Scientia Horticulturae 353 (2025) 114465

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Research Paper

Application of priming agents in red raspberries prior to transplantation and at pre-flowering stages results in improved yield efficiency and enhanced secondary metabolism

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ARTICLE INFO

Keywords:
Rubus idaeus
Primocane
Production models
Sustainability
Primary metabolism
Secondary metabolism

ABSTRACT

Red raspberry (*Rubus idaeus* L) fruit has high nutritional value and there is an increasing demand in its global cultivation, highlighting the need for sustainable practices to improve both fruit and plant productivity. Chemical priming has recently gained attention as a sustainable horticultural crop management approach to enhance plant performance. In the current study, the effects of multiple chemical priming agents were investigated on their potential to improve yield efficiency, enhance antioxidant potential and fruit quality attributes, with special reference to aroma of 'Vica Abril' raspberry plants. Treatments included: (1) NOSH-aspirin (NOSH-A, 100 μM), (2) melatonin (Mel, 100 μM), (3) sodium alginate (NaA, 0.5 % w/v), (4) sodium alginate-melatonin conjugate (NaA/Melatonin, 100 μM/0.5 % w/v), and (5) glycine-betaine (GB, 10 mM). Additionally, control treatments included application of water (hydro-primed) and DMSO (0.1 % v/v) (solvent control for NOSH-A). Treatment application was initially performed pre-planting at the root zone and subsequently at 27, 46 and 74 days after planting (DAP). Melatonin treatment significantly enhanced fruit yield, particularly during the early harvests, while NOSH-A enhanced sucrose and ascorbic acid content and all priming agents increased total flavonoid content. Treatments with NaA alone or in conjugated form with Mel led to a considerable increment of kaempferol, several anthocyanins and ellagic acid derivatives, among the 13 polyphenolic compounds identified. The analysis of volatile organic compounds (VOCs) in raspberry fruits identified a total of 98 distinct compounds. Besides d-limonene content, no striking differences in aroma composition was monitored among treatments tested. The application of priming agents, most promptly melatonin, is a promising technological approach that needs to be further exploited towards increased crop productivity and/or enhanced raspberry fruit quality.

1. Introduction

High nutritional properties of red raspberry (*Rubus idaeus* L.) are widely recognised in the horticultural sector that has led to an exponential growth of its demand on global market. However, the cultivation of raspberry is capital- and knowledge-intensive, requiring optimum cultivation protocols (Manganaris et al., 2024). Advanced breeding programs have led to the development of heat- and low-chilling tolerant cultivars which can withstand an array of adverse climatic conditions. Noteworthy, the development of new-cultivar types, namely "true primocanes", which do not have any chilling requirements to be productive, have a significant positive impact towards year-round production. Modern cultivars, are being tested in different meso-climates and can be highly productive when combined with advanced production systems, i.e. soilless production and plastic tunnels (Sønsteby et al., 2013; Demchak and Hanson, 2013). Despite recent advances, raspberry cultivation is particularly vulnerable at exposure to high temperatures (≥35 °C) that poses a significant threat to plant productivity (Sønsteby and Heide,

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https://doi.org/10.1016/j.scienta.2025.114465
Received 23 September 2025; Received in revised form 13 October 2025; Accepted 22 October 2025
Available online 5 November 2025

2008). This is a particular challenge to Mediterranean climates, since heat stress during developmental stages can affect flowering and hence plant productivity and fruit quality. Heat-sensitive cultivars may prolong their vegetative stage until milder temperatures are present, while in heat-tolerant cultivars, flowering is induced prematurely at early developmental stages.

Plant priming has emerged as a promising approach to improve crop resilience and combat negative impacts on crop productivity induced by adverse environmental conditions. Priming refers to the physiological memory of plants when being exposed to an environmental stressor which triggers the induction of several metabolic and molecular pathways that allow them to respond more effectively upon exposure to future stressors (Savvides et al., 2016; Guzmán et al., 2022; Gohari et al., 2024). Priming can be achieved using various natural or synthetic compounds in small, non-toxic doses. Priming agents (PAs) are generally recognized as safe, since they do not leave any harmful residues on fruits and are naturally present. Furthermore, they have been shown to trigger defense mechanisms against biotic and abiotic stress factors in an array of agricultural commodities.

Chemical priming agents, like melatonin, are increasingly recognised not only as antioxidant molecules, but as plant growth regulators, that have the ability to up-regulate genes related to physiological plant responses to biotic and abiotic stress, thus acting as stress elicitors, while also often leading to improvements in nutritional value of treated crops (Agathokleous et al., 2021). Organic and inorganic nanoparticles and polymers are another distinct category of priming agents. Additionally, NOSH-A, a novel synthetic donor of nitric oxide (NO), hydrogen sulfide (H_2S) and acetylsalicylic acid, represents a promising tool for plant priming to develop stress-resilient crops and advance sustainable production systems, thanks to its functional role in stress tolerance mechanisms involving oxidative stress avoidance, stomata regulation, and gene expression regulation (Antoniou et al., 2020). Notably, the use of nanocarriers (including biopolymers such as sodium alginate) as smart delivery vectors for priming agents towards improved plant performance is recently attracting increasing attention (reviewed in Gohari et al., 2024). However, such compounds have been scarcely analysed.

Towards the need for sustainable solutions, the incorporation of PAs in early developmental stages of raspberry cultivation presents a promising technological approach. The current study aimed to offer insights into the practical application of chemical PAs in raspberry cultivation under Mediterranean conditions, including melatonin, glycine-betaine, and NOSH-A and sodium alginate (alone or as conjugate with melatonin), along with appropriate solvent controls. Our working hypothesis was to dissect whether early-stage priming with different chemical agents, either through direct application or through smart delivery with the use of biodegradable polymers, will improve physiological responses of raspberry plants in the field and can potentially lead to the improvement of yield and phytochemical fruit attributes associated with taste, aroma and antioxidant capacity.

