42 ± 0.07 a	5.42 ± 0.07 a	10.30 ± 1.9 a	10.30 ± 1.9 a	12.34 ± 0.4 a	12.20 ± 0.4 a
	t ₁	4.29 ± 0.15 a	4.13 ± 0.14 a	8.47 ± 0.3 a	6.69 ± 1.6 b	16.00 ± 1.4 a	13.57 ± 1.1 b
	t ₂	4.55 ± 0.06 a	3.89 ± 0.31 b	14.02 ± 0.3 a	13.25 ± 0.9 a	22.36 ± 2.0 a	22.27 ± 4.9 a

Means (\pm SE, $n = 10$) followed by distinct letters within the same parameter and row are significantly different according to the LSD test ($p \leq 0.05$). Measurements were conducted at t₀, t₁ and t₂.

4. Discussion

4.1. Consequences of Water Deficit on Pinot Noir Physiology and Berry Skin Anthocyanin Content

Important changes in physiological responses are typically observed in *Vitis vinifera* under different levels of water stress, such as decrease of leaf stomatal conductance and photosynthetic rate [1,11,56], which often affect berry sugar accumulation [57–59]. A moderate restriction of water supply improves the quality of red wines by achieving optimal sugar levels, due to the competition for carbon assimilates between berry ripening and shoot growth and to a lower sugar concentration in berries because of increased berry size [60]. When the limitation in vine water uptake is severe, the stomatal closure restricts photosynthesis, reducing berry weight, accumulation of sugars, and yield [2,61,62]. In our study, as expected, P_n , g_s , Ψ_{pd} , Ψ_{stem} , and RWC were significantly reduced by drought in WS vines with respect to WW ones; moreover, low water availability decreased titratable acidity, pH, berry weight, and, consequently, cluster weight and yield. However, water availability did not significantly affect berry sugar accumulation. This result is in agreement with the results observed by Poni et al. [63] on Pinot noir cv. in pot conditions, whereas the same cultivar showed a decrease in berry sugar content induced by high soil water availability compared to water stresses plants in field conditions [64]. This discrepancy between pot and field trials can be linked to the space limitation for root development impaired by the pot, which does not occur in the open field. This restraint may have restricted carbohydrates reserves stored in grapevine roots, which are known to contribute to berry sugar accumulation, after translocation to berries, by hydrolyzation of the starch into soluble sugars [65,66].

At biochemical level, an increment in malvidin and in total anthocyanin contents was found in water stressed vines, suggesting that low water availability drove a major flux of carbon in the anthocyanin pathway, particularly towards more stable malvidin-based derivatives [67]. This effect partly compensated the reduction in anthocyanin biosynthesis caused by the reduction in P_n because of stomatal closure [23]. Moreover, in WS berry skins, we observed an increase in the methoxylated to nonmethoxylated anthocyanin ratio, confirming that water stress boosts methoxylation, thus anthocyanin stability, as an adaptive strategy to adverse climatic conditions, like heat stress and high radiation load [23,68,69].

4.2. Timing of Action of *A. nodosum* Extract in Improving Plant Physiological Performance and Water Relations

Our results confirm that *A. nodosum* treatments during the last stages of berry development allow to improve leaf gas exchanges of *V. vinifera* and show for the first time that the beneficial effect of these treatments can be observed under water stress conditions. Indeed, in SWE vines subjected to water stress, despite the occurrence of a severe reduction in stomatal conductance ($g_s < 50 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) [1,11], diffusional limitation to photosynthesis were less pronounced compared to CTRL

vines, thus resulting in a higher WUE. The effectiveness of *A. nodosum* treatment on vine physiology could be also discerned at t_1 under water stress conditions, since SWE plants maintained higher water potentials (Ψ_{pd} , Ψ_{stem}) than in CTRL vines. At this sampling point, the high air temperature induced a strong decrease in RWC; however, this decline was much more evident in nontreated plants, supporting the hypothesis of a positive effect of *A. nodosum* in preventing excessive leaf dehydration [70]. The effect of the treatments on plant water status persisted and was intensified with water stress progression. Indeed, at t_2 , SWE vines showed a better K_{plant} than CTRL vines. The maintenance of an adequate K_{plant} may have allowed SWE leaf to prevent stomatal closure and, consequently, an excessive restriction in carbon gain [71]. In addition, since the decline in K_{plant} is associated with hydraulic dysfunction, our results suggest that SWE leaf are less vulnerable to conduit embolism and collapse [56,72,73].

