



# Effect of zeolite and irrigation treatments on grapevine leaves, an interdisciplinary approach

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## Abstract

**Aims** Global warming depicts a real challenge for viticulture. As found by PubMed results, a 90% increase in the abiotic stress publications number from 2015 onwards is registered. Soil and grapevine response interaction to abiotic stress is arbitrated by an intricate signal transduction network that determines adaptive changes and modifications in gene expression mediated by the transcription factors (WRKY proteins).

Briefly, (i) Does zeolite application affect canopy and biochemical leaf components? (ii) Is it possible to start a gene expression approach in an open-field vineyard, without fixed and stable external parameters obtaining an interconnected net of interdisciplinary data? (iii) Could the zeolite application be a corroborant tool to maintain a state of homeostasis in grapevine?

**Methods** After a soil clinoptilolite treatment (the "Roca magica" well known as water moderator) and/or irrigation utilizing vines presented to drought and high sun exposure, we investigated leaf biochemical variations (proline, chlorophyll, and quercetins) and we analyzed with rt-PCR approach the expression of selected genes (VvWRKY47 and VvWRKY39).

**Results** Lower water potential and leaf temperature were recorded in plants subjected to treatments against abiotic stress together with greater chlorophyll a, b, and less quercetin-3-O-glucoside. A down-regulation in VvWRKY47 gene and an up-regulation in VvWRKY39 gene were found. The VvWRKY47 showed interactions from the beginning with the zeolitic treatment.

**Conclusion** The zeolite in our experiment acted as a water flywheel, mitigating the effects of climate change; plant-soil interactions were positively emphasized by clinoptilolite. Finally, results suggest that VvWRKY47 could be a valid candidate in the evaluation of drought and temperature stress in the open-field.

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## Introduction

In the past, higher mild temperatures in addition to gentle water stress enhanced grape quality in a lot of wine-growing regions and at the same time did not

exacerbate the general aspect and behavior of the vine (Zsófi et al. 2011). Nevertheless, looking at the current climate projections worldwide, these patterns will undoubtedly shift. In fact, empirical evidence clearly asserts that global warming depicts a real challenge for viticulture in the upcoming decade (Hannah et al. 2013). As found by the PubMed results, data that confirms this concern is the 90% increase in publications' number relating to abiotic stress on *Vitis vinifera* L. from 2015 onwards (Bernardo et al. 2018). This shows how scientific efforts are driven towards grapevine climate change adaptation, as well as to find possible corroborate or strategies to counterbalance environmental damages (Cataldo et al. 2023a). Owing to the expected overall hits of global warming on viticulture, an assessment of the extent of the potential risk for the plant itself would help the concrete development of adaptation strategies for winemakers (Iglesias et al. 2007). Hence, adaptation strategies should be explored to uphold grapevines towards sustainable agriculture which can make progress in the resilience of agroecosystems under stiff conditions (Brunori et al. 2016).

Zeolites are natural minerals chemically known as "hydrated allumino-silicates of alkaline and alkaline earth elements" ( tectosilicates group). They can have interesting effect on health and plant development owing to their physical and chemical peculiarities like high cation exchange capacity, revocable hydra/dehydration, and selective molecular absorption (Domenico 2020). The pronounced selectivity of these minerals for  $\text{NH}_4^{4+}$  and  $\text{K}^+$  was taken advantage of in the fertilizers preparation for improving the land retention capacity of nutritive elements by upgrading a slower release in rice fields (Wulandari et al. 2019) or in vineyards (Cataldo et al. 2023b). According to several authors, a single soil zeolite application can ameliorate land aggregates, check the ammonium ions release, and support soil moisture (Cholili et al. 2019; Sastiono 2004). Zeolites, in fact, are extensively employed as a land amendment and as a "water moderator" in the substrate due to their reversible hydration/dehydration skill. Their adjunct to soil could notably diminish water and fertilizer costs by holding advantageous nourishing in the root zone. The effects of zeolite on land characteristics are commonly employed for explanations of its positive consequences on plant production, growing, and abiotic stress persistence (such as drought) (Szatanik-Kloc

et al. 2021). *Opuntia ficus-indica* L. plants were distinguished by a general ameliorating of all agronomic parameters when treated with chabazite (Domenico 2020). The property of interacting positively with water was also observed from a disease protection point of view. Soil zeolitic applications could trigger the ethylene/jasmonate-dependent defense mechanism against crop pathogen attack (e.g., *Sclerotinia sclerotiorum* and *Rhizoctonia solani*) (Poulaki et al. 2020).

At biochemical and physiological levels, plant tolerance to abiotic stresses is mediated by a complex signal transduction network, which results in adaptive shifts correlated with distinct modifications in gene expression that are mediated by the transcription factors' action (Katagiri 2004; Wang et al. 2014a, b). WRKY proteins, for example, delineate a fundamental class of transcriptional regulators in *Vitis vinifera* L. and in general in the higher plants (Ülker and Somssich 2004); they are proteins that tie up to specific DNA sequences in the genes' promoter regions, hereby settling their transcription (Guo et al. 2014). WRKY family was described for the first time in 1994 on *Ipomoea batatas* L. (Ishiguro and Nakamura 1994) (identifying the first WRKYTF (SPF 1) known as a negative regulator of sporamin and  $\beta$ -amylase expression), after that on *Arabidopsis thaliana* L. (Eulgem et al. 2000; Dong et al. 2003), *Oryza sativa* L. (Ryu et al. 2006; Wu et al. 2005), *Hordeum vulgare* L. (Mangelsen et al. 2008), *Populus nigra* L. (He et al. 2012), *Solanum lycopersicum* L. (Huang et al. 2012), *Cucumis sativus* L. (Ling et al. 2011), *Coffea arabica* L. (Ramiro et al. 2010), *Fragaria vesca* L. (Wei et al. 2016), *Triticum aestivum* L. (Niu et al. 2012), and *Vitis vinifera* L. (Guo et al. 2014; Hou et al. 2020; Merz et al. 2015; Wang et al. 2014b). WRKY TFs, involved in biotic and abiotic stress responses, contain a WRKY domain addition to a zinc finger motif (Rushton et al. 2010). According to these features, they are divided into groups (i.e., I, II, and III) (Chen et al. 2017). The mechanism of WRKY genes involved in the vine's defense reaction has not been entirely clarified (Zhu et al. 2012). We have some information on greenhouse experiments where 59 WRKY genes were identified (Wang et al. 2014a, b). VvWRKY1 seems to be implicated in grapevine reply to *Plasmopara viticola* infection by jasmonic acid-mediated transcriptional reprogramming (Marchive et al. 2007). VvWRKY2 acts

in lignification regulation activating the promoter of VvC4H (Guillaumie et al. 2010). VvWRKY11 appears to play a salient piece in drought stress response (Liu et al. 2011). A rapid upregulation of VvWRKY08, VvWRKY14, VvWRKY16, and VvWRKY24 posterior to the imposition of cold stress was observed while the greater part of genes induced by salt and drought were up-regulated in the wake of 24 h (Wang et al. 2014a, b). Considering semiquantitative RT-PCR analysis, VvWRKY16, 25, 28, and 35 could be associated with salt stress, VvWRKY3, 25, and 35 with water stress, and VvWRKY19, 27, 48, and 52 with *Erysiphe necator* infection (Guo et al. 2014).

However, it was indicated that only certain components inside the same cluster share an analogous expression profile in vine organs or tissues in growth. For example, in the second group, VvWRKY14, VvWRKY19, and VvWRKY52 were highly expressed in berry and rachy in or after veraison. Other components of this cluster, such as VvWRKY46 and 23, exhibited a high level of expression in buds and stems, showing a cold acclimation role (Wang et al. 2014a, b).

In light of all this, we wanted to focus on the plant and its well-being, investigating at a biochemical and genetic level (WRKY transcriptional factors) the responses that a new possible adaptation strategy (e.g., zeolite) could have in comparison to plants subject to standard irrigation or no anthropogenic help. As regards the genetic approach, this open-field experiment aims to address a broader and more representative consideration of the concept of viticulture, aiming to lay the very first guidelines for future studies that do not exclusively consider the greenhouse with its controlled conditions, and fully embracing the pragmatic nature of the vineyard.

Briefly, our study aims to answer four primary questions: (i) Does zeolite application affect the canopy status, the biochemical leaf components, as well as the proportion of each one? (ii) Is it possible to try to start a gene expression approach for WRKY transcriptional factors in an open-field vineyard, without fixed and stable external parameters (i.e., no greenhouse conditions) and obtain an interconnected net of interdisciplinary data? (iii) Which gene factors best describe water and temperature stress? (iv) soil zeolite application could be a valid corroborate for grapevine homeostasis?

## Materials and methods

The experimental design took place in an Italian Sangiovese vineyard (2022 and 2023 harvests). The location has the following GPS coordinates 43° 67' 78" N and 11° 17' 43" E (San Casciano Val di Pesa, FI). The CCL 2000/1 grapevines were planted on 8th April 2016 on 775P rootstock on clay soil.