2. Materials and methods

2.1. Chemicals and standards

Chemical priming agents were obtained from Sigma-Aldrich (glycine-betaine and sodium alginate) and from Chem Cruz (melatonin). NOSH-A was provided by Avicenna Pharmaceuticals Inc., NY, USA. The analytical grade solvents, used to extract the samples, methanol and acetone, were obtained from Sigma-Aldrich and Scharlau, respectively. The HPLC grade water and acetonitrile solvents were purchased from Merck. Folin-Ciocalteu reagent, sodium hydroxide, potassium chloride, 2,6-dichloroindophenol sodium salt hydrate, aluminum chloride, trichloroacetic acid, thiobarbituric acid, metaphosphoric acid, and potassium acetate were from Sigma-Aldrich. Sodium carbonate, sodium acetate and hydrogen peroxide were obtained from Fluka, Scharlau and Supelco, respectively. Absolute ethanol was from Scharlau, hydrochloric acid and DMSO from Supelco.

The purity of all standards was higher than 95 %. Fructose, glucose and sucrose standards were obtained from Sigma-Aldrich, Himedia and Melford, respectively. Ascorbic acid, pelargonidin, and gallic acid were purchased from Sigma-Aldrich.

For the determination of volatile compounds, sodium chloride (NaCl) and SPME fiber assembly Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) was used and purchased from Sigma-Aldrich and Supelco, respectively.

2.2. Plant material, experimental design and treatment application

Experiment was conducted in Chandria village, Limassol District Cyprus located 1250 m above sea level. The climate is continental in terms of winter and chilling accumulation, with typical Mediterrenean conditions during spring and autumn. Annual rainfall ranges from 800–1200 mm depending on the season. Over the last decade, the summer period has shifted from mild Mediterrenean temperatures and occasional rains, to very hot and dry environment, similar to the coastal areas of the island.

Plant material (cv. 'Vica Abril', Viveros California, Spain) was received as tray plants in Cyprus in June 2024. In order to combat transplantation stress, first treatment application was performed in the root zone with 20 mL of respective formulation, three days prior to the placemnet of plantlets under field conditions into 10 L pots with coco coir substrate (June 18, 2024). Three foliar applications were followed after transplantation in the field at different intervals [27,46 and 74 days after planting (DAP)]. The last application took place just before flowering.

In total, the experiment consisted of the following treatments: (1) NOSH-Acetylsalicylic acid (NOSH-A, 100 μM), (2) DMSO (0.1 % v/v) (solvent for NOSH-A) (3) melatonin (Mel, 100 μM), (4) sodium alginate (NaA, 0.5 % w/v), (5) sodium alginate/melatonin (NaA/Melatonin, 100 μM/0.5 % w/v), (6) glycine-betaine (GB, 10 mM) and (7) hydro-primed which acted as the control. All treatment solutions contained 0.1 % v/v Tween-20 surfactant to facilitate the wetting and spreading of the priming agents onto the leaves. Application volumes per plant per application were 20 mL at root zone and 24 mL, 38 mL and 46 mL for the three successive sprayings, respectively.

The potted plants were used to set up a randomized complete block design (RCBD) to account for the spacial variation observed across the experimental area, particularly related to the sun exposure at different times during the day due to the topography. Experimental set-up consisted of four blocks in which treatments were randomly assigned in each block and were present only once per block. Each treatment replicate consisted of six raspberry plants serving as one biological replicate. Plants were kept under black netting of 30 % shadow and atmospheric temperature during midday constantly reach 30 °C over the period of vegetative growth of the plants. Plants were irrigated during 4 intervals within the day to collect 20–25 % drainage with fertigated values of EC (μS/cm) of no more than 1500 and pH value of 6.7.

2.3. Agronomic and physiological attributes

A set of agronomic attributes, namely sucker development, branching of laterals on ground level (Bottom Laterals-BL), total laterals (TL) per cane and of inflorescence laterals (IL: TL-BT), cane height (cm) and leaf number were assessed. Additionally, yield per plant was quantified starting on September 30 (101 DAP) and ending November 18 (150 DAP). Specifically, 13 harvests were carried out at 101, 104, 112, 116, 118, 123, 126, 130, 133, 137, 140, 143, 150 DAP. Cumulative yield was expressed in weight (g) per plant; in addition, the number of berries per plant to generate the index of average berry weight (g) was determined.

LI-600 Porometer/Fluorometer (LI-COR, USA) was used to measure gas stomatal conductance (mol m^{-2} s^{-1}) and transpiration (mmol m^{-2} s^{-1}). Fluorescence measurements were conducted during light

conditions to represent photosystem II chlorophyll fluorescence, while electron transport rate was additionally measured (μmol m^{-2} s^{-1}). LI-600 was also used to measure parameters quantifying the heat stress, such as Vapor Pressure Deficit (kPa), Leaf Temperature (°C) and Light Intensity (μmol m^{-2} s^{-}). The physiological responses of the plants were measured weekly during the vegetative stage and biweekly, during the harvest phase (chlorophyll fluorescence, stomatal conductance), along with Normalized Difference Vegetation Index (NDVI) (Molina-Bravo et al., 2011; Rungrat et al., 2016).

Data from canopy reflectance were collected twice a month starting in August, when the vegetation indices were adequate to start recording the NDVI and flowering induction has initiated. Crop vigour, represented by NDVI, was assessed with handheld proximal crop sensor Greenseeker (TRIMBLE Inc. Sunnyvale, CA, USA) at a height of ca. 0.3 m from plant canopy and at a different height from the soil according to the developmental stage. Both, proximal sensors including NDVI and LI-600 measurements were collected starting in July 26 (35 DAP) and ending in November 1 (133 DAP), 2024. Overall, measurements took place at leaf level during 35, 41, 51, 62, 72, 82, 91, 112, 118 and 133 DAP, respectively.

2.4. Cellular damage indicators

In order to assess any cellular damage induced by variations in temperature prior to flowering, the degree of oxidative stress was assessed through the quantification of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents. Six fully expanded raspberry leaves per biological replication per treatment were collected once-off before flowering and immediately placed in dry ice in the field, before stored in −80 °C until further biochemical analysis. The extent of lipid peroxidation was determined by measuring MDA content resulting from the thiobarbituric acid (TBA) reaction (Filippou et al., 2011). The absorption was measured at 532 and 600 nm (TECAN, Infinite 200® PRO) and MDA was estimated using the Lambert-Beer law, with extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nmol g^{-1} fresh weight (FW).

