



Effects of foliar applications of a proline-rich specific yeast derivative on physiological and productive performances of field-grown grapevines (*Vitis vinifera* L.)

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ABSTRACT

The occurrence of heat waves in vineyards can damage the integrity of photosynthetic systems, impair yield and affect fruit quality. The frequency of these events is increasing due to global warming, and this is the subject of growing concern among the players in the wine sector. Canopy applications of specific yeast derivatives (SYD) are proposed to enhance plant physiology under abiotic stresses and preserve yield and fruit composition. This study aimed at assessing the effects of foliar application of a proline-rich SYD formulate, LalVigne ProHydro™ (LPH), on field-grown vines cv. Barbera. In a two-year experiment, vines subjected to multiple LPH foliar applications were compared to untreated vines (C). Gas exchange rates and vegetative and productive performances were monitored together with leaf proline, carotenoids and H₂O₂ concentrations. The data demonstrate that the formulate improved midday leaf water potential (+0.14 MPa), stomatal conductance (+0.15 mol m⁻² s⁻¹) and assimilation rates (+2.8 μmol m⁻² s⁻¹) under summer stress conditions. Interestingly, LPH leaves showed a dramatically higher concentration of proline (+600 %) and a significantly lower H₂O₂ content (-26 %) as compared to C leaves. The data suggest that this could be related to a priming effect of LPH application. Moreover, in SYD vines, cluster sunburn occurrence was reduced by 69 %, compared to C vines. At harvest, LPH improved vine yield (+1.27 kg), and LPH grapes had a significantly lower sugar concentration (-2.09 °Brix). The study demonstrates for the first time the potential benefits of proline-rich SYD application in vineyards subjected to multiple summer stress. Further studies should focus on the treatment effects on proline biosynthesis and gene expression.

1. Introduction

The increasing global temperature and unequal rainfall distribution caused by climate change (Calvin et al., 2023) are likely to make vineyard summer stresses a frequent phenomenon. Vines subjected to multiple summer stresses reduce their CO₂ assimilation rates and dry matter partitioning to sink organs. If limiting conditions persist for long periods, Photosystem II loses its functionality, and leaves exhibit typical yellowings, leading to necrosis and abscissions. This results in reduced berry growth and yield. At the same time, sun exposed grapes are subjected to sunburn when air temperature is higher than 35 °C and air relative humidity is low. In such conditions flavonols are accumulated in

the berry skin to protect from UV radiations, and anthocyanins are subjected to progressive degradation. At the same time, the high and swift evaporative flux from the berry to the extra-cuticular environment can provoke a permanent shriveling (Bondada and Keller, 2012). Sunburn susceptibility can be dependent on the cultivar and, specifically, on skin thickness. At given air-to-berry vapor pressure deficit levels, the thicker the berry skin, the lower the cuticular transpiration (Bondada and Keller, 2012).

Under heat stresses or drought exposure, vines enact different mechanisms to prevent water losses, thus maintaining cellular turgor. Stomatal closure, leaf structural changes, paraheliotropism and early basal leaf senescence are just a part of them. In addition, plants respond

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through biosynthesis and accumulation of molecules called osmolytes that may be able to increase cell water potential and preserve cell turgor (Hare and Cress, 1997). Among the several osmolytes, proline is reckoned to be involved in the tolerance of many abiotic stresses and to be accumulated in many plant species (Szabados and Savouré, 2010). In addition to osmotic regulation, some studies highlight that day/night proline turnover allows for the dissipation of energy excess by generating NADP⁺ and that proline itself can directly scavenge reactive oxygen species (ROS) that usually accumulate during stress exposure (Apel and Hirt, 2004; Szabados and Savouré, 2010).

In recent years, a multitude of so-called biostimulants and naturally obtained formulatates have been proposed for the improvement of vineyard tolerance to abiotic stress (Gutiérrez-Gamboa et al., 2019; Monteiro et al., 2022; Rouphael and Colla, 2020; Samuels et al., 2022). Although evidence for a variable improvement of vine physiological performances under controlled conditions are available today, some doubts exist about their mechanisms of action and in-field efficacy, in relation to the costs of the application (Frioni et al., 2021, 2018; Gutiérrez-Gamboa et al., 2019; Rouphael and Colla, 2020). Inactivated specific yeast derivatives (SYD) are a new class of formulatates containing significant concentrations of mannoproteins, β -glucans and chitin (Kapteyn et al., 1999). Foliar SYD applications exert an elicitor function when they get in contact with plant tissues, simulating a pathogen-plant reaction and therefore triggering hypersensitivity-related gene expression (Ferrari, 2010; Portu et al., 2016). The efficacy of some SYD in improving grape phenolics concentration (Pastore et al., 2020), aromatic profile (Šuklje et al., 2016) and skin thickness (Giacosa et al., 2019; Villangó et al., 2015) has been recently demonstrated. However, the composition of the available SYD formulatates is largely variable, and the effects on plant tissue varies accordingly. Today, new proline-rich SYD formulatates are available. Their proline concentration could directly provide osmolytes to plant tissues and/or induce a priming effect, triggering an osmotic stress reaction into plant organs that promotes endogenous osmolytes accumulation (Gohari et al., 2021; Rouphael et al., 2021, 2017). However, these formulatates have never been tested before on grapevine, and their efficacy needs to be demonstrated.

The aims of this study were: (i) to evaluate the effects of proline-rich yeast (*Saccharomyces cerevisiae*) derivative applications on grapevine physiology and grape composition under field conditions; (ii) to quantify the trend of proline and stress-related metabolites accumulation in leaves according to the foliar treatments. Our general hypothesis was that the alteration of natural leaf proline concentration could interact with plant water status and canopy functioning, thus preserving dry matter partitioning to sink organs under limiting conditions.

2. Materials and methods

2.1. Site, weather data, and experimental design

The experiment was carried out in 2020 and 2022 in a non-irrigated vineyard of cv. Barbera (*Vitis vinifera* L.), clone AT84, grafted on 420A, planted in 2003 in Bacedasco Basso, Vernasca (PC), at 'Azienda Vitivinicola Villa Rosa', Italy (44°50' N, 9°54' E; 183 m a.s.l.). The vineyard was planted on a 15 % northwest facing slope. In 2021, data were not collected due to a spring frost event that killed 75% of developing shoots and dramatically reduced yield (Del Zozzo et al., 2022).

Row orientation was NW-SE and vine plant density was 3333 vines/ha, with a spacing of 2.5 m x 1.2 m. The vines were trained to a vertically shoot-positioned, bilateral double cane pruned Guyot trellis, with 12 buds on the two canes and four nodes on the two renewal spurs. Canes were tied to the first wire at 0.80 m from the ground, with three pairs of surmounted catching wires forming a canopy wall extending approximately 1.2 m above the first wire.

The daily maximum, minimum, and mean temperatures (°C) and daily rainfall (mm) were recorded by a weather station located near the

vineyard. Growing Degree Days (GDD) accumulation from 1st April to 31st October were calculated for 2020 and 2022 on a 10 °C base temperature (Baskerville and Emin, 1969).

In a plot of 96 vines, divided in four complete randomized blocks of 24 vines each (RCBD), the following two treatments were arranged: 48 vines (12 per block) were assigned to the multiple treatments with the SYD LalVigne ProHydro™ (LPH) (Patent Application No. EP21382178.8 by Lallemand Inc., Canada); the remaining 48 vines were assigned to the untreated control vines (C). LalVigne ProHydro™ is a SYD formulate obtained by *Saccharomyces cerevisiae* and containing L-proline derived from *Corynebacterium glutamicum*. On Day of Year (DOY) 125 (4 May), six randomly chosen vines per treatment per block were tagged as sub-replicates and monitored throughout the experiment duration. LPH was foliarly applied five times during the season from groat-sized phenological stage to full veraison (BBCH 73 and 81, respectively) (Lorenz et al., 1995), at the dosage of 3.33 g/L. In 2020, the dates of application were DOY 167, 178, 192, 203, and 220 (15 June, 26 June, 10 July, 21 July, and 7 August), while in 2022, they were 173, 182, 193, 202, and 206 (22 June; 1, 12, 21 and 25 July) (Fig. 1). The phenological stages were determined according to Lorenz et al. (1995).