Adopting three blocks per treatment inside an experimental randomized block design, ten healthy-central grapevines were chosen for measurements (Fig. 1). The following four treatments were established:

- o WWt=well-irrigated (with water stress threshold -1.4 MPa until veraison and -1.6 MPa until ripening) and soil-treated grapevines (1 kg per vine of clinoptilolite; BIG-Zeo Agricola Internazionale s.r.l.) (the zeolite dose was based on a preliminary potted study; Cataldo et al. 2022),
- o WW=well-irrigated grapevines (with water stress threshold -1.4 MPa until veraison and -1.6 MPa until ripening),
- o WSt=soil-treated (1 kg per vine of clinoptilolite; BIG-Zeo Agricola Internazionale s.r.l.) and no irrigated grapevines,
- o WS=control vines, no irrigated and no soil-treated grapevines.

Leaf physiological parameters, leaf biochemical analysis, and gene expression analysis were performed at three different times, as follows, 3/07/2022 and 3/07/2023 (pre-bunch closure, time one; T1); 28/07/2022 and 01/08/2023 (veraison, time two; T2); 16/08/2022 and 16/08/2023 (maturation, time three; T3).

Everyday temperatures and precipitation were annotated by a meteorological station.

10 healthy-developed leaves per treatment were chosen from 10 grapevines (into the three blocks).

Photosynthesis (PN), leaves' temperature (Tleaf), stomatal conductance ( $g_s$ ), chlorophyll fluorescence ( $F_v/F_m$ ), and water potential ( $\Psi$ ) were measured on ten basal leaves per treatment.

A gas analyzer Ciras 3, PP Systems was employed for quantifying PN, Tleaf, and  $g_s$  parameters.

A Hansatech PEA fluorometer with leaf clips was used to calculate the  $F_v/F_m$  ratio (Bucher et al. 2018).



**Fig. 1** Photographic image of plants treated with zeolite together with irrigation

Stem water potential was calculated from mid-day leaf potential (Scholander pressure chamber) following Williams and Araujo (2002) (i.e.,  $\Psi_{\text{Leaf}} = -0.37 + 0.91 \Psi_{\text{Stem}}$ ).

In addition, other leaves were taken from the same 10 grapevines to quantify the proline amount according to Carillo and Gibon's (2011) methodology.

Chemicals such as acetonitrile, acetone, methanol, acetic acid, and formic acid were all HPLC grade and purchased from Carlo Erba (Italy). Ultra-pure water was produced using a Smart2Pure PRO water purification system (ThermoFisher, USA). Flavone (99%) was obtained from Alfa Aesar (USA). Caffeic acid and coumaric acid, used for the determination of their respective cinnamyl tartaric acids, were supplied by Sigma-Aldrich (USA).

Quercetin-3-O-glucoside (qu-3-O-glu), qu-3-O-galattoside, rutin, qu-3-O-glucuronide, epigallocatechin, procyanidinB1, myricetiin-3-O-glucoside, Isorhamnetin-3-O-glucoside, and kaempferol-3-O-glucoside were purchased from Extrasynthese (France).

Before analysis, the samples were lyophilized using a Lio 5P lyophilizer (Cinquepascal s.r.l., Italy). The lyophilized samples were ground into a fine powder with a TissueLyser II ball mill (Qiagen, Netherlands) and stored at  $-25^{\circ}\text{C}$  until the time of analyses.

Approximately 2 g of lyophilized powder were accurately weighed into a 50 mL screw cap plastic

tube. 250  $\mu\text{L}$  of internal standard (50 mg of Flavone in 50 mL of methanol) and 30 mL of acidic methanol (3% formic acid in methanol) were added, and the mixture was placed on an orbital shaker for 30 min. The mix was then centrifuged at 4000 rpm for 5.0 min, and the supernatant was collected in a 50 mL volumetric flask. The pellet was resuspended in another 20 mL of acidic methanol and agitated for a second extraction. After centrifugation, the supernatants were joined, and the volume was adjusted to 50 mL with the acidic methanol solution. 2 mL of the sample was then passed through a 0.45  $\mu\text{m}$  RC syringe filter (Cole-Parmer Instrument Co. Europe, United Kingdom) and collected in a glass vial with a screw cap for HPLC analysis.

For the HPLC determination of resveratrol glucoside, the extract was concentrated: 20 mL of the methanolic extract was dried with a Heidolph 2000 VV rotary evaporator (Heidolph Instrument, Germany) maintaining the bath temperature at  $35^{\circ}\text{C}$ . Once dry, the extract was resuspended in 2 mL of acidic methanol and filtered as previously described.

The HPLC system (Agilent, U.S.A) consisted of an autosampler (Series 1260), degasser (Series 1100), column thermostat (Series 1100), quaternary pump (Series 1100), and diode array detector (DAD, Series 1200). Phenolic compounds were separated using an Omega Polar C18 5  $\mu\text{m}$  250  $\times$  4.6 mm Column (Phenomenex, USA) preceded by a guard column packed

with the same material and maintained at 10 °C. The chromatographic separation method and mobile phases composition are reported in Table 1.

Chromatograms were recorded at 280 nm (for flavans), 360 nm (for flavonols and stilbenes), 300 nm (for flavone), and 320 nm (for hydroxycinnamic tartaric acids).

Pure standards of the phenolic compounds were analyzed to achieve calibration curves, determine retention times, and record their UV–VIS spectra.

For the extraction of chlorophylls and carotenoids (6 repetitions per treatment), approximately 250 mg of lyophilized powder was placed in a 15 mL plastic tube with 2.5 mL of acetone, vortexed, and subjected to ultrasound bath for 5 min. After centrifugation (5 min at 4000 rpm), the supernatant was transferred to a 5 mL volumetric flask, and a second extraction was performed on the pellet. Once the supernatants were combined, the volume was adjusted to 5 mL, and the extract was filtered with a 0.45 µm RC syringe filter. The liquid was promptly analysed using an Agilent 5483 spectrophotometer (Agilent, USA), recording the spectrum from 230 to 750 nm, with an optical path of 0.1 cm and using acetone as blank. The content, expressed in µg/mL of chlorophyll a ( $Ch_a$ ), chlorophyll b ( $Ch_b$ ), and total carotenoids ( $Ch_{x+c}$ ) in the extracts were calculated using the formulas proposed by Lichtenthaler (1987).

$$Ch_a = 11.24 A_{661} - 2.04 A_{644h}$$

$$Ch_b = 20.13 A_{645} - 4.19 A_{662}$$

**Table 1** HPLC elution and flow gradient program. Where: a 2% acetic acid in water, b 20% mobile phase A in acetonitrile

Time (min)	Flow rate (mL/min)	% of mobile phase A <sup>a</sup>	% of mobile phase B <sup>b</sup>
0	0.8	100	0
5	-	94	6
35	-	77	23
50	0.9	74	26
60	-	45	55
80	-	30	70
95	1.2	0	100
100	1.2	0	100
101	0.8	100	0

$$Ch_{x+c} = \frac{1000 A_{470} - 1.90 Ch_a - 63.14 Ch_b}{214}$$

To convert the values to µg/mg of dry matter, the values were multiplied by  $K = v/wl$  where  $v$  was the final volume of the extract in mL,  $w$  was the exact weight in mg of the leaf powder, and  $l$  was the light path length in cm.

Leaves were taken in the open field with gloves and placed in liquid nitrogen directly in the open field and then stored at -80 °C. Total RNA was extracted starting from 50.00 mg of fresh leaves adopting the RNeasy PowerPlant Kit (Qiagen). After on-column DNase digestion (Qiagen), the single-strand cDNA was synthesized starting from 200 ng of RNA in a 20.00 µL reaction using QuantiTect Reverse Transcription Kit (Qiagen). The quantitative reverse transcription (RT)-PCR was performed in a 20 µL reaction according to PowerTrack SYBR Green Master mix protocol (ThermoFisher Scientific). Briefly, 1 ng of cDNA was combined with 400 mM of specific forward and reverse primer, 0.5 µL of Yellow Sample Buffer (1X), 10 µL of PowerTrack™ SYBR™ Green Master Mix (1X), and Nuclease-free water up to 20.00 µL. The thermal cycling conditions used consisted in of 1 cycle for the enzyme activation at 95.0 °C for two min, then 40 cycles at 95.0 °C (denaturation) for fifteen seconds and 58.0 °C (annealing) for sixty seconds. Expression analysis of selected genes (VvWRKY47 and VvWRKY39) was monitored in three time points (T1-T2-T3) during the 2022 and 2023 seasons (6 repetitions per treatment). Gene expression analysis was performed using 2 technical replicates for each of the six biological replicates. Relative quantifications of gene expression were carried out adopting the comparative method with the  $2^{-\Delta\Delta Ct}$  formula (Livak and Schmittgen 2001) where  $\Delta Ct = Ct \text{ target gene} - Ct \text{ endogenous ctrl}$  and  $\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ calibrator}$ .

A preliminary phase of the concrete real-time work was carried out. Both housekeeping and target genes were screened.