Hydrogen peroxide (H_2O_2) content was calculated spectrophotometrically based on the oxidation of iodide (I^{-1}) to iodine (I), after the reaction of H_2O_2 with potassium iodide (SSKI oral solution), using the procedure described by Loreto and Velikova, 2001. The content of H_2O_2 was measured at 390 nm and was estimated based on a standard curve of known concentrations of H_2O_2 and expressed as μmol g^{-1} F.W.

2.5. Fruit quality attributes

2.5.1. Soluble solid content (SSC), titratable acidity (TA) and sugar content

During harvest peak (133 DAP), approximately 200 g of raspberries were collected per biological replicate from each treatment and directly transferred to the laboratory. One-half of the amount (100 g) was frozen in liquid nitrogen and stored in −80 °C until further analysis while the other half was homogenized using IKA® T25 digital ULTRA-TURRAX® for 1–2 min and transferred into 50 mL falcon tubes. The homogenate was then centrifuged (Sigma 4K15, Germany, 21,190 g, 10 min, 4 °C) and the supernatant was collected. The supernatant was initially used to assess soluble solid content (SSC) and total acidity (TA) and then stored in −20 °C until further analysis. SSC was quantified with the employment of a refractometer (Atago, PR-32α, Japan) and results expressed as °Brix. TA was determined using an automatic titrator with multiple positions (862 Compact Titrosampler, Metrohm AG, Switzerland). For each measurement, 1 mL of diluted juice in 49 mL distilled H_2O was used for titrating with 0.1 N NaOH to a pH end point of 8.1. Results were expressed as % citric acid, and the ripening index (RI) was calculated as the SSC/TA ratio.

Fructose, glucose and sucrose contents were quantified using the homogenate samples which were thawed before the analysis. From the homogenate, 2 mL were transferred into an Eppendorf tube and centrifuged at 6163 g speed for 10 min and 1 mL of the supernatant was transferred to a new Eppendorf tube. Following this step, 1 mL of the solvent mixture (acetonitrile/water, 80:20) was added and vortexed. The mixture was then filtered through PVDF 0.45 μm filters. Glucose, fructose and sucrose contents were quantified using a high-performance liquid chromatography system (Waters 1525–2707) coupled with a dual λ absorbance detector (Waters 2487) following a method by Jalaludin and Kim (2021) with some modifications. Briefly, the chromatographic separation of the analytes was achieved on a Waters SPHERISORB® NH_2 column (4.6 × 150 mm, 3 μm), and the mobile phase consisted of water: acetonitrile (80:20). The flow rate was set at 1.0 mL/min, and the elution was isocratic. The injection volume was 15 μL and the column temperature set at 25 °C. The detection wavelength was set at 190 nm.

The estimation of sugar concentrations was achieved by constructing a calibration curve for each analyte within the concentration range of 0.3–15 mg/mL. All measurements were performed in triplicate. Method validation included evaluation of linearity (correlation coefficients, slopes, and intercepts), limits of detection (LOD) and quantification (LOQ), calculated as $LOD = \frac{3.3\sigma}{S}$ and $LOQ = \frac{10\sigma}{S}$, where σ is the standard deviation of response, and S is the slope of calibration curve. The precision of an analytical method is expressed as the relative standard deviation, %RSD of the repeatability (intra-day) and intermediate precisions (inter-day) of three analyses ($n = 3$) during the same day and over three days studied, respectively. Precision was assessed at a concentration of 5 mg/mL of each analyte standard. The method was considered acceptable when the %RSD value was lower than 15 %. For recovery evaluation, a concentration of 6.25 mg/mL of each sugar standard was spiked into a raspberry sample matrix. The results of the validation parameters are presented in **Supplementary Table 3**. Additionally, quality control (QC) standards (10 mg/mL) analyzed between sample runs showed percent errors of 0.3–5.4 % for fructose, 1.9–4.7 % for glucose, and 2.4–5.1 % for sucrose.

2.5.2. Quantification of total phenolic, reduced ascorbic acid, anthocyanin and flavonoid content

Previously flash-frozen raspberry fruit samples were ground into fine powder using the basic A11 analytical mill (IKA Mills) and liquid nitrogen. Extraction procedure of total phenolic compounds was followed as described by Shehata et al. (2020) with some alterations: 1.5 mL of 50 % v/v methanol was added to 0.05 g of ground raspberry fruit and vortexed. Next, the mixtures were placed at −20 °C for 24 h. Subsequently, samples were centrifuged for 10 min at 16 000 g at 4 °C (Eppendorf Centrifuge 5415 R), and the supernatant was stored at −20 °C. The total phenolic content was estimated by the method of Georgiadou et al. (2018) with slight alterations. The reaction mixture consisted of 100 μl of the diluted 50 % v/v methanol extract, 0.5 ml of distilled water, and 0.05 ml of the Folin-Ciocalteu reagent. After 3 min, 0.1 ml of saturated sodium carbonate solution was added, and the mixture was made up to 1 ml with distilled water. The mixture was thoroughly mixed, left to stand for 1 h in the dark at room temperature, and the absorbance was measured at 765 nm. Each measurement was repeated three times, and the results were expressed as mg gallic acid equivalent (GAE) 100 g^{-1} F.W.

Reduced ascorbic acid content was quantified in accordance to Habibzadeh et al. (2019) with some modifications. In short, 0.2 g was vortexed with 1.5 mL 2 % w/v metaphosphoric acid. Afterward, samples were centrifuged for 1 min at 16 000 g at 4 °C (Eppendorf Centrifuge 5415 R), and the supernatant was used for the analysis. The reduced ascorbic acid was estimated by the method of Georgiadou et al. (2018) with some alterations. First, 500 μL of the diluted 2 % w/v metaphosphoric acid extract was added to 900 μL of 50 mmol L^{-1} 2,6-dichloroindophenol and the absorbance was monitored at 520 nm. Ascorbic acid (AsA) content was quantified using a standard curve and expressed as mg 100 g^{-1} F.W.