2.2. Vine physiology, thermal images, and vegetative growth

Leaf gas exchanges parameters, midday leaf water potential (ψ_{leaf}), and leaf photosystem II (PSII) efficiency (Fv/Fm) were measured on five dates in 2020, DOY 191, 202, 209, 218, and 229 (9, 20, and 27 July; 5 and 16 August), and on two dates in 2022, DOY 195 and 217 (14 July and 5 August).

As for gas exchange parameter, leaf assimilation rate (A) and stomatal conductance (g_s) were measured between 12:00 and 13:00 on 12 vines per treatment, using a LCi T Pro (ADC Bioscientific Ltd., Hoddesdon, Herts., UK) on one main leaf inserted on nodes 3–6, under saturating light conditions ($\text{PAR} > 1400 \mu\text{mol m}^{-2} \text{s}^{-1}$) and ambient relative

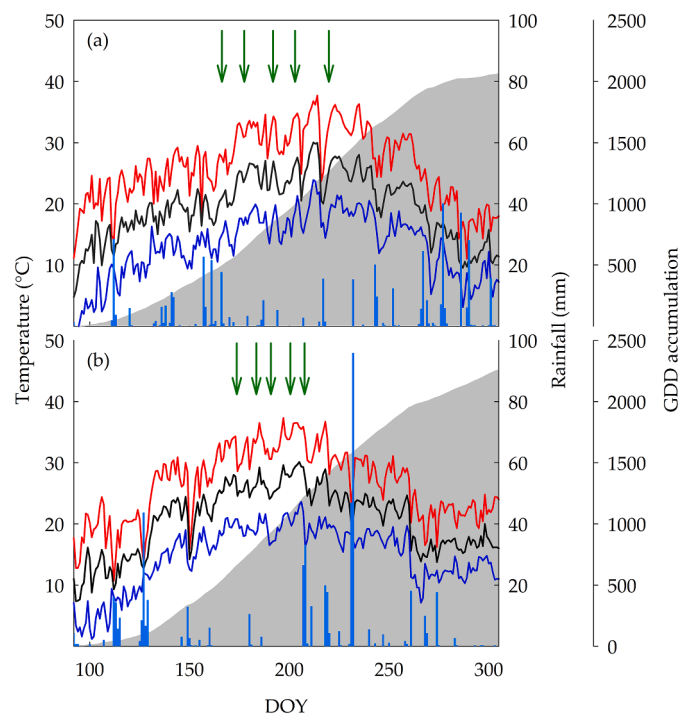


Fig. 1. Seasonal evolution of maximum (—), mean (—), and minimum temperature (—), rainfall (↓) and growing degree days (GDD, —) from 1 April to 31 October at the experimental location in 2020 (a) and 2022 (b). The arrows indicate foliar application of the specific yeast derivative LalVigne ProHydro™. DOY = Day of Year.

humidity, with the adjustment of airflow to 350 mL min^{-1} . The leaf cuvette chamber had a 6.25 cm^2 window. The intrinsic leaf water use efficiency (WUE) was calculated as the ratio between leaf assimilation and stomatal conductance (A/g_s). PSII efficiency was assessed on the same leaves with the HandyPea chlorophyll fluorescence system (Hansatech Instruments Ltd. Narborough Road, Pentney, King's Lynn, Norfolk, UK). The installation of the leaf clips for dark adaptation of the photosystems took place 1 h before the measurement. The PSII health status was expressed as the ratio between the variable (Fv) and maximum fluorescence (Fm) (Baker and Oxborough, 2004). Immediately after Fv/Fm analysis, ψ_{leaf} was measured on the same leaves with a Scholander pressure bomb (Soilmoisture Corp, Santa Barbara, CA, USA).

On DOY 202, 209, and 218 (20 July, 27 July, and 5 August) in 2020 and on DOY 195 and 217 (14 July and 5 August) in 2022, thermal images of one leaf (the same used for analysis of leaf gas exchange parameters) and one cluster per vine were taken using a FLIR i60 infra-red thermal imaging camera (FLIR Systems Inc., Wilsonville, OR, USA). The thermal images were elaborated with Flir Tools software (FLIR Systems Inc., Wilsonville, OR, USA) and the leaf and cluster maximum (Tmax), mean (Tmean), and minimum (Tmin) temperatures were then calculated.

At harvest, one shoot per each tagged vine was collected, and all the leaves were detached from the stem, counted, and then, making a distinction of main and lateral leaves, the area of each leaf was measured with a LI-3000A leaf area meter (LI-COR Biosciences, Lincoln, NE, USA). The average surface of one main leaf and that of one lateral shoot leaf was then obtained. After leaf fall, for each tagged vine, the total number of nodes on the cane and on the lateral shoots were counted for total leaf area estimation based on the number of nodes and average area of the leaf, after Gatti et al. (2018).

2.3. Leaf metabolites

On DOY 209, 229, and 239 (26 July, 16 August, and 26 August) of 2020 and on DOY 195 and 223 (14 July and 11 August) of 2022, one primary mature leaf per tagged vine was sampled, washed under deionized water and stored at -80°C . The frozen leaves were subjected to a 48 h lyophilization process and were then finely ground using a Tube Mill control (IKA®-Werke GmbH & CO. KG, Staufen, Germany) for a minute at 6000 rpm. The samples were utilized to determine leaf soluble sugars, starch concentration, chlorophylls, carotenoids, proline and hydrogen peroxide concentration.

For proline determination, the samples were prepared according to Hummel et al. (2010): 0.05 g of the finely milled leaf in screw cap tubes was mixed with 500 μL of ethanol and water (70:30 v/v) extraction solution and 1 ml of 1 % ninhydrin (w/v) in acetic acid 60 % (v/v), ethanol 20 % (v/v) reaction mix according to Carillo and Gibon (2011). The obtained sample was heated at 95°C for 20 min, centrifuged at 10000 rpm for a minute and the analysis of absorbance was performed at 520 nm on a JascoV-530 spectrophotometer (Jasco Analytical Instruments, Easton, MD, USA).

Leaf chlorophylls and carotenoids were analyzed according to Yuan and Qian (2016) with some adaptations. Samples were prepared weighing 0.1 g of lyophilized leaf and mixed with 100 mg/L β -apo-8'-carotenol, as internal standard. The extraction buffer consisted of 1 mL of ethyl acetate 0.1 % of butylated hydroxytoluene (BHT) and was added to the prepared sample and agitated for 30 min. The resulting sample was then centrifuged for 5 min at 1500 rpm, and the supernatant was collected, followed by a second extraction of the bottom residual adding 1 mL of ethyl acetate 0.1 % BHT. Afterwards, the two supernatants were mixed and dried out at 30°C under vacuum. The resultant residual was redissolved with 1 mL of acetone 0.1 % BHT and then centrifuged for 5 min at 11,000 rpm; the supernatant was collected and analyzed by HPLC. The samples were extracted in triplicates, and all the phases from extraction to final analysis were conducted under minimal light and cold conditions to reduce light-induced degradation of