The following target genes were tested in the laboratory VvWRKY03 (Guo et al. 2014), VvWRKY07 (Guo et al. 2014), VvWRKY08 (Wang et al. 2014a, b), VvWRKY13 (Hou et al. 2020), VvWRKY25 (Wang et al. 2014a, b), VvWRKY26 (Guo et al. 2014), VvWRKY30 (Guo et al. 2014), VvWRKY35 (Guo et al. 2014), VvWRKY39 (Guo et al. 2014),

VvWRKY47 (Wang et al. 2014a, b), VvWRKY51 (Guo et al. 2014), VvWRKY52 (Guo et al. 2014); VvWRKY39 and VvWRKY47 were chosen for their stability and efficiency (2.14 efficiency VvWRKY39;  $y = -3.0225x + 24.388$ ;  $R^2 = 0.9992$  and 1.95 efficiency VvWRKY47;  $y = -3.4525x + 19.712$ ;  $R^2 = 0.9931$ ) in the laboratory. In addition, multiple housekeeping genes (i.e., Actin3 (Wei et al. 2021), Actin4 (Borges et al. 2014), Actin5 (Reid et al. 2006), Elongation Factor 1 $\alpha$  a (Wei et al. 2021), Elongation Factor 1 $\alpha$  b (Katayama-Ikegami et al. 2016), Elongation Factor 1 $\alpha$  c (Wei et al. 2021), GAPDH (Wei et al. 2021)) were tested and from the preliminary analysis; Elongation Factor 1 $\alpha$  a was chosen for its stability and efficiency (2.03 efficiency;  $y = -3.2525x + 21.33$ ;  $R^2 = 0.9989$ ) in the laboratory for the open field conditions tested (Table 2).

To understand the significant relevance of the single factors (i.e., zeolitic-soil treatment and irrigation schedule), the values were subjected to a two-way analysis of variance using RStudio software (4.0.3. version) and TukeyHSD test for mean values. A statistically interesting difference was admitted for  $p \leq 0.05$ . All graphs were made by RStudio and XLstat.

## Results

The climate scenario is represented in Figs. 2 and 3. The monitoring of average hourly temperatures during vegetative growth up to post-harvest (from April to September) is drawn in Fig. 2.

The daily and monthly rainfall trend is shown below in Fig. 3.

The trend of the two seasons turns out to be different during the crucial months of photosynthetic activity of the vine. The 2022 season was characterized by an early arrival of warm temperatures since May, which peaked during the month of July. On the

contrary, the arrival of the hot season during 2023 took place around June, reaching the thermal peak in August. This can also be appreciated from the temperature difference between the two years ( $\Delta^\circ\text{C}$ ).

Even from the rainfall point of view, there were differences although the annual sum of both can be considered similar (around 850/900 mm). In 2023, the months of May, June, and July were considerably rainier than in 2022 season. Water availability in 2023 was more constant and assiduous.

On the contrary, during 2022 we witnessed more extreme phenomena: summer heat and water bombs from August to September, with a peak of 78.1 mm on September 24.

The ecophysiological and biochemical parameters, and their correlations, are expressed in Figs. 4, 5 and 6 and in Tables 3 and 4.

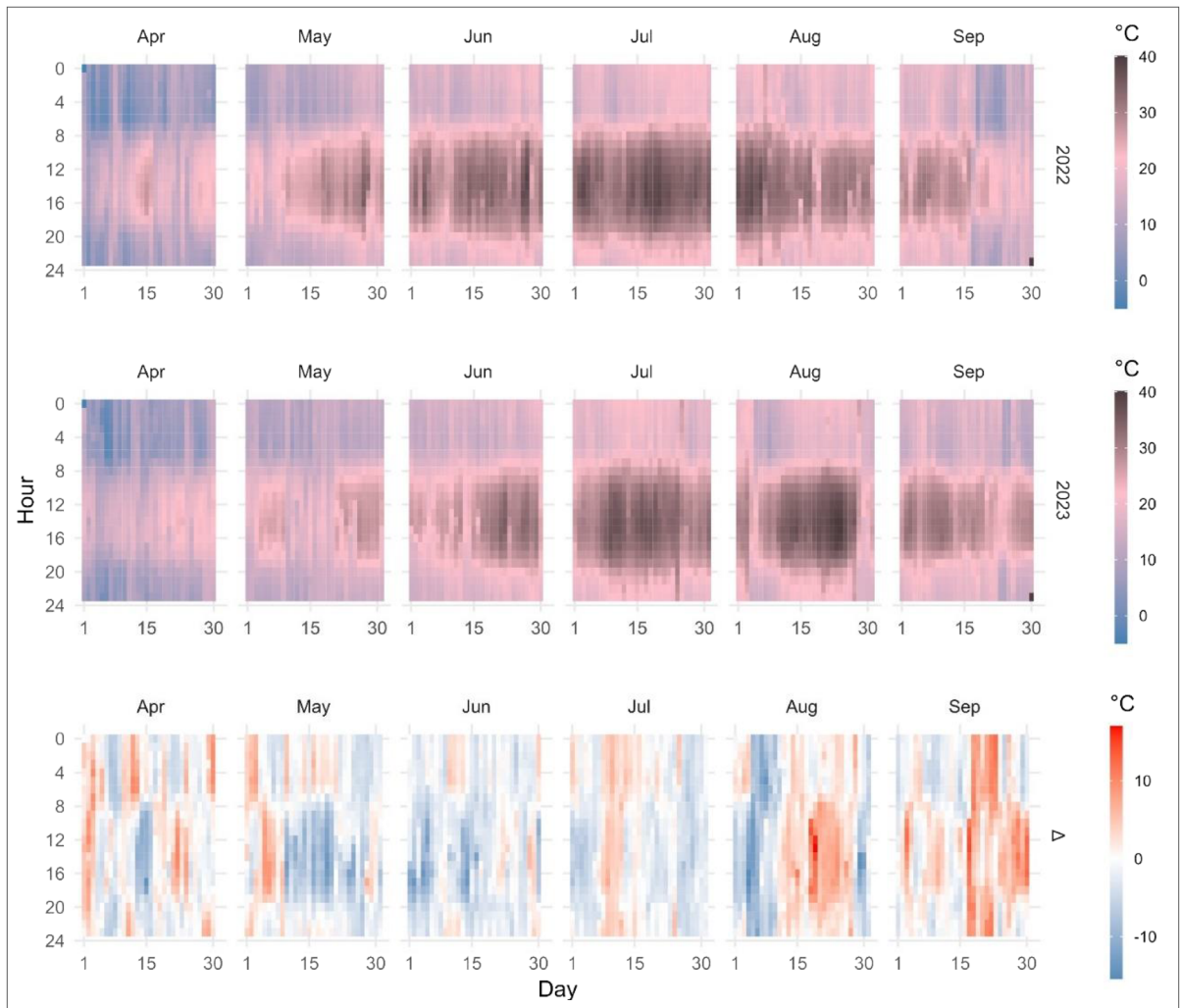
The strength and direction of the linear relationship between the continuous variables (PN and  $Fv/Fm$ ) are explained with the following correlation coefficients 0.53 in 2022 and 0.46 in 2023. A stronger correlation was found in the drier growing season (i.e., 2022); however, a similar trend was found in the measurements referring to 2023.

As regards gas exchange on single leaves, the most striking differences were monitored during the 2022 season. In fact, here at pre-bunch closure, véraison, and harvest the following leaf temperature increase percentages were respectively recorded in WS treatment compared to WWt, WSt, and WW: +4.69%, +6.94%, and +5.08%; +4.25%, +3.29%, and +2.37%; +5.52%, +7.95%, and +2.91%. In contrast, during the three measurements in 2023, no significant differences in leaf temperature were found at bunch closure. A significant difference was recorded between the WS and WWt treatments at véraison and a clear and substantial difference at harvest among treatments.