Extraction of total anthocyanin content was performed following the

procedure of Bal & Ürün (2021) with some modifications: 1 mL of 95 % v/v ethanol: 0.1 N HCl (85:15) was added to 0.1 g of ground frozen raspberry fruit and vortexed. Successively, the mixtures were placed at −20 °C for 24 h, then centrifuged for 10 min at 16 000 g at 4 °C (Eppendorf Centrifuge 5415 R), and the supernatant was stored at −20 °C. Total anthocyanin content was estimated by the pH-differential assay, using two buffer systems: potassium chloride buffer (0.025 M) at pH 1.0 and sodium acetate buffer (0.4 M) at pH 4.5 (Georgiadou et al., 2018). Samples were diluted in pH 1.0 and pH 4.5 buffers and their absorbances were subsequently measured at 510 and 700 nm. Anthocyanin concentration was computed as mg cyanidin-3-glucoside 100 g^{-1} of F.W.

Total flavonoid content was estimated according to the method described by Meyers et al. (2003) with minor modifications. 10 mL of acetone was added to 1 g of ground frozen raspberry fruit and vortexed. Next, the mixtures were placed at −20 °C for 24 h. Subsequently, samples were centrifuged for 10 min at 16 000 g at 4 °C (Eppendorf Centrifuge 5415 R), and the supernatant was stored at −20 °C. Total flavonoids content was estimated by the method of Chang et al. (2020) with slight modifications. The reaction mixture consisted of 0.5 mL plant extract, 1.5 mL of 95 % v/v ethanol, 0.1 mL of 10 % w/v aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The absorbance of the reaction mixture was measured at 415 nm after incubation at room temperature for 30 min. The results were expressed as mg quercetin 100 g^{-1} F.W.

2.6. Polyphenolic compound analysis

The polyphenolic compound analysis by HPLC-DAD-ESI-MS/MS was performed according to Salazar-Orbea et al. (2022). Phenolics identification and quantification were carried out on an Agilent 1100 HPLC system equipped with a photodiode array detector (G1315D) and coupled in series to an HCT Ultra Bruker Daltonics ion trap mass spectrometer through an electrospray ionization (ESI) interface HPLC-DAD-ESI-MS/MS. The chromatographic separation was performed using a Poroshell 120 EC column (3 × 100 mm, 2.7 μm) from Agilent Technologies (Waldbronn, Germany). Phenolic compounds were identified by their UV spectra, retention time, molecular weight, and MS/MS fragmentation pattern. Phenolic compounds quantification was performed using the authentic standards of castalagin (280 nm), catechin (280 nm), *p*-coumaric acid (320 nm), pelargonidin (520 nm), ellagic acid (360 nm) and quercetin (360 nm) to quantify ellagitannins, flavan-3-ols, hydroxycinnamic acids, anthocyanins, ellagic acid conjugates and flavonols respectively.

2.7. Melatonin content

Melatonin extraction and quantification was carried out using a Melatonin ELISA Kit following the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY, USA).

2.8. Volatile organic compounds

Five grams of frozen powdered raspberry fruit tissue was placed in 50 mL tube and 5 mL of 1 M NaCl solution was added. The mixture was homogenized using the IKA® T25 digital ULTRA-TURRAX® (IKA Mills) and samples were stored at −80 °C until further analysis. Prior to the analysis, samples were transferred to a headspace vial (20 mL) and incubated in a water bath (40 °C) for 35 min. After the incubation, the volatile compounds were extracted by SPME fiber (50/30 μm DVB/CAR/PDMS, Stableflex (2 cm), 23Ga) for 40 min. Immediately after the 40 min of extraction, the SPME fibre was injected manually into the inlet of the GC–MS.

A Shimadzu GCMS-QP2010 Plus System was used for the determination of the VOC composition of raspberries aroma, following the protocol described by Vandendriessche et al. (2013) with some modifications. GL Sciences InertCap 5MS 30 m - 025 mm - 0.25 μm film thickness capillary column was used. Helium (He) was used as carrier gas (1.2 mL/min) at high purity. The samples were injected manually in spitless mode. The injection temperature was set at 250 °C. The oven was programmed as follows: hold at 35 °C for 5 min, increase to 150 °C in 4 °C/min increments, then to 240 °C in 50 °C/min increments. The temperature remained at 240 °C for 5 min. The MS Detector operated in EI mode (full scan, m/z range: 30–350). The EI-mass spectra were compared with the NIST library spectra.

2.9. Statistical analysis

Statistical analysis for quality attributes involved first one-way ANOVA analysis and then Tukey-HSD post-hoc pairwise comparison test at $p \leq 0.05$ using SPSS v.25 (SPSS Inc., Chicago, IL, USA). Biochemical data were statistically analysed using one-way ANOVA followed by Tukey-HSD post-hoc test ($p \leq 0.05$) both performed in GraphPad version 10.4.1 (GraphPad Software, San Diego, CA, USA). Principal component analysis (PCA) and heatmap were created using ClustVis 2.0 according to Metsalu and Vilo (2015). For metabolites the data were normalized to the control. For VOCs the data matrices of the original component data (VOCs concentrations versus treatment) were standardized in order to present (via a hierarchical clustering analysis heatmap) differences in the relative VOCs content. Euclidean distance was used as the clustering distance metric.

3. Results and discussion

3.1. Morphophysiological responses

The effect of PAs on plant architecture was assessed in this study to evaluate the impact of priming on canopy size. Canopy size as well as total number of laterals determines the yield efficiency of primocane raspberry plants. The priming agents tested did not significantly influence canopy development and plant architecture when compared with control samples (**Supplementary Figure 1**). Over the 5-month experimental period, physiological measurements did not show any striking differences among treatments applied, although the pattern was variable (Fig. 1). Leaf temperature and leaf vapour pressure deficit play an important role on the stomatal conductance and transpiration rate. Transpiration as well as stomatal conductance were slightly higher at 35 DAP and decreased along the developmental period and closer to the flowering period (Fig. 1).

Leaf vapour pressure deficit (VPD) can be defined as the difference between the moisture holding capacity of the atmosphere (saturation vapor pressure) and moisture currently present in the atmosphere (vapor pressure). This is highly influenced by changes in temperature as higher temperatures increase the saturation capacity of the atmosphere at a faster rate compared to actual vapor pressure, leading to higher VPD (Grossiord et.al., 2020). Higher values of VPD negatively influence stomatal conductance and transpiration as plants activate water-conservation mechanisms to minimize water loss. Therefore, fluctuations observed in stomatal conductance and transpiration are highly driven by temperature and VPD as well as the transition along the different developmental stages.