carotenoids. Analysis of carotenoids was performed on an Agilent 1260 Infinity HPLC (Santa Clara, CA, USA) with a Luna 3 μm C8 (2) 100 A, $100 \times 4.6 \text{ mm}$ column (Phenomenex, Castel Maggiore, Italy) set at 60°C . Mobile phases consisted of 1 M ammonium acetate (70:30) (A) and 100 % methanol (B). The injection volume was 5 μL and the run flow rate 1 mL/min. The mobile phases were utilized under a double gradient from 0 min (95 % A and 5 % B) to 60 min (5 % A and 95 % B) and absorbance was detected at 450 nm for compound quantification. The assessment of β -Carotene was performed by the comparison of retention time and UV spectra of commercial β -carotene standard (95 % purity) (Sigma-Aldrich, St. Louis, MO, USA). For the identification of Violaxanthin (Vx), Antheraxanthin (Ax), Zeaxanthin (Zx) and Neoxanthin (Nx), retention time and UV-visible photodiode array spectra were compared with authentic standards. Vx, Ax, Zx and Nx standards were purchased from CaroteNature (Münsingen, Switzerland). The epoxidation state (EPS) was determined with the following calculation:

$$\text{EPS} = \text{Vx} + \text{Ax} + \text{Zx}$$

The determination of de-epoxidation state (DPS) was obtained with the following equation:

$$\text{DPS} = \frac{(\text{Zx} + 0.5 \text{Ax})}{(\text{Vx} + \text{Ax} + \text{Zx})}$$

Hydrogen peroxide (H_2O_2) was determined after a slight modification of the methods described by Palliotti et al. (2015) and Loreto and Velikova (2001). 0.05 g of dry leaf were mixed with 1 mL 1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 5 min at 4°C . Then, 0.75 mL of the supernatant were added to 0.75 mL of phosphate buffer (pH 7.0) and 1.5 mL of 1 M KI solution. The absorbance was read at 390 nm.

2.4. Vine yield, fruit composition and sunburn

Harvest was executed at the achievement of technological maturity, intended as total soluble solids (TSS) above 23° Brix and titratable acidity (TA) $\sim 8 \text{ g/L}$. At harvest, vine yield (kg/vine) and the number of clusters per vine was recorded. A sample of three representative clusters from each vine was collected and returned to the laboratory in a cooler for cluster morphology and fruit composition analysis. Cluster weight and rachis length were measured, and the resulting ratio expresses cluster compactness (g/cm). The number and weight of berries per cluster was determined, and berry mass was calculated. Sixty randomly selected berries per sample were frozen and stored for the determination of total anthocyanins and phenolics.

The remaining berries were crushed, and the resulting juice was analyzed using the method described by Iland (2004). The TSS concentration was determined with a digital refractometer SMART-1 (Atago, Bellevue, WA, USA), while pH analysis was performed with a pH-meter (pH 60 VioLab Giorgio Bormac, Carpi, MO, Italy). TA, expressed as g/L of tartaric acid equivalents, was determined by titration with 0.1 N NaOH to a pH 8.2 end point and expressed as g/L of tartaric acid equivalents, using an AT 1000 Series Potentiometric Titrator (Hach company, Loveland, CO, USA). Malic and tartaric acid concentrations were quantified via HPLC (Agilent Technologies, Santa Clara, CA, USA) into auto-sampler vials through a Synergy 4 u Hydro-RP80A column (Phenomenex Inc., Torrance, CA, USA), $250 \times 4.6 \text{ mm}$, after juice dilution and 0.22 μm polypropylene syringe filtration. The buffer solution utilized for separation was a 0.2 M KH_2PO_4 adjusted to 2.4 pH with ortho-phosphoric acid. The 15 μL sample ran through the column maintained at $30^\circ\text{C} \pm 0.1^\circ\text{C}$. The run was monitored at 200 nm–700 nm with diode array detector (DAD) at 210 nm UV. The calibration curves were built with authentic standards, and the concentration of organic acids was quantified determining areas of peaks corresponding to malic and tartaric acid.

The total anthocyanins and phenolics were determined after Iland

and Coombe (1988). Berries were homogenized at 24,000 rpm with an Ultra-Turrax T25 (Rose Scientific Ltd., Edmonton, Canada) homogenizer for 5 min; afterwards, 2 g of the homogenate were put into a centrifuge tube and 10 mL of aqueous ethanol extraction solution (50 %, pH 5) were added. It was kept for 1 h, mixing every 10 min. After the extraction period, the solution was centrifuged at 3,500 rpm, and after 5 min, 0.5 mL supernatant was added to 10 mL 1 M HCl. After three hours, the absorbance was read at 520 nm for total anthocyanins and 280 nm for total phenolics on a JascoV-530 spectrophotometer (Jasco Analytical Instruments, Easton, MD, USA). The total anthocyanins and phenolics concentration were expressed as mg per g of fresh weight.

Starting at veraison (BBCH 81), sunburn incidence and its severity on clusters was visually assessed on a weekly basis. Sunburn incidence was recorded for each experimental vine as the number of clusters showing symptoms, expressed as a percentage (%). Sunburn severity was instead recorded as the average percentage of berries affected by sunburn on symptomatic clusters. The McKinney index was then calculated after McKinney (1923):

$$\text{McKinney index} = \frac{(i \times s)}{(I \times S)} 10$$

Where “i” is the incidence, the percentage of clusters affected with sunburn symptoms, “s” is the severity, the percentage of the cluster area with sunburn symptoms, “I” is the maximum incidence (100 %), and “S” is the maximum severity (100 %).

2.5. Data elaboration and statistical analysis

The data were processed using Microsoft Office Excel (Microsoft Corporation, Redmond, WA, USA). The results were plotted with Sigma Plot 12 (Systat Software Inc., San Jose, CA, USA). Yield components and

composition means were subjected to a two-way ANOVA (Treatment, Year) using the IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp, Armonk, NY, USA), assuming the treatment (T) as a fixed factor and the year as a random factor. When the Treatment x Year (TxY) interaction resulted significant, means were separated by Student's *t*-test ($p < 0.05$). Plotted data means were separated using the student's *t*-test ($p < 0.05$), using the same software. Correlation analysis was performed with Sigma Plot 12 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Weather conditions

In 2020, 2062 GDD were calculated from 1 April to 31 October. The warmest period of the year was recorded from DOY 210 (28 July) to 216 (03 August) and on DOY 214 (01 August), air temperatures peaked to 37.7 °C. Afterwards, on DOY 216, the temperature decreased to 25 °C due to a rainfall event (18 mm) and then rose again above 30 °C after DOY 219. The total rainfall from 01 April to 31 October was 437 mm, mainly occurring in spring and fall. In 2022, 2255 GDD were recorded in the same period, and the total rainfall was 520 mm, with 188 mm occurring between DOYs 218 and 232 (8 August–20 August). The warmest period occurred between DOYs 192 and 217 (11 July – 5 August), peaking at 37.3 °C on DOY 197 (16 July, Fig. 1).