Transpiration in both years reflected the climatic framework (available temperatures and rainfall) and the water system of the plants, highlighting reduced

**Table 2** Primers and house-keeping used in the experiment

Gene name	Forward Primer (5'-3')	Reverse Primer (5'-3')	References
VvWRKY47	TCGCCTATTGGGGACTACAGTTTC	CTTTATGGGTTCGTCTCACTTGG	(Wang et al. 2014a, b)
VvWRKY39	GCCACTCCGACCGATAAGC	CCTTGGATTGTATTGCCTTT	(Guo et al. 2014)
EF 1 $\alpha$ a	GAAGTGGGTGCTTGATAGGC	AACCAAAATATCCGGAGTAAAAGA	(Wei et al. 2021)



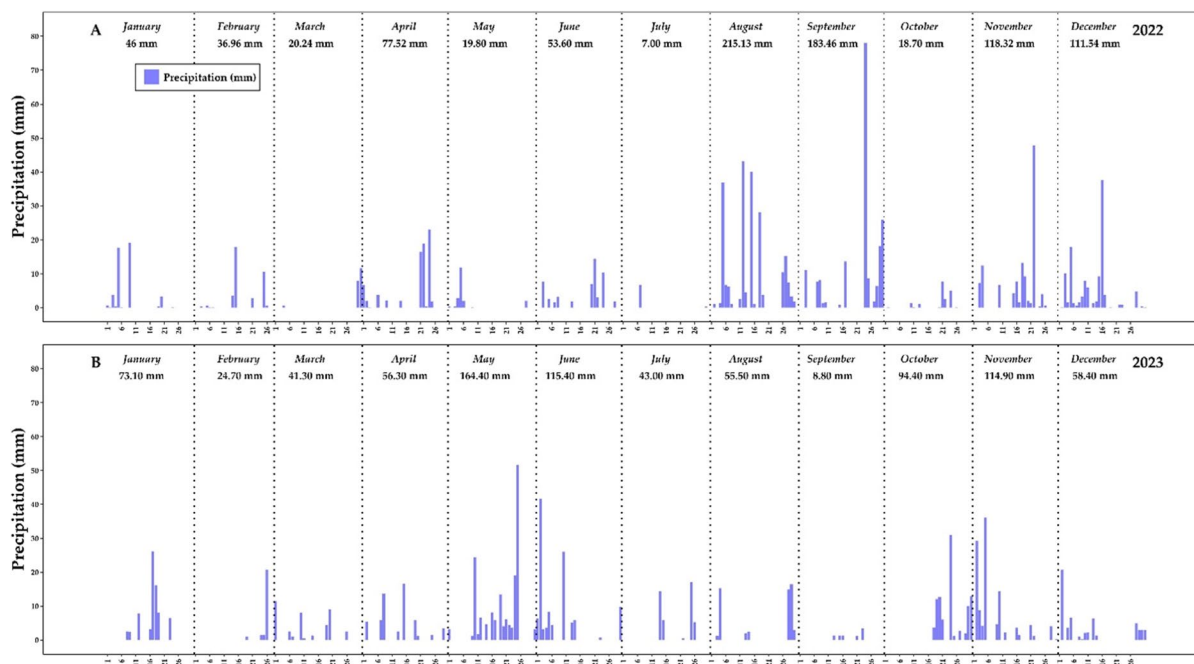
**Fig. 2** Representation of average hourly temperatures during the 2022 and 2023 seasons from April to September and representation of the thermal delta, understood as the difference in degrees centigrade between the two seasons ( $\Delta^{\circ}\text{C} = 2023 - 2022$ )

activity in WS plants (stressed plants with less water availability).

As regards stomatal conductance during 2022, the following percentage decreases compared to WWt, WSt, and WW were recorded during the hottest period in veraison in WS plants: -57.90%, -36.12%, and -57.89%. Here, although no difference was found between WS and WSt, only the WS treatment reached the critical stress threshold (i.e. 56.80  $\text{mmol}/\text{m}^2\text{s}$  WS vs 88.90  $\text{mmol}/\text{m}^2\text{s}$  WSt). In addition, during 2023, the following percentage decreases compared to WWt, WSt, and WW were recorded during the drier period at harvest in WS

plants: -62.56%, -59.57%, and -62.20%. Here, only the WS treatment reached the critical stress threshold (i.e. 52.20  $\text{mmol}/\text{m}^2\text{s}$ ).

The strength and direction of the linear relationship between the continuous variables ( $\Psi_{\text{stem}}$  and Proline) are explained with the following correlation coefficients 0.56 in 2022 and 0.43 in 2023. A stronger correlation was found in the drier growing season (i.e., 2022); however, a similar linearity was found in the measurements referring to 2023. In 2023, the evident graphic bipartition is given by the difference among the three sampling times. In fact, here the harvest samplings differ (the driest period) from others,



**Fig. 3** Representation of the daily rainfall trend from January to December during the two seasons 2022 and 2023

shifting their values toward stress thresholds (e.g.,  $-1.8$  Mpa).

The analyzed chlorophyll a and b amount was significantly lower in both years in the WS treatment.

In particular, looking at chlorophyll a, the following percentage increases were recorded respectively for WWt, WW, and WSt compared to WS treatment in 2022:  $+38.38\%$ ,  $+52.02\%$ ,  $+42.42\%$  (T1),  $+16.94\%$ ,  $+9.04\%$ ,  $+7.90\%$  (T2), and  $+5.43\%$ ,  $+21.26\%$ ,  $+19.90\%$  (T3); moreover, in 2023 these percentages picks  $+85.16\%$ ,  $+73.07\%$ ,  $+74.72\%$  (T1),  $+29.38\%$ ,  $+55.67\%$ ,  $+56.70\%$  (T2), and  $+56.11\%$ ,  $+65.00\%$ ,  $+88.88\%$  (T3) were noted.

Instead, looking at chlorophyll b, the following percentage increases were recorded respectively for WWt, WW, and WSt compared to WS treatment in 2022:  $+35.29\%$ ,  $+54.11\%$ ,  $+45.88\%$  (T1),  $+17.33\%$ ,  $+8.00\%$ ,  $+40.00\%$  (T2), and  $+19.54\%$ ,  $+18.39\%$ ,  $+14.94\%$  (T3); moreover, in 2023 these percentages picks  $+92.30\%$ ,  $+72.30\%$ ,  $+75.38\%$  (T1),  $+42.02\%$ ,  $+56.52\%$ ,  $+57.97\%$  (T2), and  $+56.25\%$ ,  $+60.93\%$ ,  $+93.75\%$  (T3) were noted.

In total carotenoids, the most relevant significant differences were found in the driest and warmest sampling times, at veraison in 2022 and pre-bunch

closure in 2023. On both dates, a smaller carotenoid content was found in the WS treatment.

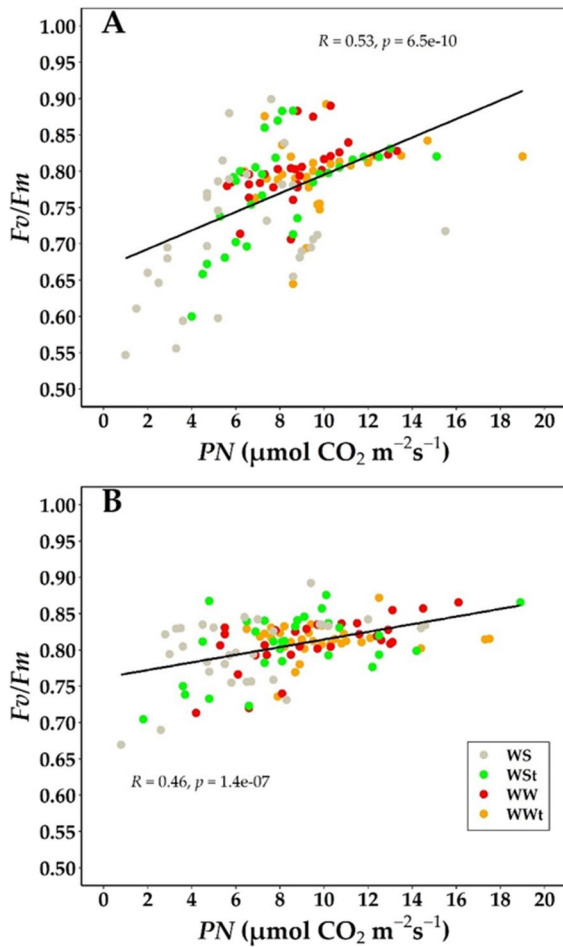
The PCA analysis is expressed in Fig. 7 (2022 and 2023 seasons).

The model engendered by PCA analysis is gathered by four components that account for 85.24% of the total variance. The PC 1 and the PC 2 exploited for the scatter plot account for 68.13% of the total variance.

The second dimension describes the following variables very well: caftaric (88.00% factor loadings) and Qgluglucu (78.60% factor loadings). While PrB1 (76.70% factor loadings), Epigallocatechin (82.80% factor loadings), fertaric (88.70% factor loadings), tcoutaric (86.40% factor loadings), Myricetin (94.50% factor loadings), and Isorhamnetin (71.10% factor loadings) are described by the first dimension.