This effect could be particularly important in Pinot noir cv. which adopts an anisohydric strategy to cope with water stress, since anisohydric species are usually reported as more vulnerable to hydraulic failure [74]. The typical anisohydric behavior is evident from the slope (0.902) of the linear correlation between predawn and midday leaf water potential, which is close to 1 for extreme anisohydric species [75]. Our result suggests that *A. nodosum* treatment could help maintaining a better water status of the plant preventing excessive stomatal closure without affecting the plant hydraulic strategy. The strong anisohydric behavior with great variations in leaf gas exchange and water relations during water stress progression could have been exacerbated by pot conditions, which imply the impossibility to access deeper soil water as in field conditions, where minor reductions in Ψ_{leaf} are usually observed [76].

However, controlled water stress conditions allowed us to validate the effect of *A. nodosum* treatments on vine physiology previously documented in the field trial [46] and to highlight a timing of action of these treatments. Indeed, the effects of *A. nodosum* treatments resulted particularly effective over the last part of the growing season, allowing treated plants to cope better with prolonged stress and leaf senescence. We hypothesize that the amino acids of the *A. nodosum* extract, in particular proline, which is involved in drought tolerance acting both as cytoplasmic osmolyte and as ROS-scavenging compound [36,37], may have played a key role in the enhancement of physiological performances and the maintenance of optimal water status. This mechanism could be particularly important under atmospheric stress conditions in adult leaves, which, differently from apical young tissues, are less able to accumulate actively solutes during the day and are subjected to oscillations of RWC [77]. These changes induced by *A. nodosum* treatments on plant physiology did not have relevant consequences on berry ripening, indeed the evolution of total sugars, titratable acidity, and pH were unaffected by *A. nodosum* treatments. This observation is in agreement with the results obtained by Frioni et al. [49,50], but it is somewhat contrasting with the previous observations conducted in field conditions [46]. This discrepancy could be related to multiple factors, such as biological (cultivar, rootstock), methodological (pot or field studies), and environmental (season) factors, which may influence grape ripening pattern [78].

In addition, berry weight and vine productivity (data not shown) were not affected by medium-late *A. nodosum* extract applications. Conversely, biostimulant applications during early stages of berry development could lead to an improvement in berry weight and vine yield, as formerly mentioned for table grape [41–44].

4.3. *A. nodosum* Treatments Influenced the Biosynthesis of Berry Skin Anthocyanins Irrespective of Irrigation Regime

The *A. nodosum* treatment induced higher content of delphinidin, petunidin, cyanidin, and peonidin derivatives under both irrigation regimes both at t_1 and at t_2 . This treatment-induced increment of anthocyanins in *Vitis vinifera* is consistent in both pot and field conditions [46,50] and is reported also in other species [79–81]. In addition, we observed a reduction of the methoxylated to nonmethoxylated anthocyanin ratio and of the percentage of methoxylated anthocyanins on total anthocyanins in berry skin induced by *A. nodosum* treatments. This result is also supported by enzymatic activities, which generally showed lower 3'-OMT and 5'-OMT values in SWE than CTRL