3.2. Leaf water status, gas exchange parameters and temperature

In 2020, C vines ψ_{leaf} progressively decreased during the season, passing from -0.87 MPa on DOY 191 to -1.56 MPa shown on DOY 209 (Fig. 2). On DOY 218, after a rainfall of 18 mm, C vines ψ_{leaf} rose to -1.43 MPa. LPH vines had a higher ψ_{leaf} than C on DOY 202 ($+0.13$

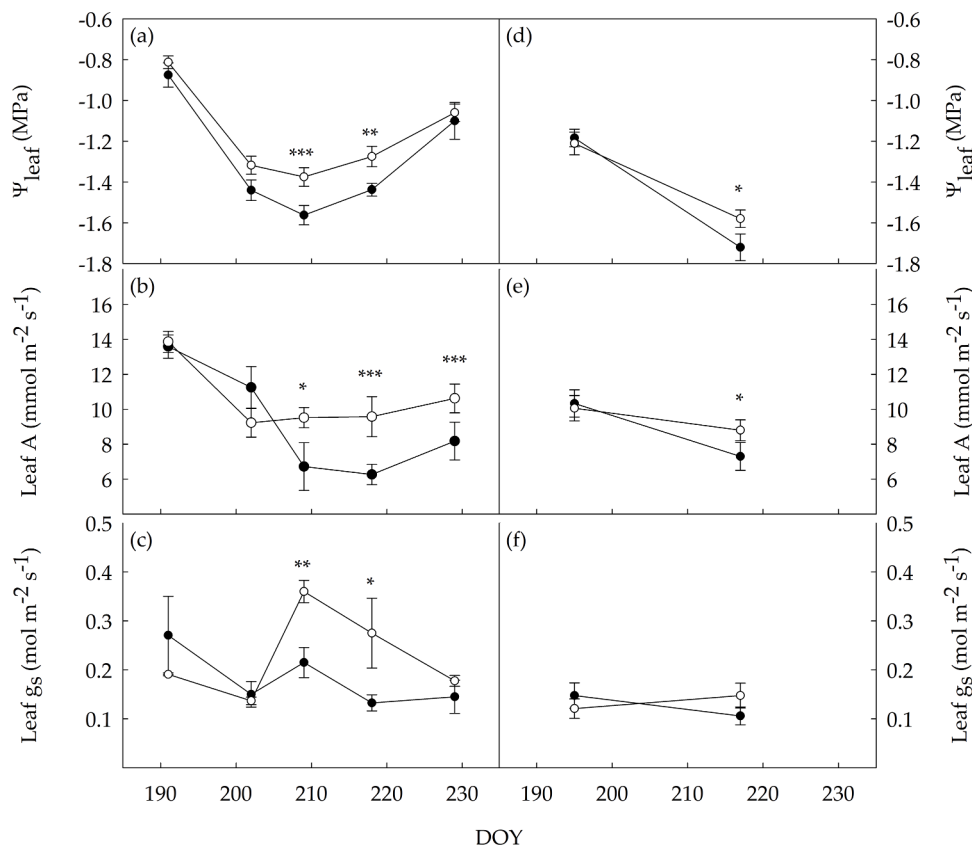


Fig. 2. Leaf water potential (ψ_{leaf}) (a, d), assimilation (A) (b, e) and stomatal conductance (g_s) (c, f), of grapevines cv. Barbera subjected to multiple application of the SYD LalVigne ProHydro™ (LPH, ○) and of untreated vines (C, ●) in 2020 (a, b, c) and 2022 (d, e, f). *, ** and *** indicate significant mean differences at <0.05 , <0.01 and <0.001 , respectively ($n = 12$). DOY = Day Of Year.

MPa), 209 (+0.19 MPa), and 218 (+0.16 MPa). On DOY 229, the air temperature decreased, and the water potential of both treatments resumed higher values (-1.06 to -1.10 MPa). In 2022, no difference between the treatments was found on DOY 195, whereas on DOY 217, LPH vines showed again a higher ψ_{leaf} (+0.13 MPa).

Leaf A and g_s were similar between treatments from DOYs 191 to 209. On DOY 218 and 229, LPH vines showed higher leaf A than C vines ($+3.31 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $+2.44 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively) (Fig. 2). C vines also showed a significantly lower g_s ($-0.14 \text{ mol m}^{-2} \text{s}^{-1}$) on DOY 209. As for intrinsic WUE (A/g_s), no significant differences were observed among treatments in both years of field investigations (Fig. 3). In 2022, while no differences were observed in leaf g_s or WUE, LPH vines exhibited higher leaf A than C vines on DOY 217 ($+2.32 \mu\text{mol m}^{-2} \text{s}^{-1}$). There were no differences between treatments in leaf Fv/Fm until DOY 229, when LPH vines exhibited a higher Fv/Fm value (0.74 vs 0.67) than C (Fig. 3). Similarly, in 2022, no differences were observed on DOY 195, while on DOY 217, LPH vines had a higher Fv/Fm than C (+0.1).

In 2020, on DOY 218, the LPH vines showed a lower leaf Tmax (-1.9°C) as compared to C vines (Table 1). Conversely, on DOYs 202 and 209, no significant differences were found between treatments. As for Tmean and Tmin, no differences were observed in any of the dates evaluated. Regarding 2022, on DOY 195, both Tmax and Tmean were significantly lower in LPH vines (-2.93°C and -1.8°C , respectively), and no differences were observed in leaf Tmin. On DOY 217, no differences were found between treatments for any of the parameters evaluated (Tmax, Tmean, and Tmin).

3.3. Leaf proline, H_2O_2 , chlorophylls and carotenoids concentrations

Foliar LPH application dramatically increased leaf proline concentration (Table 2). On DOY 209, the LPH vines had a leaf proline concentration of $108.96 \mu\text{mol/g}$ dry weight (DW), vs $33.15 \mu\text{mol/g}$ DW exhibited by C vines. The difference between treatments peaked on DOY 229, when the leaf proline concentration of the LPH vines was +600 %

than that of the C. Afterwards, on DOY 239, the LPH vines leaf proline concentration decreased to $59.98 \mu\text{mol/g}$ DW yet remaining significantly higher than that of C (+119 %). Similarly, in 2022, the leaf proline concentration was higher in LPH vines and on both DOY 195 (+188 %) and 223 (+22 %).

In 2020, the LPH leaf H_2O_2 concentration was lower on DOY 229 (-10%) and 239 (-26%), while no differences were observed on DOY 209. In 2022, there were no differences between treatments on DOY 195, whereas on DOY 223, the LPH H_2O_2 concentration was significantly lower (-21%). The LPH leaf β -Carotene and chlorophyll a and b concentrations were significantly higher than that of C on DOY 229 ($+23\%$, $+39\%$, and $+35\%$, respectively) and 239 ($+16\%$, $+21\%$, and $+21\%$, respectively) of 2020 and DOY 223 ($+17\%$, $+14\%$, and $+18\%$) of 2022.

On DOY 229 of 2020, the LPH vines had a significantly higher leaf concentration of Nx ($+34\%$), Vx ($+27\%$), Zx ($+38\%$), and Lutein ($+36\%$), as compared to that of the C vines (Table 3). On DOY 239, the LPH vines had higher amounts of Vx ($+48\%$), Ax ($+12\%$), Zx ($+42\%$), and Lutein ($+29\%$) than did the C vines. On the same dates, the LPH vines had a higher EPS ($+26\%$ and $+32\%$, respectively) than C, while no differences were observed in DPS. In 2022, on DOY 195, there were no significant differences between LPH and C vines in terms of leaf carotenoids concentration. On DOY 223, a higher amount of Lutein ($+20\%$) was observed on LPH vines.