WS has higher values in the y-axis than the other treatments. Furthermore, observing the widths of the angles, the following variables are positively correlated with each other: PrB1, Epigallocatechin, tcoutaric, fertaric, Resveratrol, Myricetin, Kaempferol, and Isorhamnetin. On the contrary, caftaric and ccoutaric are inversely correlated variables. The WS plants



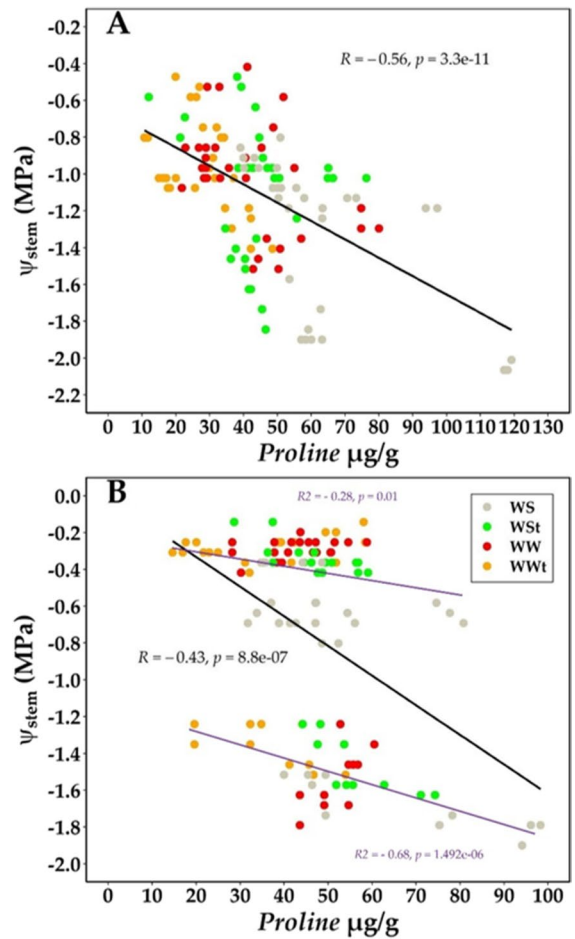


**Fig. 4** Linear correlation between chlorophyll fluorescence ( $F_v/F_m$ ; y-axis) and net photosynthesis (PN; x-axis). A (2022); B (2023)

differ from the other treatments and move to the right of the x-axis with positive values. Overall, the WWt, WW, and WSt treatments are similar for the considered variables, deviating from the WS treatment with a few overlapping ellipses.

The gene expression analyses resulting from the (RT)-PCR of the two WRKY genes (39 and 47) are depicted in Figs. 8 and 9. The elongation factor (housekeeping gene) was employed as a reference gene owing to its stability in response to treatments.

The  $2^{-\Delta\Delta CT}$  method was applied as a relative quantification strategy for quantitative realtime polymerase chain reaction data analysis. The boxes surrounded by brown color are not statistically significant ( $0.5 < x < 2$ ) (Rao et al. 2013).

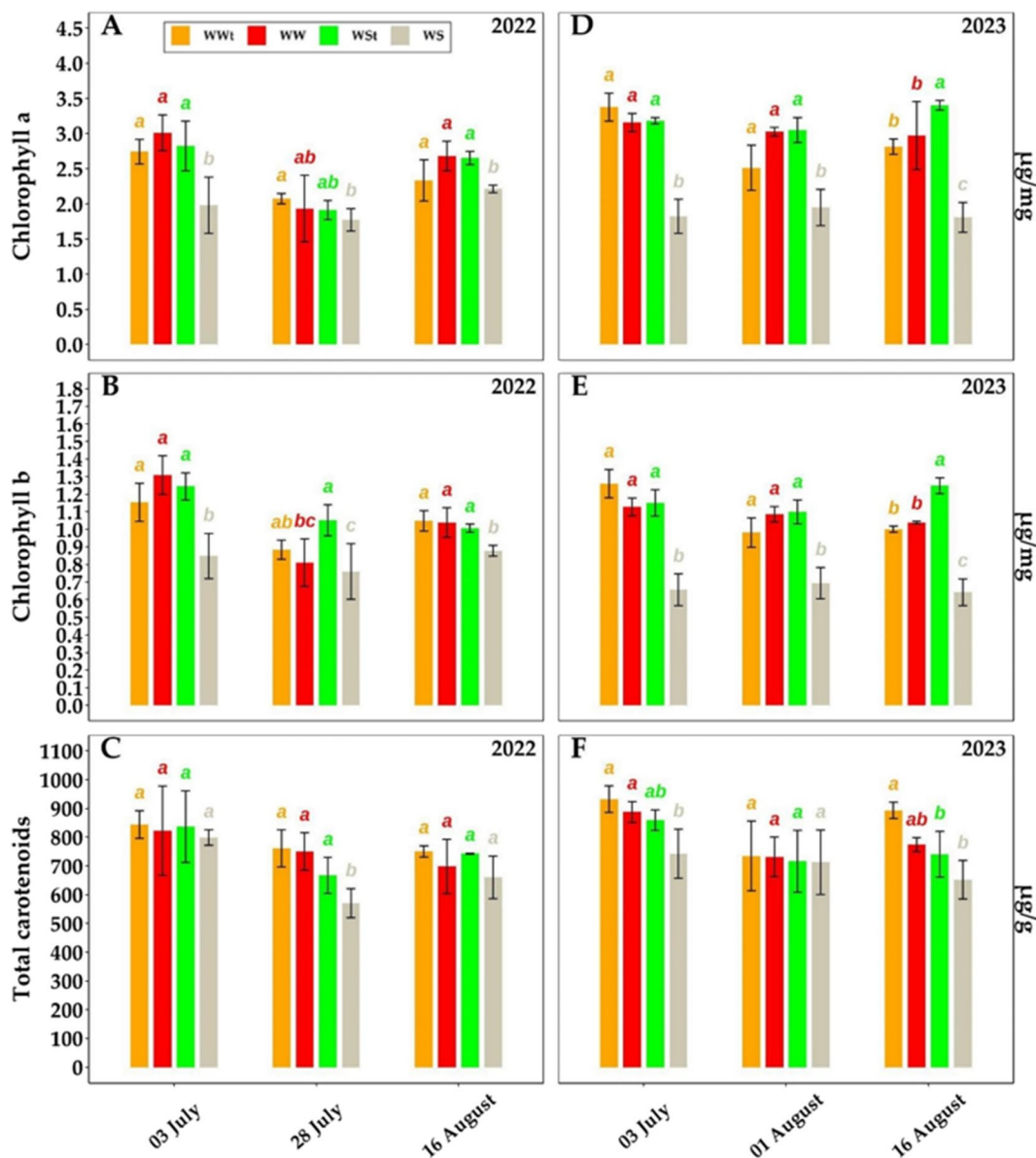


**Fig. 5** Linear correlation between stem water potential ( $\Psi_{stem}$ ; y-axis) and proline content (Proline; x-axis). A (2022); B (2023)

From the results obtained, a down-regulation emerged for the *VvWRKY47* gene and an up-regulation for the *VvWRKY39* gene.

Considering the WRKY 39 gene, during 2022 only the WSt treatment differed significantly (values  $< 0.5$  in WSt treatment; higher expression in WS plants = up-regulation) from the stressed plants (WS) at times T1 and T2. In a completely similar way, during 2023 only the WSt treatment differed in the driest period from the stressed plants (T1).

Considering the WRKY 47 gene, during 2022 only the WWt and WSt treatments (clinoptilolite application) differed significantly (values  $> 2$  in WWt and WSt treatments; lower expression in WS plants = down-regulation) from the stressed (WS)



**Fig. 6** Biochemical parameters of the treatments WWt (orange color), WW (red color), WSt (green color), and WS (grey color) during 3/07/2022 and 3/07/2023 (pre-bunch closure, time one; T1), 28/07/2023 and 01/08/2023 (véraison, time two; T2), and 16/08/2023 and 16/08/2023 (maturation,

time three; T3). The following parameters are reported: chlorophyll a, b, and total carotenoids. The significant difference is expressed as letters. The standard deviation is expressed as dark bars

plants at the T1 and T2 times. In a very similar way, during 2023 only the treatments with zeolitic supply differed in the driest period from the stressed plants (T1).

For a more accurate global picture, the trends during the three sampling times of the gene expressions

of two genes are reported below with the microclimatic scenario of the 10 days preceding the survey. In particular, natural rainfall, applied irrigation, and maximum temperatures (recorded above 34 °C) are reported.

**Table 3** Ecophysiological parameters of the treatments WWt (orange color), WW (red color), WSt (green color), WS (grey color) during 3/07/2022 (pre-bunch closure, time one; T1), 28/07/2022 (véraison, time two; T2), and 16/08/2022 (matura-

tion, time three; T3). The following parameters are reported: leaf temperature (Leaf T), stomatal conductance (*gs*), and transpiration (E). The significant difference is also expressed in the table

	2 July 2022			28 July 2022			16 August 2022		
	Leaf T °C	<i>gs</i> mmol/ m <sup>2</sup> s	E mmol/m <sup>2</sup> s	Leaf T °C	<i>gs</i> mmol/m <sup>2</sup> s	E mmol/m <sup>2</sup> s	Leaf T °C	<i>gs</i> mmol/m <sup>2</sup> s	E mmol/m <sup>2</sup> s
Zeolite Treatment (T)									
WWt	37.90 ± 1.27 b	133.40 ± 44.54 ab	4.47 ± 0.31 b	35.83 ± 0.55 b	134.80 ± 29.19 a	4.58 ± 0.60 a	30.70 ± 0.53 a	286.60 ± 70.59 a	5.25 ± 0.95 a
WSt	38.13 ± 0.98 b	138.00 ± 16.04 ab	6.84 ± 0.92 a	35.27 ± 0.54 b	88.90 ± 26.48 b	3.28 ± 0.72 b	30.62 ± 0.28 a	295.50 ± 27.02 a	5.75 ± 0.33 a
Irrigation Regime (IR)									
WW	37.42 ± 1.22 b	156.50 ± 35.00 a	5.16 ± 0.70 b	35.88 ± 0.29 b	134.90 ± 26.86 a	4.70 ± 0.69 a	30.43 ± 0.29 a	309.00 ± 48.35 a	6.01 ± 0.58 a
WS	40.72 ± 1.10 a	111.40 ± 23.86 b	5.01 ± 0.97 b	37.41 ± 0.62 a	56.80 ± 14.18 b	2.50 ± 0.48 c	30.71 ± 1.31 a	135.10 ± 48.56 b	3.37 ± 0.89 b
Significance Pr(>F)									
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05									
T	0.007 **	0.862	0.026 *	0.000 ***	0.049 *	0.108	0.703	0.000 ***	0.003 **
IR	0.000 ***	0.051	0.000 ***	0.005 **	0.000 ***	0.000 ***	0.671	0.000 ***	0.000 ***
T × IR	0.000 ***	0.018 *	0.000 ***	0.000 ***	0.048 *	0.030 *	0.447	0.000 ***	0.000 ***