grapes, thus a higher enzymatic activity towards the biosynthesis of nonmethoxylated anthocyanins (cyanidin and delphinidin) than methoxylated ones (peonidin and malvidin) following *A. nodosum* treatment. The reduced investment in methoxylated anthocyanins was observed both under well-watered and water stress conditions, differently from the results conducted without the application of biostimulants by Castellarin et al. [82], which showed a more effective increase of expression of some enzymes involved in the biosynthesis of tri-hydroxylated anthocyanins as a consequence of water stress application. On this basis, we hypothesize that the activity of flavonoid 3'-hydroxylases (F3'H) enzyme may have been stimulated by *A. nodosum* treatments, hence activating the synthesis of cyanidin-based anthocyanins, rather than inducing the activity of F3'5'OH enzyme and the delphinidin parallel branch [35,83]. Indeed, the treatment may reduce the investment in more stable methoxylated anthocyanins, commonly stored in berries to confer protection against severe climate conditions [84,85]. In addition, under both irrigation regimes, we observed an increase in flavonols and hydroxycinnamic acids in berry skin after *A. nodosum* treatments. In particular, the increment in quercetin derivatives, which are di-hydroxy B-ring-substituted flavonols and confer protection against reactive oxygen species, may concur to wine color by acting as copigmentation factors [86], positively affecting wine color stability [87]. The biosynthesis of flavonols and anthocyanins in the berry are mutually related, because both classes of compounds derive from the same metabolic pathway [19]: F3'H and F3'5'OH activate both the biosynthesis of cyanidin- and delphinidin-based anthocyanins, as well as of quercetin and myricetin derivatives, respectively [88]. Quercetin derivatives were abundantly detected in our berry samples, whereas myricetin derivatives were recognized only in traces. These findings may further support the hypothesis that *A. nodosum* treatments selectively promoted F3'H enzyme activation, instead of F3'5'OH, thus the biosynthesis of di-hydroxy flavonoids in place of their tri-hydroxy B-ring-substituted. These metabolic and enzymatic results perfectly match recent findings on *A. nodosum* capability of inducing the expression of specific genes involved in secondary metabolites pathways in *Vitis vinifera* after veraison [50], also hypothesized before by other authors [35,83]. On this basis, we can speculate that the *A. nodosum* extract may have helped to mitigate environmental stress conditions by affecting the activation of specific genes related to the biosynthesis of less carbon expensive anthocyanins and flavonols [89]. In fact, the early accumulation of these antioxidant compounds in the berry skin may have offered protection to treated vines against environmental conditions [90], as demonstrated by higher physiological and hydraulic performances observed at t.

5. Conclusions

The increments of leaf gas exchanges and water potentials following *A. nodosum* treatments were previously reported in other species. However, this research provides new evidences that *A. nodosum* treatments may affect both eco-physiological traits and berry skin metabolism of Pinot noir cv., conferring a better ability to counteract low water availability during post-veraison stages.

In summary, *A. nodosum* enhanced vines water stress tolerance by improving leaf water status and hydraulic conductance, thus maintaining optimal gas exchanges. Furthermore, in both water regimes, the biochemistry of berry skin was profoundly affected by *A. nodosum* treatments. We demonstrate a selectivity action of these treatments on lowering the enzyme activities involved in the synthesis of methoxylated anthocyanins in treated vines. Moreover, *A. nodosum* may have a significant effect on wine color stability, increasing the content of antioxidant flavonoids (quercetin derivatives) involved in copigmentation in berry skin. Overall, the data obtained from this experiment indicated that the effects and the protocol of application of this *A. nodosum* extract have been validated, and that late treatments with *A. nodosum* can be a practical approach for wineries to improve the eco-physiology of *V. vinifera* and to positively affect berry biochemical composition.

Supplementary Materials: The following are available online at www.mdpi.com/2076-3417/10/13/4473/s1. Figure S1: HPLC-DAD chromatograms of hydroalcoholic extract of berry skins. Figure S2: Relationship between pre-dawn (Ψ_{pd}) and midday (Ψ_{leaf}) water potential. Figure S3: Experimental site pictures. Table S1: Total sugars, titratable acidity (TA), pH and berry weight. Table S2: Two-way ANOVA ($p < 0.05$) for biochemical parameters

in berry skin and enzymatic activities. Table S3: Shapiro-Wilk's test ($p < 0.05$) (George and Mallery, 2010) results for each parameter.

Author Contributions: The experiment was designed and arranged by G.B.M., L.S., and C.B. L.S. and E.C. sprayed *A. nodosum* extract, executed gas exchange and water potentials measurements, and picked berry samples. L.S. and E.C. also processed data and carried out statistical analysis. L.S. and C.B. accomplished the phenylpropanoids HPLC analysis. L.S. and C.B. wrote the original draft, which was edited and improved by P.S. and G.B.M. All authors have read and agreed to the published version of the manuscript.

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