3.4. Cluster temperature and sunburn

In 2020, the cluster Tmax was lower in the LPH vines on DOY 209 (-2°C) (Table 1). No significant differences were observed on the other dates in both years. In 2020, the LPH treated clusters had a lower sunburn incidence than the C on DOY 218 (16% vs 32 %), 229 (42% vs 64 %), and 239 (85% vs 61 %) (Fig. 4). The McKinney index of the LPH clusters was significantly lower than that exhibited by the C clusters on DOY 229 (11 vs 21) and 239 (20 vs 35). In terms of sunburn severity, no

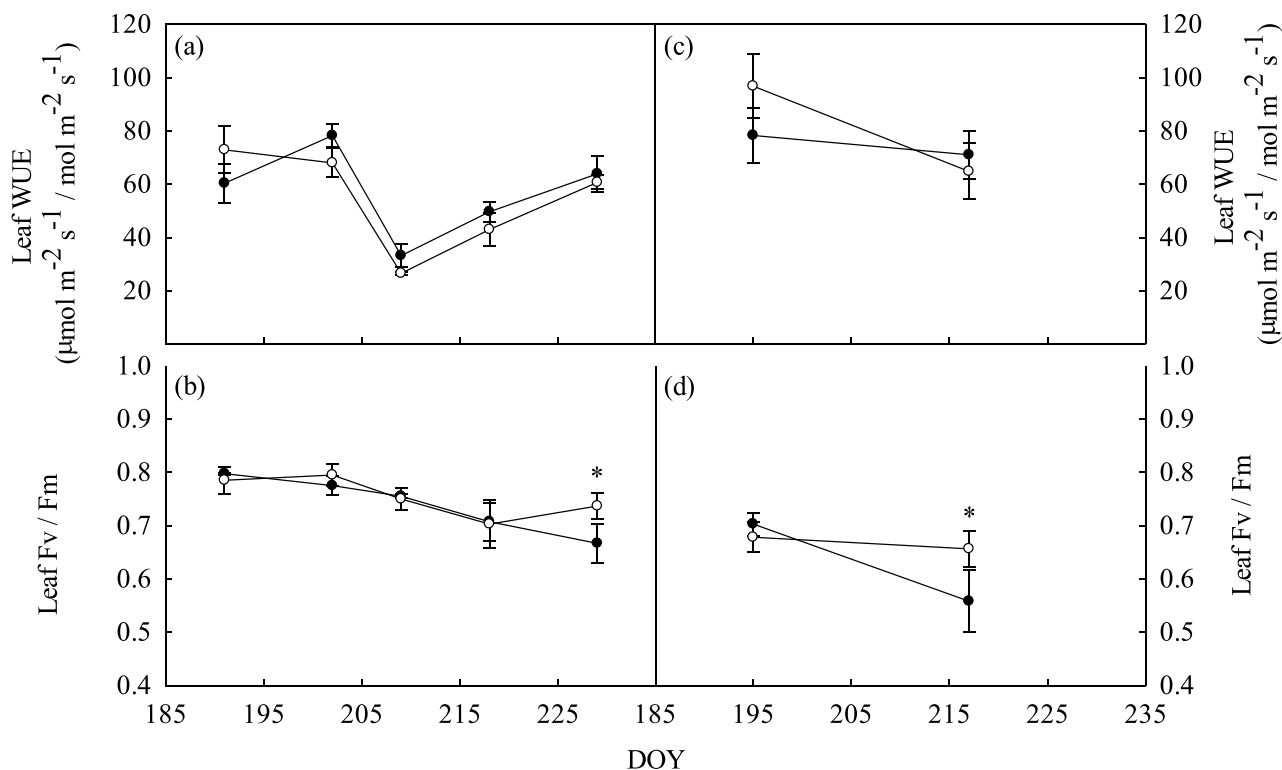


Fig. 3. Leaf WUE (a, c) and Fv/Fm (b, d) of field grown cv. Barbera grapevine subjected to multiple application of the SYD LalVigne ProHydro™ (LPH, ○) and untreated vines (C, ●) in 2020 (a, b) and 2022 (c, d). *, ** and *** indicate significant mean differences at <0.05 , <0.01 and <0.001 , respectively ($n = 12$). DOY = Day Of Year.

Table 1

Maximum (Tmax), mean (Tmean) and minimum (Tmin) leaf and cluster temperature in grapevines cv. Barbera subjected to multiple applications of the SYD LalVigne ProHydro™ (LPH) and untreated vines (C) in 2020 and 2022. *, ** and *** indicate significant mean differences at <0.05, <0.01 and <0.001, respectively (n = 12). ns = no difference.

Year (Y)	DOY	Treatment (T)	Leaf Tmax (°C)	Leaf Tmean (°C)	Leaf Tmin (°C)	Cluster Tmax (°C)	Cluster Tmean (°C)	Cluster Tmin (°C)
2020	202	C	39.9	36.7	32.5	42.1	38.6	34.9
		LPH	38.4	35.2	32.2	40.7	37.8	33.9
		Sig.	ns	ns	ns	ns	ns	ns
	209	C	40.9	37.6	34.6	44.6	39.3	35.9
		LPH	40.1	36.3	33.9	42.6	38.9	35.1
		Sig.	ns	ns	ns	*	ns	ns
	218	C	38.3	33.9	28.4	42.4	36.9	32.9
		LPH	36.3	32.9	28.7	45.7	40.3	32.8
		Sig.	*	ns	ns	ns	ns	ns
2022	195	C	47.7	41.9	35.8	47.2	38.8	33.8
		LPH	44.8	40.1	34.5	43.7	39.0	34.3
		Sig.	*	*	ns	ns	ns	ns
	217	C	45.5	40.2	34.3	41.2	38.5	33.4
		LPH	43.6	39.0	34.2	44.0	39.8	34.0
		Sig.	ns	ns	ns	ns	ns	ns

Table 2

Leaf proline, H₂O₂, β-Carotene, Chlorophyll A and Chlorophyll B concentration in grapevines cv. Barbera subjected to multiple application of the SYD LalVigne ProHydro™ (LPH) and in untreated vines (C) in 2020 and 2022. *, ** and *** indicate significant mean differences at <0.05, <0.01 and <0.001, respectively (n = 12). ns = no difference.

Year (Y)	DOY	Treatment (T)	Proline (μmol/g DW)	H ₂ O ₂ (μmol/g)	β-Carotene (μg/g)	Chlorophyll A (μg/g)	Chlorophyll B (μg/g)
2020	208	C	33.15	3.59	289.65	1441.23	3064.80
		LPH	108.96	3.25	271.00	1359.65	2785.67
		Sig.	***		ns	ns	ns
	229	C	24.37	3.99	252.29	1148.51	2423.94
		LPH	170.58	3.17	312.55	1605.86	3275.74
		Sig.	***	*	*	***	**
	239	C	27.35	4.00	243.29	1177.30	2516.98
		LPH	59.98	3.53	284.51	1430.52	3068.81
		Sig.	*	**	*	**	*
2022	195	C	21.44	2.35	280.58	1121.93	1084.47
		LPH	61.76	2.32	296.36	1143.71	1095.30
		Sig.	*	ns	ns	ns	ns
	223	C	25.79	2.76	237.17	1182.82	1177.71
		LPH	39.42	2.28	278.44	1351.71	1390.86
		Sig.	*	*	**	**	*

Table 3

Leaf xanthophylls concentration, epoxidation (EPS) and de-epoxidation state (DPS) in grapevines cv. Barbera subjected to multiple application of the SYD LalVigne ProHydro™ (LPH) and in untreated vines (C) in 2020 and 2022. *, ** and *** indicate significant mean differences at <0.05, <0.01 and <0.001, respectively (n = 12). ns = no difference.

Year (Y)	DOY	Treatment (T)	Neoxanthin (μg/g)	Violaxanthin (μg/g)	Antheraxanthin (μg/g)	Zeaxanthin (μg/g)	EPS (μg/g)	DPS (μg/g)	Lutein (μg/g)
2020	208	C	234.76	88.45	26.62	51.12	166.19	0.39	629.99
		LPH	206.48	76.04	23.97	44.34	144.35	0.39	596.54
		Sig.	ns	ns	ns	ns	ns	ns	ns
	229	C	186.47	83.75	20.57	32.36	136.68	0.31	492.48
		LPH	249.96	106.31	21.79	44.63	172.73	0.32	674.81
		Sig.	*	*	ns	*	**	ns	**
	239	C	190.2	77.92	33.32	28.18	139.42	0.32	491.13
		LPH	219.82	114.59	29.55	40.15	184.29	0.29	638.10
		Sig.	ns	*	*	**	*	ns	*
2022	195	C	138.11	30.85	9.26	35.39	75.50	0.53	563.88
		LPH	140.11	29.4	9.61	35.75	74.76	0.54	585.42
		Sig.	ns	ns	ns	ns	ns	ns	ns
	223	C	137.31	22.86	10.43	35.28	68.57	0.59	523.90
		LPH	157.32	21.83	11.19	40.15	73.17	0.63	630.33
		Sig.	ns	ns	ns	ns	ns	ns	**

difference was observed. In 2022, the LPH and C vines had a similar pattern of sunburn incidence and severity, whereas the LPH vines had a lower McKinney index than the C vines on DOY 236 (−50 %).