## Discussion

Grapevine crop is vulnerable to several atmospheric coefficients that act over a vast gamma of timescales, for example, frosts or hail (short-duration weather occurrences), heat stress, drought (medium-range occurrences), and fluctuations in temperatures and precipitation (longer-term trends). Without targeted anthropic changes aimed at a planet-friendly approach, emissions will continue to increase with the consequence of a rise in the average annual global temperature (up to 6 °C or more by the end of this century) (Jones et al. 2022).

This experimentation arises both from the need to find sustainable and compatible solutions with the company's perspective to make the plant in balance with the system and with the microclimate and from the desire to find a pragmatic and more realistic genetic approach towards this crop by investigating

some imputed transcriptional factors to abiotic stresses.

From a general perspective, the results of the study demonstrated how the treatments (zeolites and/or irrigation) modified the ecophysiological structure of the plant by changing the biochemical contents in quantitative terms compared to stressed plants. The changes recorded in gas exchange and leaf phenolic parameters showed in WWt, WW, and WSt plants an ecophysiological organization of the plant of adaptation and harmony towards abiotic stresses. Even at the gene expression level, these differences have been appreciated in the study of the two transcriptional factors.

The accumulations of proline (an osmoprotectant that has a role in counterbalancing the osmotic stress consequence (Liang et al. 2013)) in dehydrated and stressed grapevines (WS) could be generated both by activation of its biosynthesis and by its inactivation of

**Table 4** Ecophysiological parameters of the treatments WWt (orange color), WW (red color), WSt (green color), WS (grey color) during 3/07/2023 (pre-bunch closure, time one; T1), 01/08/2023 (véraison, time two; T2), and 16/08/2023 (matura-

tion, time three; T3). The following parameters are reported: leaf temperature (Leaf T), stomatal conductance (gs), and transpiration (E). The significant difference is also expressed in the table

	3 July 2023			01 August 2023			16 August 2023		
	Leaf T °C	gs mmol/m <sup>2</sup> s	E mmol/ m <sup>2</sup> s	Leaf T °C	gs mmol/m <sup>2</sup> s	E mmol/m <sup>2</sup> s	Leaf T °C	gs mmol/m <sup>2</sup> s	E mmol/m <sup>2</sup> s
Zeolite Treatment (T)									
WWt	31.79	135.90	3.43	31.05	146.40	3.35	35.14	138.30	3.22
	± 0.49	± 24.97	± 0.59	± 0.74	± 36.76	± 0.74	± 0.62	± 32.55	± 0.84
	a	a	a	b	ab	a	b	a	a
WSt	31.12	159.00	3.61	31.34	174.00	4.16	34.31	129.10	3.32
	± 1.76	± 37.76	± 0.71	± 1.11	± 43.94	± 0.65	± 0.78	± 80.96	± 1.58
	a	a	a	ab	a	b	c	a	a
Irrigation Regime (IR)									
WW	31.67	146.90	3.43	31.62	153.10	3.36	35.99	138.10	3.98
	± 1.72	± 36.29	± 0.44	± 0.65	± 37.80	± 0.57	± 1.00	± 57.02	± 0.93
	a	a	a	ab	a	a	b	a	a
WS	33.28	129.60	3.20	32.37	105.50	2.57	37.04	52.20	1.84
	± 0.66	± 32.34	± 0.51	± 1.42	± 43.11	± 0.58	± 0.39	± 27.46	± 0.74
	a	a	a	a	b	c	a	b	b
Significance Pr(>F)									
Signif. codes: 0 '****' 0.001 '***' 0.01 '**' 0.05									
T	0.017 *	0.384	0.259	0.019 *	0.021 *	0.000 ***	0.000 ***	0.038 *	0.293
IR	0.260	0.780	0.863	0.119	0.440	0.961	0.640	0.011 *	0.004 **
T × IR	0.008 **	0.061	0.263	0.485	0.005 **	0.000 ***	0.000 ***	0.039 *	0.002 **

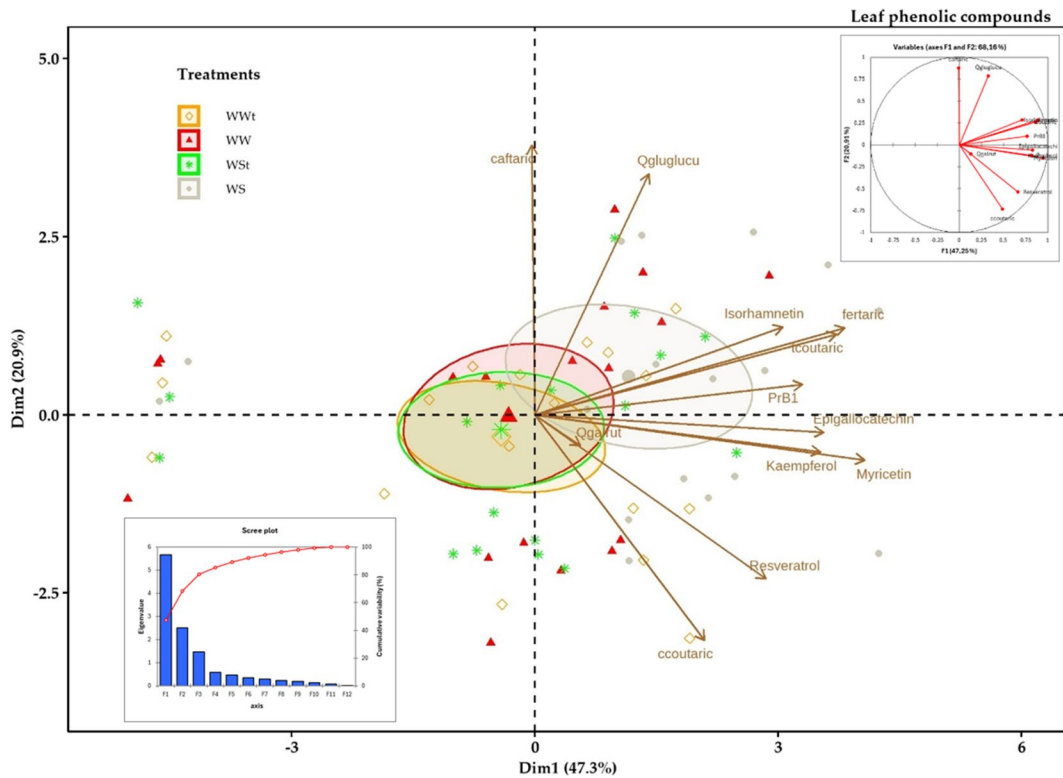
the degradation. There may have been an arrest of the degradation metabolism whose responsibility is borne of the two following enzymes proline dehydrogenase/oxidase and  $\Delta 1$ -pyroline-carboxylate dehydrogenase. In fact, under dehydration conditions, when gene expression for  $\Delta 1$ -pyroline-carboxylate synthetase is heavily induced, gene expression for proline dehydrogenase is hindered (Yoshida et al. 1997).

The osmotic adjustment and the increase in leaf abscisic acid (ABA) probably allowed the plants to balance the water shortage in the WS treatment during the beginning of the 2022 season. However, the decrease in photosynthesis rate under severe drought stress (July 2022 and August 2023) is related to the abnormalities in the internal structure of chloroplast (Miyashita et al. 2005) as observed in the reduction of chlorophyll fluorescence (0.55–0.56  $F_v/F_m$  in WS treatment). Foliar ABA suggests a mechanism of long-term down-regulation of transpiration in WSt and WS plants (reduction in transpiration rate; 3.28 and 2.50 mmol/m<sup>2</sup>s, 28 July 2022) to preserve water

under repeated drought circumstances (Tombesi et al. 2015).

In fact, stomatal regulation represents one of the notable mechanisms allowing crops to settle and optimise CO<sub>2</sub> assimilation versus evaporative H<sub>2</sub>O leak. Under soil water limitation or elevated atmospheric evaporative request conditions, the partial or entire stomatal closure lets vines keep a useful water balance while restricting carbon gain (Franks 2013).