Biochemical analysis of cellular damage indicators from leaves harvested prior to flowering revealed no significant differences among treatments tested. Such results can be considered as an indicator that no harmful effect due to the priming treatments on leaves occurred (**Supplementary Figure 2**). Yet, additionally time points need to be tested to confirm this hypothesis.

3.2. Yield performance

Significant improvements due to melatonin pre-treatment were observed in the yield performance of the primocane raspberry 'Vica

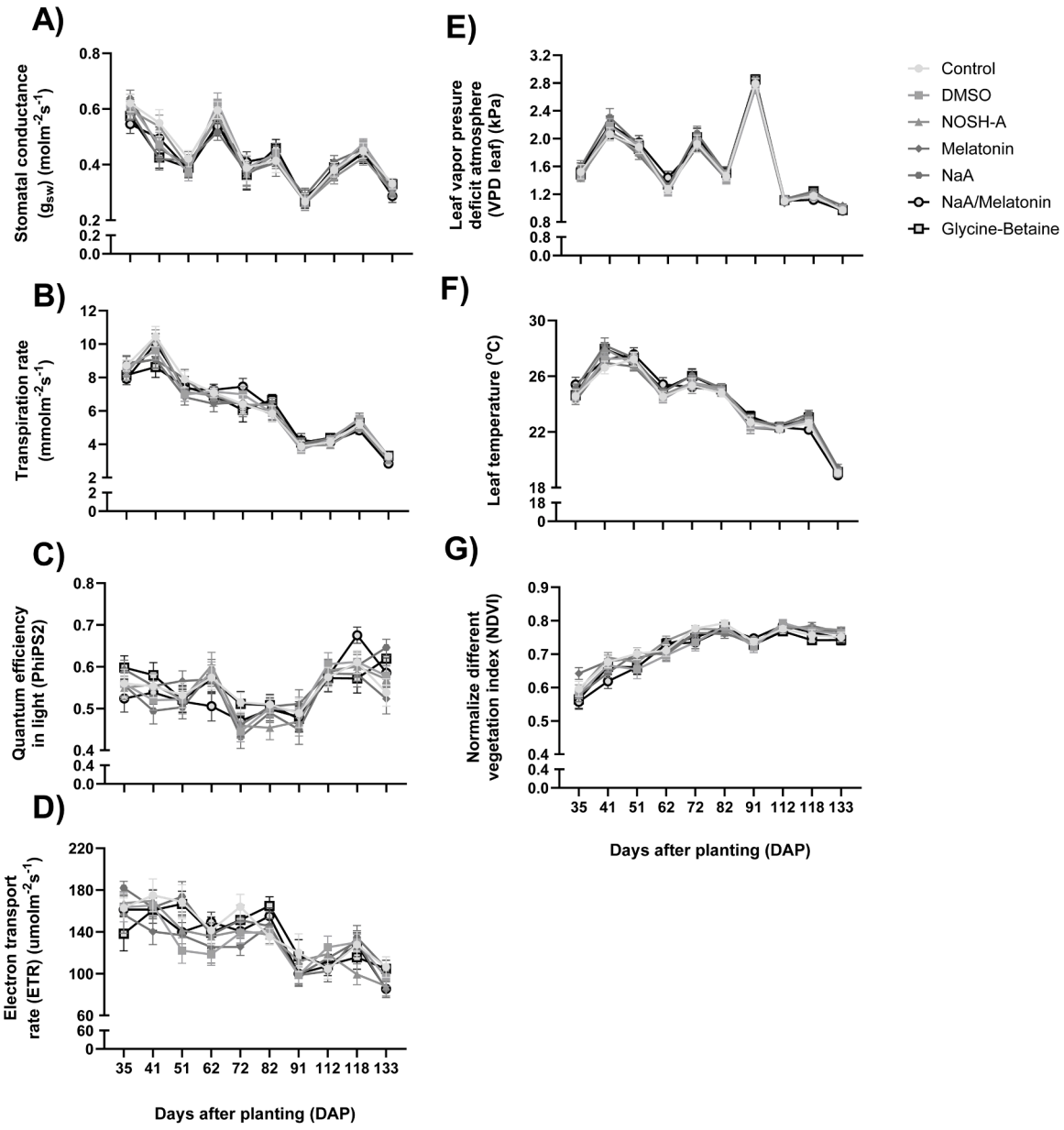

Fig. 1. The effect of pre-harvest application of DMSO, NOSH-A, melatonin, NaA, NaA/melatonin and glycine-betaine on A) stomatal conductance (g_{sw}), B) transpiration rate, C) quantum efficiency in light (PhiPS2), D) electron transport rate (ETR), E) Leaf vapor presure deficit atmosphere (VPD leaf), F) Leaf temperature, G) Normalize different vegetation index (NDVI) of raspberry leaves (cv. 'Vica Abril').

Abril' (Fig. 2). Early in the harvest period, at 118 DAP, melatonin primed plants showed pronounced differences in cumulative yield (ca. a 4-fold increase) compared with the hydro-primed control (Fig. 2**C**). By the end of the harvesting period (150 DAP), cumulative yield in melatonin-treated plants maintained a significant higher yield compared with the hydro-primed plants (Fig. 2**D**). Notably, yield improvements observed due to melatonin declined over time suggesting that the effectiveness of priming was transient (Fig. 2**B**). Overall, melatonin can serve as a sustainable strategy to improve raspberry productivity early in the harvesting period, which is particularly important during periods of limited fruit availability. In another soft fruit crop, strawberry, the melatonin application mitigated the negative effects of salinity stress, leading to increased fruit yield and quality. This was achieved by enhancing the plant's antioxidant defense systems and improving photosynthetic efficiency (Zahedi et al., 2020).

3.3. Fruit quality attributes

Titratable acidity (TA) of fruits refers to the concentration of all organic acids present and significantly impacts fruit's taste, texture, and overall quality. In the present study, plants pre-treated with melatonin, NOSH-A and NaA, exhibited significantly higher TA compared with the hydro-primed controls (Table 1). Soluble solids content (SSC) ranged from 8.5–9.7 % which is consistent with other studies (Ancos et al., 1999; Pantelidis et al., 2007). Current findings showed no significant impact of priming on SSC of raspberries, aligning with previous reports where the pre-harvest application of melatonin and glycine betaine on raspberries and cherries, respectively, did not result to any significant change in SSC (Li et al. 2019; Shah et al., 2024).