3.5. Vine yield and fruit composition

The harvest date was 02 September in 2020 and 15 September in 2022. The 2022 season resulted in a higher yield, number of clusters per vine, and cluster and berry weight, compared to 2020 (Table 4). Pooling the two years, the C vines had a significantly lower yield (−1.27 kg)

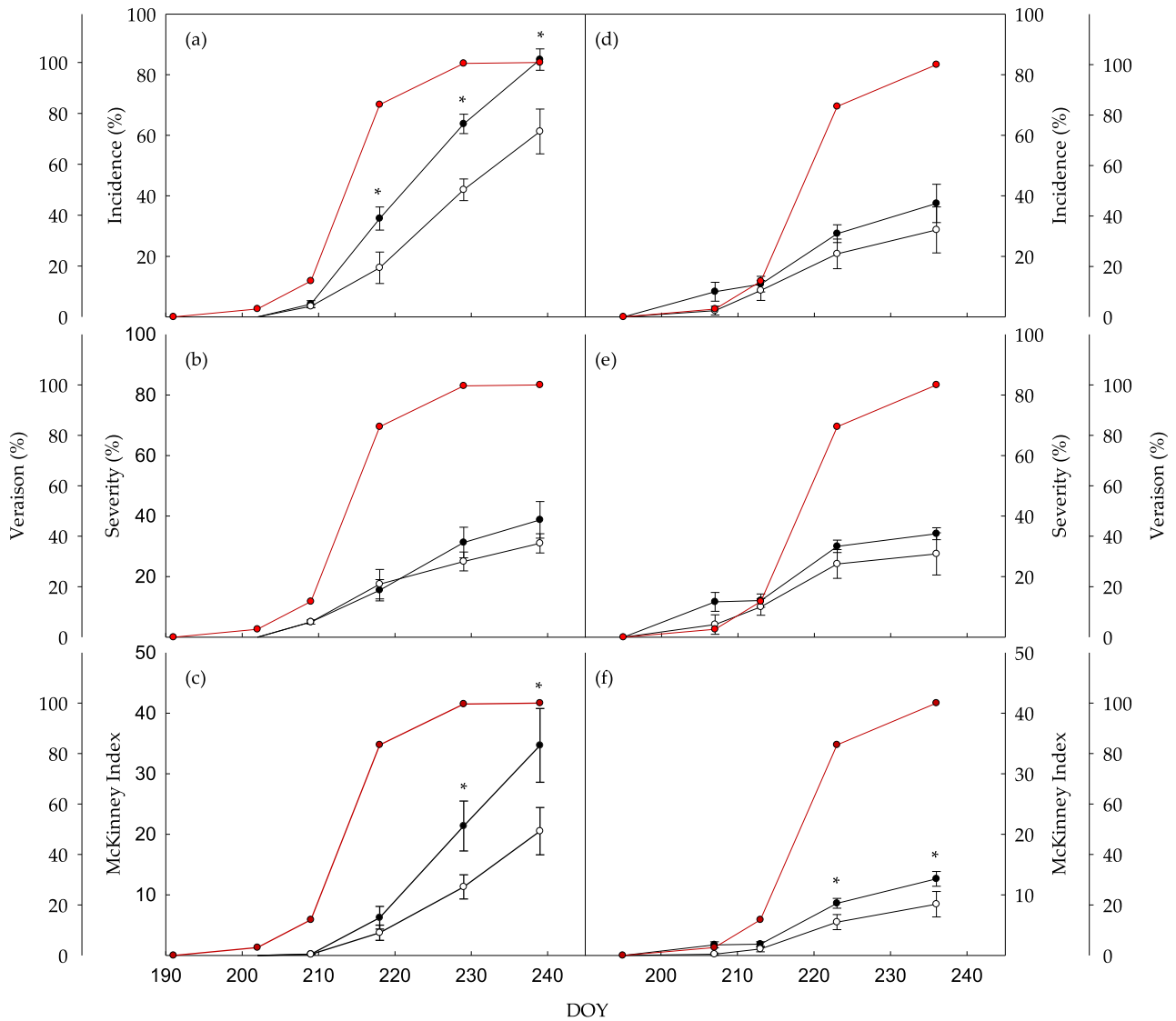


Fig. 4. Seasonal dynamics of grape sunburn incidence (a, d), severity (b, e) and McKinney index (c, f) in vines cv. Barbera subjected to multiple application of the SYD LalVigne ProHydro™ (LPH, ○) and in untreated vines (C, ●), in 2020 (a, b, c) and 2022 (d, e, f). *, ** and *** indicate significant mean differences at <0.05, <0.01 and <0.001, respectively (n = 24). Red dots and lines indicate the seasonal progression of veraison. DOY = Day Of Year.

Table 4

Yield components, cluster anatomy, vegetative growth and vine balance of grapevines cv. Barbera subjected to multiple application of the SYD LalVigne ProHydro™ (LPH) and in untreated vines (C), in 2020 and 2022. *, ** and *** indicate significant mean differences at <0.05, <0.01 and <0.001, respectively (n = 24). ns= no difference.

Treatment (T)	Yield (kg/vine)	Shoot fruitfulness (n)	Clusters/vine (n.)	Cluster weight (g)	Cluster compactness (g/cm)	Berry weight (g)	Skin weight (g)	Skin/pulp ratio	LA (m ²)	LA/Y (m ² /kg)
C	4.25	1.56	25	171	13.40	1.77	0.24	0.157	2.81	0.66
LPH	5.52	1.68	27	194	16.11	2.27	0.21	0.102	2.99	0.54
Sig.	*	ns	ns	**	*	***	ns	***	ns	*
Year (Y)										
2020	4.04	1.25	20	208	13.33	2.00	0.23	0.130	3.23	0.80
2022	6.43	1.82	32	197	16.18	2.13	0.25	0.133	2.57	0.42
Sig.	***	***	***	ns	**	*	ns	ns	*	***
TxY	ns	ns	ns	ns	ns	**	ns	ns	ns	ns

compared to LPH treatment. Indeed, in C vines, the cluster weight was lower by about 23 g and berry weight by 0.5 g, compared to the LPH vines. Being berry skin weight similar between treatments, the skin/pulp ratio was higher in LPH berries. No differences were observed in LA,

with LPH vines resulting in a higher LA/Y ratio (+0.12 m²/kg). The TSS concentration was significantly higher in C grapes (+2.09 °Brix), while pH and TA were unaffected by the treatment (Table 5). Consequently, TSS/TA (3.15) was also higher with respect to LPH (2.87). The LPH vines

Table 5

Grape composition in grapevines cv. Barbera subjected to multiple application of the SYD LalVigne ProHydro™ (LPH) and in untreated vines (C) in 2020 and 2022. *, ** and *** indicate significant mean differences at <0.05, <0.01 and <0.001, respectively (n = 24). ns = no difference.