In the moments of greatest thermal and water stress of the two seasons, only the WWt, WW, and WSt treatments managed to cope with the water deficiency and high temperatures. The increase in leaf temperature could be a reaction to the shortage of water reserve and a result of stomata closing to prevent evaporation and therefore cooling of leaves (Markulj Kulundžić et al. 2016). Like in our result, according to Pallas et al. (1967) in *Gossypium hirsutum* L. leaf, temperature was correlated to soil humidity, the cotton grown in well-watered soil showed slightly higher than ambient temperature,



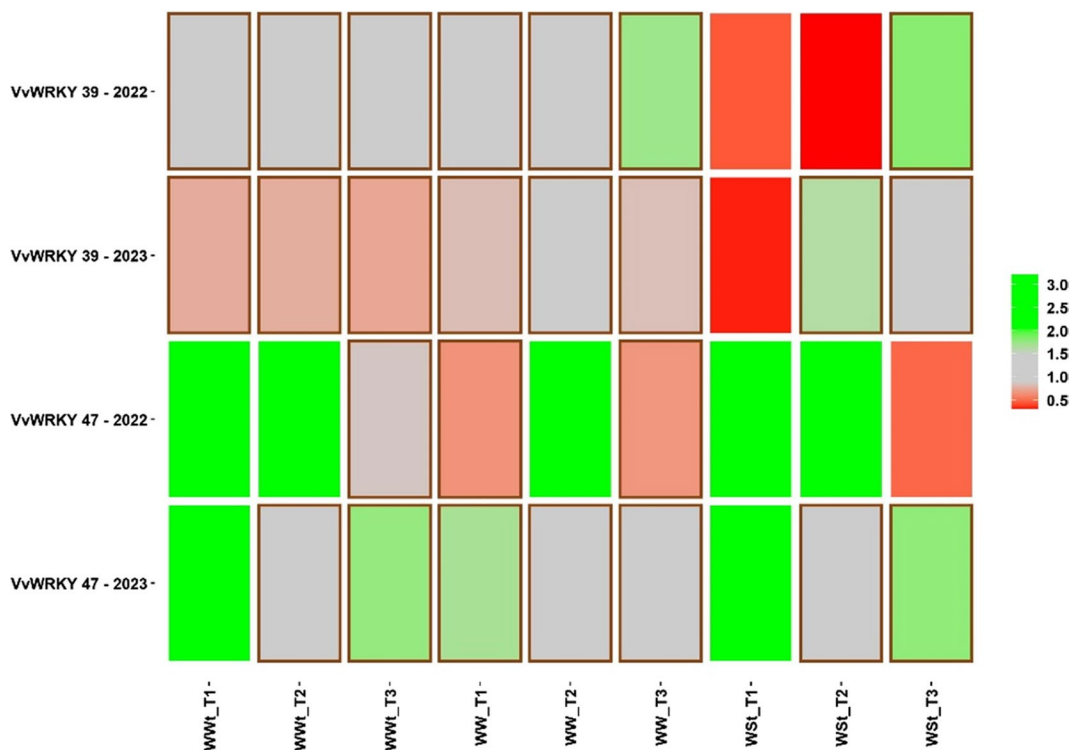
**Fig. 7** The PCA analysis was performed by examining leaf phenolic compounds during T1-2022/2023, T2-2022/2023, and T3-2022/2023. The following abbreviations correspond to the respective compounds: PrB1 (procyanidin B1), Epigallocatechin (epigallocatechin), caftaric (caftaric acid), ccoutaric (c-coutaric acid), tcoutaric (t-coutaric acid), fertaric (fertaric

acid), Resveratrol (resveratrol-glucoside), Myricetin (myricetin-3-O-glucosid), Kaempferol (kempferol-3-O-glucosid), Isorhamnetin (isorhamnetin-3-O-glucosid), Qgalrut (qu-3-O-galattosid + rutin), and Qgluglucu (qu-3-O-glucosid + qu-3-O-glucuronide)

whereas cotton grown under land drought stress exhibited 3.4 °C leaf temperature higher than environmental one. Under water-limited conditions, the lower transpiration rates limit leaf cooling, and the canopy microclimate is in general 2–3 °C higher than in well-irrigated crops (Nautiyal et al. 2008). Therefore, the occurrence of physiological adaptations furnished by biochemical modifications, water balance, and gene expression could have led to thermo-tolerance adaptation in WSt treatment (no irrigation supply).

On hemp crop, it was shown an increasing trend in superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase compared to well-watered conditions throughout the season and a drop in chlorophyll a, b content under drought stress. Here, under well-watered states, the zeolite skill prevented an augment in enzyme activity. At the same time, zeolite

compensated for the drop in chlorophyll pigments (Bahador and Tadayon 2020). In accordance with our work, it was indicated a reduction in photosynthetic pigment content under drought stress for *Helianthus annuus* L. (a reduction in Chl a, b by 29.00% and 32.00% respectively was noted in drought-stressed conditions) (Shehzad et al. 2023). The drop in carotenoid content in WS treatment was probably owing to their susceptibility to oxidative destruction. Water limit can diminish the chlorophyll tissue concentrations and carotenoids, chiefly with the reactive oxygen species production in the thylakoids (Murtaza et al. 2016). Water stress produced a depletion of photosynthetic reaction centers (i.e., chlorophyll a). Whereas carotenoids are principally detected in conjunction with photosynthetic reaction centers, this found-carotenoid impoverishment was to forecast. Other authors also confirmed a fall in the quantum



**Fig. 8** General view of expression profile of VvWRKY39 and 47 responses to zeolite and/or irrigation treatments (2022 and 2023 seasons). Gene expression analysis was performed at three different times, as follows, 3/07/2022 and 3/07/2023

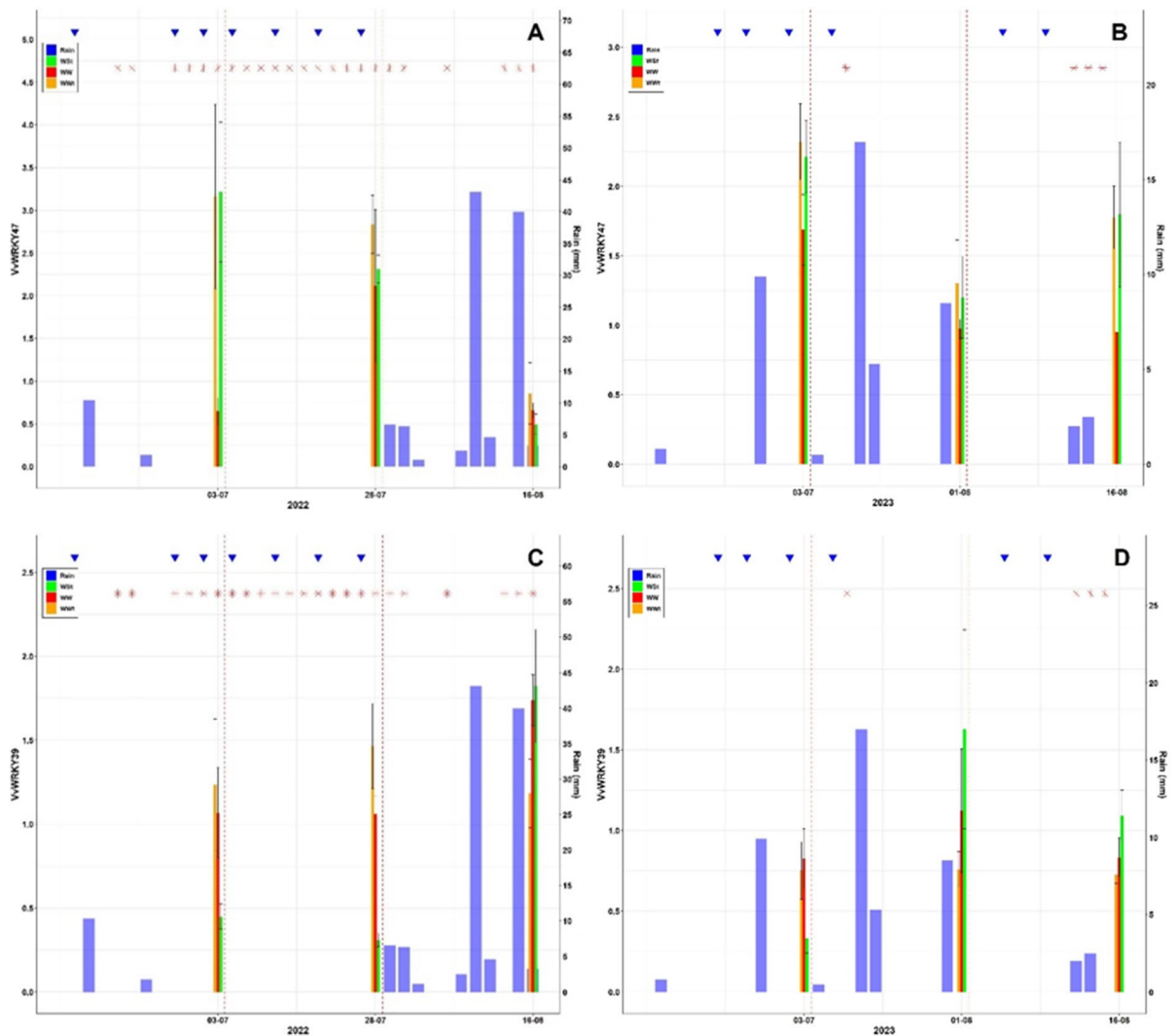
(pre-bunch closure, time one; T1); 28/07/2022 and 01/08/2023 (véraison, time two; T2); 16/08/2022 and 16/08/2023 (maturation, time three; T3)

yield of PSII electron transport (quenching) with a significant reduction under drought in carotenoid content (Efeoğlu et al. 2009). In fact, it was reported that in plants simultaneously displaced by drought stress and elevated sunshine irradiance or/and temperature (i.e., midday hours), the enzyme activity pitched at detoxifying  $H_2O_2$  slumped remarkably (Fini et al. 2012).