While no significant differences were observed in SSC among treatments, fructose, sucrose and glucose content were quantified to assess potential differences in sugar composition which may have a significant impact on fruit flavour (Fig. 3**E, F, G**). Concentration of the sugars in

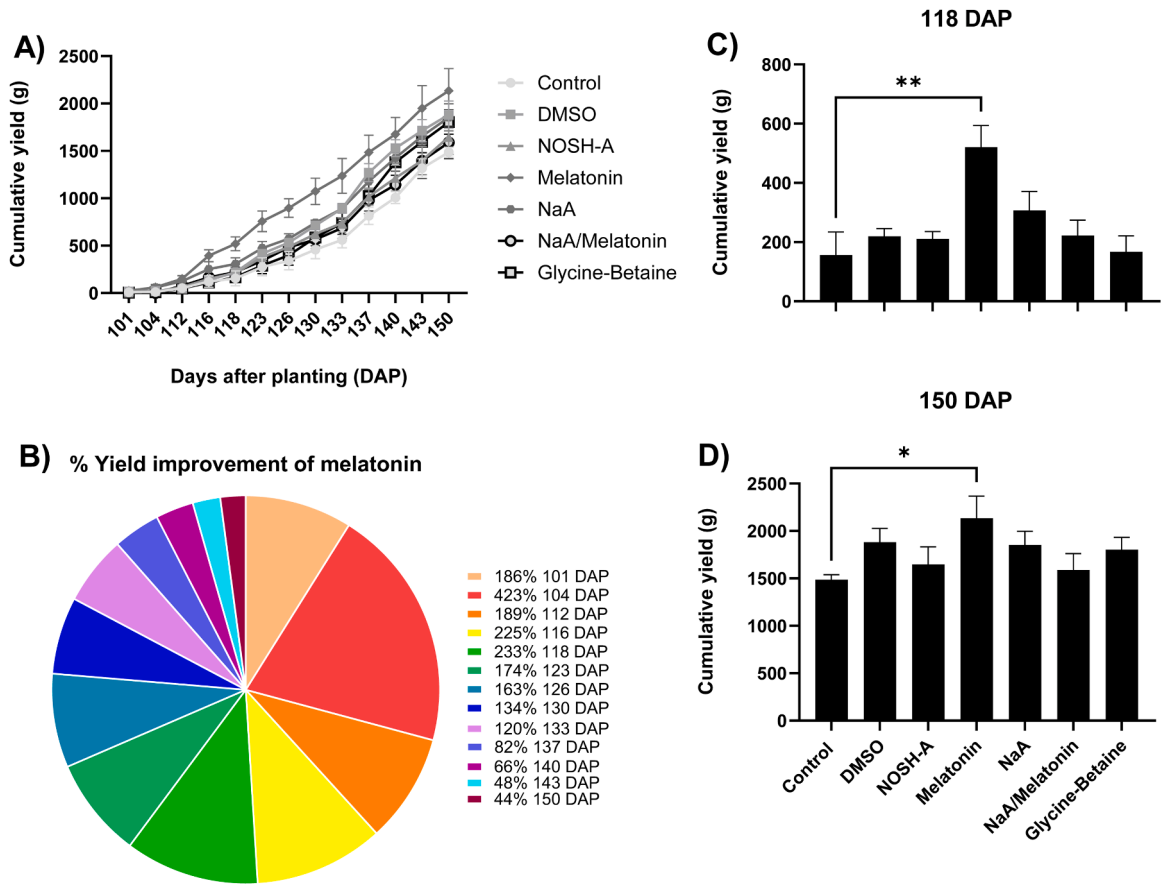

Fig. 2. A) Average cumulative weight (g) for the thirteen harvests along the harvest season, B) % yield improvement of melatonin per harvest, C) average cumulative weight (g) for the first five harvests per treatment and D) average cumulative weight (g) for the thirteen harvests per treatment of harvested raspberry fruits (cv. 'Vica Abril'). ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Table 1
The effect of pre-harvest application of DMSO, NOSH-A, melatonin, NaA, NaA/Mel and GB on fruit quality attributes [soluble solid content (SSC), total acidity (TA) and RI (SSC/TA)] of raspberry fruits (cv. 'Vica Abril').

Treatment	SSC (° Brix)	TA (% citric acid)	RI (SSC/TA)
Control	9.25 ± 0.31 a	2.47 ± 0.07 b	3.76 ± 0.19 a
DMSO	8.45 ± 0.17 a	2.70 ± 0.04 ab	3.13 ± 0.07 a
NOSH-A	8.90 ± 0.27 a	2.77 ± 0.04 ab	3.22 ± 0.10 a
Melatonin	9.01 ± 0.36 a	2.91 ± 0.11 a	3.14 ± 0.20 a
NaA	8.70 ± 0.17 a	2.88 ± 0.11 a	3.03 ± 0.11 a
NaA/Melatonin	9.70 ± 0.53 a	2.56 ± 0.06 ab	3.80 ± 0.26 a
Glycine-Betaine	8.78 ± 0.23 a	2.58 ± 0.04 ab	3.40 ± 0.05 a

raspberry juice can be highly variable ranging from 1.0–6.0, 0.8–4.7, 0.1–3.0 g 100 mL^{-1} for fructose, glucose, and sucrose, respectively and is dependent on the cultivar as well as mesoclimate conditions (Spanos and Wrolstad, 1987; Viljakainen et al., 2002). Results presented herein, showed that NOSH-A significantly improved sucrose content up to 35 %, while NaA treatment resulted in lower sucrose contents compared with the hydro-primed (control) samples. Furthermore, NaA alone or in combination with melatonin treatments exhibited lower levels of fructose and glucose. Observed increases in sucrose content following NOSH-A treatment could be attributed to altered sucrose metabolism levels, as individual components of the donor (i.e. NO, H_2S, and aspirin) have all been shown to increase activity levels of sucrose synthase and sucrose phosphate synthase (Jiang et al., 2007; Huang et al., 2020; Gao et al., 2024). In general, the effect of priming on sugar content can be variable in different crops and conditions. Okatan et al. (2022) investigated the effect of melatonin on four strawberry cultivars with no significant effect being reported on sugar content, while reports on priming with melatonin in grapevine and plum trees, indicated enhanced sucrose content, without however the exact mechanism to be clear (Xia et al. 2021; Xiao et al., 2024). Some studies indicated that melatonin increases sucrose content by promoting the activity of sucrose phosphate synthase, while others propose that melatonin increases the activities of sucrose synthase (in the catabolic direction), sucrose phosphate synthase, glucokinase, and fructokinase (Xiao et al., 2024). However, in the present study, melatonin pre-treatment did not influence sugar content in raspberries. Nevertheless, a more in-depth understanding of the effects of different priming agents on sugar biosynthesis across different crops is needed.