Treatment (T)	TSS	pH	TA	TSS/TA	Anthocyanins (mg/g)	Anthocyanins (mg/berry)	Phenolics (mg/g)	Phenolics (mg/berry)	Tartaric acid (g/L)	Malic acid (g/L)
C	25.40	3.33	8.29	3.15	1.43	2.77	3.24	5.79	8.97	3.34
LPH	23.31	3.31	7.92	2.87	1.28	3.03	2.99	6.95	8.60	3.45
Sig.	**	ns	ns	*	ns	**	ns	**	ns	ns
Year (Y)										
2020	25.24	3.26	7.76	3.25	1.65	3.57	3.33	6.70	8.14	3.41
2022	23.47	3.38	8.45	2.77	1.05	2.23	2.90	6.04	9.42	3.38
Sig.	*	**	ns	**	***	***	ns	ns	*	ns
TxY	ns	ns	ns	ns	ns	ns	*	ns	ns	ns

had a higher concentration of grapes anthocyanins (+0.26 mg/berry) and phenolics (+1.16 mg/berry) on a single berry basis, whilst no differences were found when the content was expressed as mg per g of berry. Pooling years and treatments, the correlation between the percentage of berries affected by sunburn symptoms and the average berry mass (Fig. 5a) fitted to an inverse linear equation ($y = -0.015x + 2.401$, $R^2 = 0.641$, $p < 0.05$), while a logarithmic model described grapes TSS

at varying rates of sunburn affected berries (Fig. 5b, $y = 1.658\ln(x) + 19.477$, $R^2 = 0.65$, $p < 0.05$).

4. Discussion

In our conditions, LPH foliar applications had a significant effect on vine water status and on leaf physiological performances in both

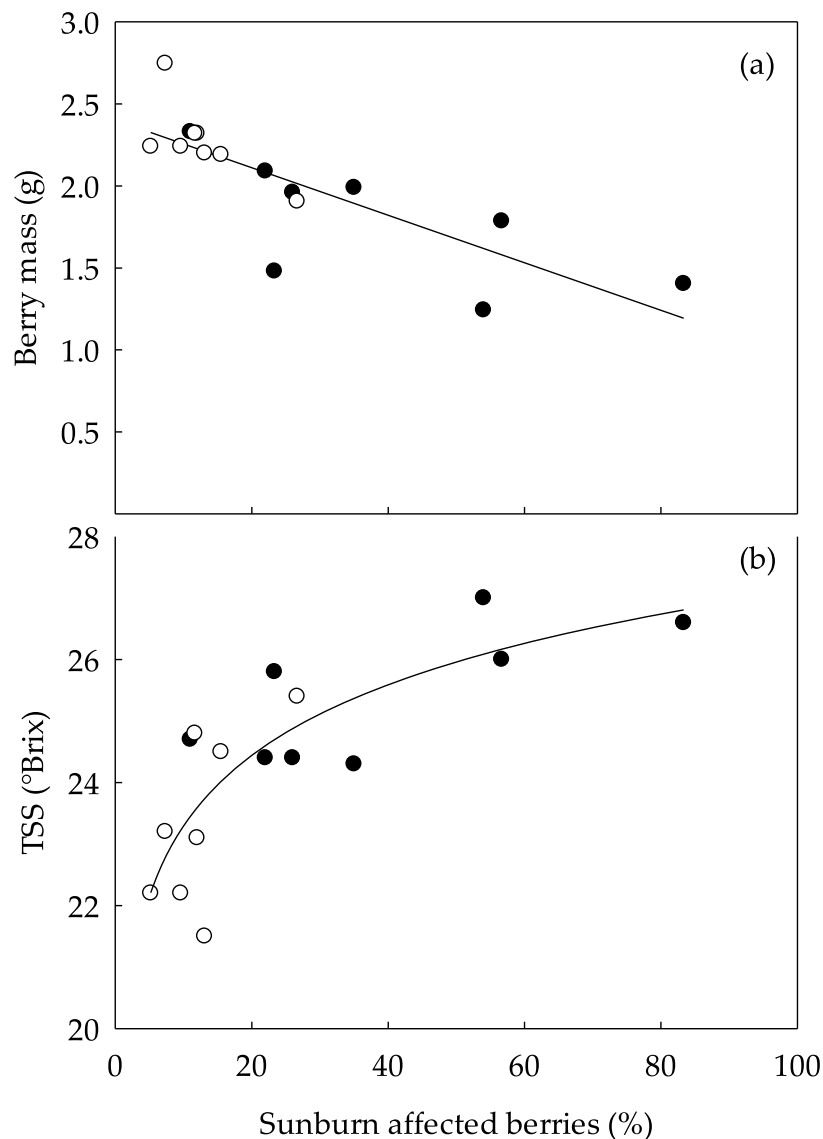


Fig. 5. Correlation fitted between berries affected by sunburn (% of the total on a cluster basis) and berry mass ($y = -0.015x + 2.401$; $R^2 = 0.64$; panel a), or grape Total Soluble Solids (TSS) ($y = 1.658\ln(x) + 19.477$; $R^2 = 0.65$; panel b) in grapevines cv. Barbera subjected to multiple application of the SYD LalVigne ProHydro™ (LPH, ○) and in untreated vines (C, ●), in 2020 and 2022.

seasons. In detail, the LPH vines were able to preserve higher ψ_{leaf} , while C vines exhibited a ψ_{leaf} lower than -1.4 MPa (Fig. 2a, d), indicating severe stress conditions (Van Leeuwen et al., 2009). In 2020, this occurred either when ψ_{leaf} was progressively decreasing between DOYs 190 and 209 or later when it was resuming higher values between DOYs 209 and 218. In grapevines, ψ_{leaf} is mainly affected by soil water availability and VPD (Suter et al., 2019). Hence, while ψ_{leaf} can be partially controlled in presence of irrigation, piloting plant water status remains a quite difficult task when water is not available. In this framework, the higher ψ_{leaf} exhibited by the LPH vines at increasing limiting condition is of special interest. Such effect was consistent even in the warmer 2022 and was associated in both years with a higher leaf A and g_s (Fig. 2) and better thermoregulation in terms of leaf Tmax (Table 1). As expected, no differences were observed in intrinsic leaf WUE (Fig. 3a). Additionally, in 2020, the higher leaf assimilation rates in the treated vines on DOY 229 suggest that LPH can be particularly efficient in promoting a rapid recovery when non-limiting conditions are restored. This was likely linked to the preservation of leaf chlorophylls concentration and PSII efficiency. While in the C vines, the leaf Fv/Fm decreased to 0.68 in 2020 and to 0.55 in 2022, in LPH vines it was significantly higher at the end of each season. The data demonstrate that LPH partially prevented the onset of leaf non-reversible photoinhibitions and yellowings, especially in the warmer 2022. While the available literature about the foliar application of SYD in grapevines explores only productive traits, fruit composition or pathogens control, in other crops, several studies reported positive effects on plant physiology and abiotic stress tolerance. On garlic, wheat, and tomato, it was demonstrated that SYD application could preserve leaf functioning and plant water status under drought conditions (Abdelaal et al., 2021; Campobenedetto et al., 2021; Hammad and Ali, 2014). The effects were mainly attributed to the action of flavonoids, amino acids, and mannoproteins triggering abiotic stress tolerance related pathways. However, unlike the mentioned paper, in our work, the SYD tested contained a fraction of microbially derived proline. Nowadays, the scientific community is debating the role of proline in plants under limiting conditions. The main hypothesis about an active role of proline on abiotic stress tolerance is related to its eventual contribution to osmotic adjustments and/or to a direct involvement in leaf redox balance through the production of reducing equivalents during its day/night turnover, as well as by acting as a singlet oxygen quencher (Dar et al., 2016; Matysik et al., 2002; Signorelli et al., 2014; Smirnov and Cumbes, 1989; Szabados and Savouré, 2010; Verbruggen and Hermans, 2008). In our work, after foliar treatments, the LPH vines exhibited a humongous increase in leaf proline concentration, compared to the C vines (Table 2), especially in 2020. The difference between treatments falls in the standard range of proline concentration changes after the imposition of an abiotic stress. Many works report that grapevine leaf proline concentration could change up to +600 % after an abiotic stress (Bertamini et al., 2006; Gohari et al., 2021; Ozden et al., 2009). In our experiment, being the leaves washed after samplings, the detected proline corresponds to within-leaf proline. The pending question is whether the leaf proline measured represents exogenous proline migrating into leaf tissues or endogenous proline synthesized in response to the treatment. Exogenous proline can be rapidly absorbed by leaves (Trotel-Aziz et al., 2008). However, the amount of proline found in one g of LPH leaves is not compatible with the exogenous proline applied (less than 9 $\mu\text{mol/g}$ leaf DW as a gross average). At the same time, exogenous proline applied at concentrations < 10 mM can promote endogenous cytosolic proline biosynthesis, fostering the production of the above mentioned NADP⁺ reducing equivalents and actively contributing to excess energy dissipation (Hayat et al., 2012). However, a dose-dependent toxic effect of exogenous proline applied at >10 mM was also observed. In fact, at high concentrations, exogenous proline has the opposite outcome, inhibiting $\Delta 1$ -pyrroline-5-carboxylate synthetase (P5CS), a key enzyme for endogenous proline biosynthesis, breaking the day/night proline turnover and the natural cytosolic production of reducing NADP⁺ (Hayat