Well-being in the balance of the exploitation of water resources can also be appreciated from the water potential parameter. Clinoptilolite intensified soil water-holding capacity and might have ameliorated soil quality in the root zone (AL-Busaidi et al. 2011) by extending the soil wetness period (Wu et al. 2019). These minerals can retain  $H_2O$  up to 60.00% of their weight owing to their porous crystal framework and hydrate and dehydrate reversibly without changes (Zheng et al. 2018). The application of zeolite alleviated the negative effects of water stress by controlling the release of anthropogenic and meteoric

water and the humidity present in the soil (WWt and WSt), improving the resilience of the plant during high temperatures. It was seen that sandy soil could be amended with around 0.300–0.050 mm particle sizes zeolite to enhance water holding capacity while still maintaining a satisfactorily elevated saturated hydraulic conductivity. The water content of zeolite-sand mixture at land matric potentials from -3 to -10 kPa increased with dwindling zeolite particle size (Huang and Petrovic 1994).

In agreement with recent works, we found a higher leaf content of epigallocatechin, quercetin, kaempferol, myricetin, fertaric, coutaric, and caftaric acids during periods of peak stress in WS plants. Newly, flavonoids were hypothesized to have antioxidant tasks in reaction to severe light stress, probably integrating the typical roles of antioxidant enzymes. Phenylpropanoids characterized by a high potential to reduce reactive oxygen species are almost solely synthesized in response to abiotic stress, such as water



**Fig. 9** A particular view of the expression profile of VvWRKY39 (C; 2022 and D; 2023 seasons) and 47 (A; 2022 and B; 2023 seasons) responses to zeolite and/or irrigation treatments is depicted. Gene expression analysis was performed at three different times, as follows, 3/07/2022 and 3/07/2023 (pre-bunch closure, time one; T1); 28/07/2022 and 01/08/2023 (véraison, time two; T2); 16/08/2022 and

16/08/2023 (maturation, time three; T3). The  $2^{-\Delta\Delta CT}$  method was applied as a relative quantification strategy for quantitative realtime polymerase chain reaction data analysis. The dashed vertical intercepts represent the separation of times (T1, T2, T3). Irrigations and maximum temperatures above 34 °C are respectively drawn by blue triangles and red asterisks. The standard deviation is represented by the black vertical bars

stress (Agati and Tattini 2010; Agati et al. 2011). It was shown a boost in catechin galloyl derivatives in tea plants as a repercussion of severe water stress; the levels of oxidized flavan-3-ols, epicatechin quinone and epigallocatechin gallate quinone enhanced with a sharp under drought (Hernández et al. 2006). In addition, as drought stress proceeded, in *Fraxinus ornus* L. leaves, higher storage of esculetin and quercetin

3-O-glycosides (phenylpropanoids characterized by scavenge  $H_2O_2$  skill) was found linked with decrements in ascorbate peroxidase and catalase activity (Fini et al. 2012). In *Chrysanthemum morifolium* L., rutin, quercetin, ferulic acid, apigenin, and luteolin amounts generally grew (by expression patterns of the following key genes pal, chi, and f3h) with drought rising (Hodaei et al. 2018). The increase in quercetin

in grape leaves subjected to stress could be explained by its antioxidant role; the OH-group situated at the 3-position of the flavonoid skeleton acts by inhibiting reactive oxygen species aggregation and making them most skillful against oxidative stress (reactive oxygen species scavengers' function) (Singh et al. 2021).

From gene expression analysis in the open field, a possible approach emerged towards WRKY genes study correlated to abiotic stress. A down-regulation emerged for the VvWRKY47 gene and an up-regulation emerged for the VvWRKY39 gene. It was demonstrated that a major part of the examined VvWRKY transcripts manifested an accumulation high level at eight days after the onset of the stress treatment (drought), approximately enhancing up to twenty-two fold for VvWRKY05. An expression top of VvWRKY51, VvWRKY35, VvWRKY03, and VvWRKY52 was noted at 12 h after *Erysiphe necator* inoculation, whilst a tardier induction was seen for the 29 (Wang et al. 2014a, b).

The VvWRKY47 showed interactions from the beginning with the zeolitic treatment. Specifically, the differences found were the following, WWt and WSt were significantly higher expressed compared to WS at time T1, T2 of 2022 and at time T1 of 2023. Only at time T2 of 2022 did the WW treatment show a similar trend. This would suggest that 47 could be a valid candidate in the evaluation of drought and temperature stress in the open field. Here the gradual release of the water resource by the clinoptilolite may have triggered an early activation response to counterbalance the water stress. At the other times, the differences were probably absent due to atmospheric rain events which homogenized the data.

Considering the VvWRKY39 it would seem that irrigation and consequently also rainwater triggered responses similar to WS plants for WW and WWt plants. The determining factor for this gene could be water; the irrigation 12 h before carrying out the sampling (T1 and T2 2022; T1 2023) and the rains (T3 2022; T2 and T3 2023) that occurred the day before the sampling would seem to have affected the output of this gene. In fact, only the treatment with zeolite alone (WSt) proved to be less expressed compared to stressed plants during T1-T2 2022 and T1 2023 (up-regulated gene in stressed plants).

According to Guo et al. (2014), from RT-PCR data, the VvWRKY family tends to be down-regulated to a higher level by salinity stress (SS) than by

water one (DS). VvWRKY 12, 14, 15, 26, 28, 31, 32, 39, 46, and 48 that whole manifested clear down-regulation by salt stress management, were up-regulated to different levels by water stress imposition, showing distinguishable dissimilar regulatory networks presence. In this study, the reaction time for the expression pattern alters was evaluated too. VvWRKY1 and 51, showed the following altered expression at 1 h under SS and 24 h under DS, whilst VvWRKY57 and 59 showed up-regulated expression at 48 h under SS and 144/168 h under DS (confirming that an inevitable reply time is mandatory for VvWRKYs to answer to water stress). In addition, VvWRKY2 was down-regulated at 1 h after the onset of the SS treatment but was up-regulated after 48 h showing two different trends: an early down-regulation but succeeding up-regulation.

All this leads to an unequivocal conclusion, there is not yet a single key to studying the behavior of these transcription factors. The subject is at the beginning of its knowledge, therefore further studies are considered necessary to clarify, explain, deny, or confirm the experiments carried out so far. All this in light of the fact that the identification and functional analysis of WRKY genes are of noticeable zest in the climate change scenario and are necessary to understand any new adaptation or resilience strategies of the plant.

## Conclusions

Briefly, our study aims to answer four primary questions: (i) Does zeolite application affect canopy status and biochemical leaf components? Leaf ecophysiology and biochemical compounds were influenced by the treatments and therefore by the zeolite. In fact, treatments with zeolite and/or irrigation reduced the content of quercetin and proline, increasing the photosynthetic pigments. (ii) Is it possible to try to start a gene expression approach in an open-field vineyard, without fixed and stable external parameters (i.e., no greenhouse conditions) and obtain an interconnected net of interdisciplinary data? The matter is at the beginning of its knowledge, therefore further studies are considered necessary to clarify, explain, deny, or confirm the greenhouse experiments carried out so far. It is possible to attempt a genetic approach in the vineyard with the WRKY genes thanks to their



non-immediate response times. However, since this is the first open-field approach, research must delve deeper into this topic and dissect every peculiarity. (iii) Which gene factors best describe water and temperature stress? VvWRKY47 showed interactions with zeolitic treatment from the beginning. This gene would appear to be the most suitable for evaluating open-field drought due to its response times and type of response. On the contrary, VvWRKY39 would seem suitable for evaluating water changes in the plant and therefore more generic responses in the plant's water balance. (iv) zeolite soil application could be a valid corroborate for grapevine homeostasis? Yes, the zeolite in our experiment acted as a water flywheel, mitigating the effects of climate change.

**Author contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Eleonora Cataldo, Sergio Puccioni, Massimo Gori, and Roberto Natale. The first draft of the manuscript was written by Eleonora Cataldo. All authors read and approved the final manuscript.

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**Data availability** The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

#### Declarations

**Conflict of interest** Authors state no conflict of interest.

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