3.4. Total phenols, flavonoids, anthocyanins and reduced ascorbic acid content

Raspberry fruits are known for their high content of polyphenolic compounds including phenolic acids, flavonoids, and anthocyanins (Sariburun et al., 2010). In the present study, priming application did not result in any significant difference on the total phenolic content with the average concentration of total phenolics being 160.7 mg gallic acid equivalents 100 g^{-1} FW (Fig. 3A). Contradictory results have been reported in a recent study, where exogenous melatonin application (50 and 200 μmol L^{-1}) led to improved TPC in raspberries (Shah et al., 2024). Overall, total phenolic content (TPC) may vary significantly depending on the cultivar and the harvest period (Liu et al., 2002; Bobinaite et al., 2012). To what extent the cultivar under study may possessed different TPC if harvested under different microclimate conditions or at other time points need to be further elucidated. This is also

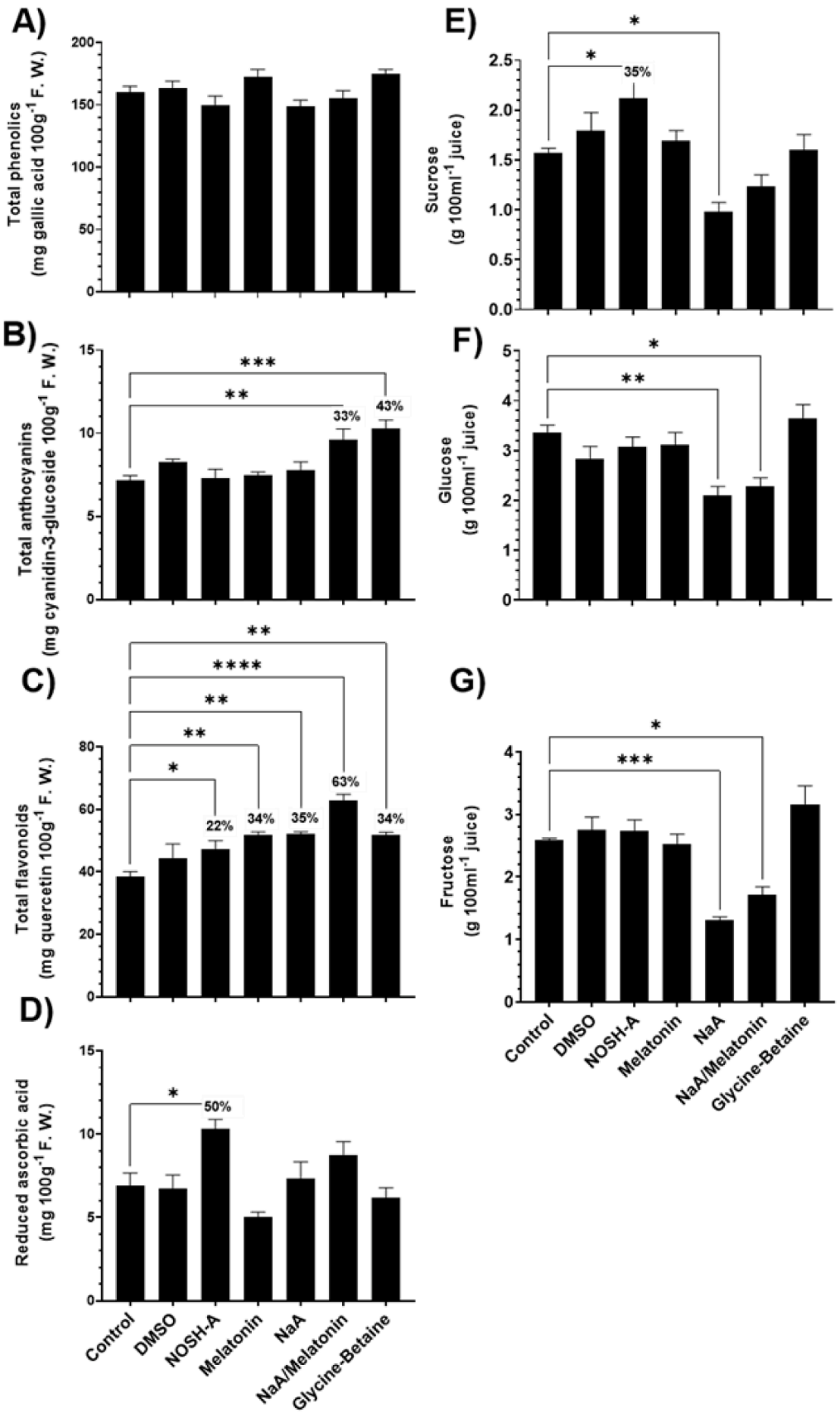

Fig. 3. The effect of pre-harvest application of DMSO, NOSH-A, melatonin, NaA, NaA/melatonin and glycine-betaine on A) total phenolics, B) total anthocyanins, C) total flavonoids, D) reduced ascorbic acid, E) sucrose, F) glucose, G) fructose of raspberry fruits (cv. 'Vica Abril'). ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

the case if analysis were conducted among different raspberry cultivars in order to explore the effect of the genetic background.

Anthocyanin content of Mel/NaA conjugate and GB priming applications led to modest, yet significant increase (Fig. 3**B**). Specifically, Mel/NaA treatment lead to 33 % increase in total anthocyanins, while GB treatment resulted in 43 % improvement in anthocyanin content (Fig. 3**B**). Raspberries contain significant levels of anthocyanins, often exceeding 35 mg cyanidin-3-glucoside equivalents 100 g^{-1} FW (Pantelidis et al., 2007), with major compounds including cyanidin-3-sophoroside, cyanidin-3--glucoside, and pelargonidin-3-sophoroside (Ancos et al., 1999).