et al., 2012; Trovato et al., 2008). Even if we cannot discern exogenous and endogenous leaf proline, our data suggest that LPH foliar application had a priming effect, boosting endogenous leaf proline biosynthesis and accumulation, with no detrimental effects on its day/night turnover. The higher ψ_{leaf} observed in the LPH vines is in agreement with the higher leaf water potential observed on other crops after exogenous proline application or after the induction of endogenous proline increase (Hayat et al., 2012). Leaf gas exchange parameters and thermoregulation seem to follow the better LPH vines water status, while the reduced leaf H₂O₂ and increased chlorophylls, carotenoids, and EPS at late sampling stages demonstrate a better dissipation of energetic excess in the LPH vines. Further studies under controlled conditions are needed to univocally clarify proline-rich SYD mechanisms of action, but our data hint that: (i). osmotic adjustments could be the basis of the improved plant water status and leaf functioning at decreasing water availability; (ii). better dissipation of energy excess could be the reason for the higher photosynthetic rates found under severe summer stress and later when non-limiting conditions were restored, thanks to the preservation of PSII and canopy functionality. To the best of our knowledge, this is the first time that the priming effect of a proline-rich SYD was observed, and it demonstrates the potentialities of such formulates on vineyards subjected to summer stresses.

Although cluster temperature was substantially unaffected by treatments (Table 1), the LPH vines exhibited a significantly lower susceptibility to berry sunburn than C in both years (Fig. 4). Interestingly, sunburn incidence and severity were higher in the cooler 2020 than in the warmer 2022. This was likely due to the advance of veraison and ripening observed in 2020 as a consequence of the lower yield and the higher LA/Y ratio (Table 4). Sunburn mainly affects grapes from berry softening onwards, when berry water influx increases and berry skin and cuticle approach final size and relative weight (Bondada and Keller, 2012). At a given phenological stage, sunburn spread is mainly due to the balance between berry cuticular transpiration and pedicel water influx. Cuticular transpiration is determined by air-to-berry VPD and skin thickness. Some papers highlight that SYD could affect berry skin thickness and change its mechanical properties. Giacosa et al. (2019) (Giacosa et al., 2019) found that a SYD was consistently increasing skin thickness and its resistance to physical damage in three different grapevine varieties. Similarly, Villangò et al. (2015) (Villangò et al., 2015) found that SYD application increased skin thickness, improving skin break force and skin break energy. In our work, skin weight was unaffected, but skin/pulp ratio was lower in the LPH vines, a consequence of the large fraction of berries affected by dehydration phenomena in the C vines (Table 4). Therefore, spread of sunburn in both treatments disguised the eventual effects of LPH on skin parameters, but an augmented resistance to cuticular transpiration is the most probable cause of the reduction of berry dehydration phenomena and of the preservation of berry size in LPH grapes. Indeed, the inverse linear correlation in Fig. 5a depicts the relationship occurring between sunburn affected berries and average berry size. Other yield components followed accordingly: the reduced berry size in C vines turned into a lower cluster weight and a lower yield.

Also, fruit composition was significantly affected by treatments. The different spread of sunburn and the berry dehydration process drove the TSS increase according to a logarithmic model towards an asymptotic threshold of about 27 °Brix (Fig. 5b). As a result, in the C grapes, TSS rose to excessive values even for a premium red wine (i.e., >25 °Brix), and TSS/TA ratio increased accordingly (Table 5). Grape's anthocyanins and phenolics were similar between treatments when expressed as mg/g. However, when total anthocyanins and phenolics were expressed on a berry basis, the LPH grapes exhibited significantly higher contents. Such outputs hint that anthocyanins were boosted by LPH treatments, but the subsequent berry dehydration that occurred in C vines caused a concentration effect, resulting in no difference between treatments. Proline-rich SYD effects on grapes were never tested before. Several studies investigated the use of other SYD formulates on grapevines, and authors

observed a consistent increase of grapes anthocyanins, stilbene, and phenolics in general in treated grapes (Giacosa et al., 2019; Gutiérrez-Gamboa et al., 2019; Kogkou et al., 2017; Pastore et al., 2020; Portu et al., 2016; Villangó et al., 2015). Pastore et al. (2020) demonstrated that SYD directly trigger many genes involved in the flavonoid pathway. In Sauvignon Blanc, the application of a different SYD favored the accumulation of grapes aromatic compounds and precursors, as well as their stability in final wines (Suklje et al. 2016). SYD are a large family of different formulates, and the literature available suggests that the origin and composition of the applied SYD cause significantly different effects. In our work, the main effect of LPH on grapes composition was linked to the reduction of berry dehydration phenomena that allowed the maintenance of a desirable TSS concentration and TSS/TA ratio when C vines showed a dramatically unbalanced biochemical profile. However, we cannot rule out the possibility that the higher photosynthetic rates of the LPH vines could have promoted an eventual increase in grapes TSS and anthocyanins concentration, disguised by the different degree of berry dehydration between treatments. The higher anthocyanins berry content seems to support this hypothesis.

5. Conclusion

Our two-year experiment demonstrates that proline-rich SYD positively affect physiological and productive performances of field-grown grapevines subjected to summer stresses. Our work suggests that the microbial proline included in the formulate exert a priming effect. Boosting leaf endogenous proline accumulation, LPH improved vine water status and physiological performances.

Furthermore, grapes subjected to multiple LPH treatments showed a reduced spread of sunburn and berry dehydration. This, in turn, allowed to preserve berry size, cluster size, yield, as well as to obtain an optimal sugar concentration at harvest, compared to untreated grapes.

Although the mechanisms of action need to be clearly elucidated, the present work suggests that proline-rich SYD deserve consideration when implementing strategies to protect the vineyard from multiple summer stresses. Further studies should dissect the role of exogenous and endogenous proline on leaf functioning, verify changes on berry skin texture, analyze effects on fruit composition in absence of the interference of severe sunburn symptoms, and evaluate the economic convenience of SYD applications.

CRedit authorship contribution statement

Filippo Del Zozzo: Writing – original draft, Investigation, Data curation, Conceptualization. **Despoina Maria Barmpa:** Methodology, Investigation, Formal analysis, Data curation. **Ginevra Canavera:** Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Lucia Giordano:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Alberto Palliotti:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Fabrizio Battista:** Writing – review & editing, Software, Resources, Methodology. **Stefano Poni:** Writing – review & editing, Visualization, Funding acquisition, Conceptualization. **Tommaso Frioni:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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