While all priming applications resulted in >22 % of improvement in total flavonoids, the impact of Mel/ NaA conjugate was most significant with improvements of up to 63 % compared with the hydro-primed control (Fig. 3**C**). A recent study demonstrated the positive impact of Mel pre-treatment in total flavonoid content (Shah et al., 2024). Flavonoids are a major group of polyphenols present in raspberries, known for their antioxidant, anti-inflammatory, and cardiovascular health benefits (Yao et al., 2004; Sariburun et al., 2010). Among them, quercetin and kaempferol are particularly dominant (Bradish et al., 2012).

A further notable result identified, was the effect of NOSH-A treatment in reduced ascorbic acid content which resulted in a 50 % increase (Fig. 3**D**). Zhang et al. (2024), demonstrated that salicylic acid impacts ascorbate-glutathione cycle in prune fruits, improving ascorbic acid content by up-regulating the activity of key metabolic enzymes. Given the presence of an acetylsalicylic acid moiety in the NOSH-A chimera, it is plausible to employ similar mechanistic action.

Raspberries are known for their high ascorbic acid (vitamin C) content which is recognized for its high antioxidant potential and ability to counteract oxidative stress (Ponder and Hallmann, 2020). Ascorbic acid content is influenced by different factors such as cultivar and harvest period, with reported levels typically exceeding 20 mg per 100 g of fresh fruit (Pantelidis et al., 2007; Ancos et al., 1999; Ponder and Hallmann, 2020). Our study demonstrates the importance of NOSH-A in the improvement of ascorbic acid content, especially in cultivars which tend to demonstrate lower ascorbic acid content.

3.5. Polyphenolic compound analysis

Polyphenol analysis conducted in this study, led to the identification and quantification of 13 polyphenolic compounds in raspberry fruits, following the application of different priming agents along plant development. Significant improvements due to priming application were observed in caffeoylglucose, cyanidin 3-O-sophoroside, cyanidin 3,5-diglucoside, kaempferol derivative, cyanidin-3-O-glucoside, ellagic acid pentoside and ellagic acid acetyl pentoside (Fig. 4). The compound corresponding to either Cyanidin 3-O-sophoroside or Cyanidin 3,5-diglucoside could not be fully determined as both metabolites share the same mass and fragmentation pattern. In addition, some ellagic acid and conjugated derivatives could not be fully distinguished due to the similarity of the spectra between them. Furthermore, various ellagitannins were also present in the samples but were below the threshold of detection and thus are not included in the metabolomics analysis. The most significant effect due to priming application was observed in the content of kaempferol derivative, with NaA and NaA/Mel treatments resulting in a three-fold increase in its content compared with the control (Fig. 4**E**). Additionally, elevated levels of kaempferol derivative were also observed as a result of Mel and GB treatments ($p < 0.01$). Among all priming applications, NaA and NaA/Mel treatments had the most significant effects on enhancing the content of different polyphenolic compounds identified in the present study (Fig. 4).

In order to evaluate the patterns observed in the metabolomics data and assess whether these patterns are associated with priming application, a principal component analysis (PCA) was performed. The approach visualizes the data in a two-dimensional space based on the directions of maximum variance. Distinct separation of different priming treatments suggests differential effects on the polyphenolic composition of raspberry fruits. The first two principal components summarize 80.5 % of total variance (PC1=58.8 %; PC2=21.7 %) (Fig. 5**A**). PCA loading values indicate the contribution of each polyphenolic compound to the principal components. Significant impact of kaempferol derivative on both PC1 and PC2 was observed. PC1 is largely influenced by kaempferol derivative (0.581), cyanidin-3-O-sophoroside/Cyanidin 3,5-diglucoside (0.437), and cyanidin-3-O-glucoside (0.423), suggesting that variation of PC1 is driven by the differences observed in anthocyanins and flavonols between treatments. Kaempferol derivative (0.581) and catechin (0.351) strongly influence the variability observed in PC2. Separation of priming agents in PCA is strongly influenced by the variation observed in the flavonol and anthocyanin content (Fig. 5**A,B**). Due to the lack of authentic standards of the cyanidin glycosides, and the almost identical fragmentation patterns, it was not possible to distinguish between cyanidin 3-O.sophoroside and cyanidin 3,5-diglucoside and thus both are considered when dealing with the cyanidin dihexosides present in raspberries. The same happens with some isomers of ellagic acid acetyl-pentoside and ellagic acid pentosides, in which MS spectrometry fragmentation does not allow the differentiation between isomers, and authentic standards are not available.

An overview of the results of the analysis of all detected anthocyanin, catechins, flavonols and phenolic acids are shown in the form of heatmap (Fig. 5**C**). Strong impact of priming is observed in the anthocyanin group as well as flavonoid group, highlighting the significant impact of melatonin, NaA, NaA/Mel conjugate and GB betaine on polyphenolic compound content (Fig. 5**C**).

Polyphenolic compounds identified through the metabolomic analysis in this study were in accordance with other publications investigating raspberry secondary metabolites (Carvalho et al., 2013; Renai et al., 2021; Zhang et al., 2018). Interestingly, NaA and NaA/Mel treatments had a significant effect on kaempferol derivative content. Kaempferol is a known flavonoid recognized for its high antioxidant and health promoting-effects, like anti-inflammatory, anticancer, antidiabetic, and neuroprotective properties (Calderon-Montano et al., 2011; Parveen et al., 2023). Similarly, NaA/Mel treatment resulted in increased cyanidin-3-O-glucoside and cyanidin 3,5-diglucoside contents. Given that NaA/Mel treatment led to a 33 % increase in total anthocyanins, it is very likely that increase is driven by these compounds (Fig. 3**B**). These two compounds belong to the anthocyanin group and have high antioxidant potential (Zhang et al., 2023). PCA further supports these trends as both kaempferol derivative and cyanidin glycosides had the biggest contribution to the principal components associated with treatment differences (Fig. 5**A,B**).

These results indicate that priming agents have the potential of influencing not only the total flavonoid and anthocyanin content, but also affect the composition of key bioactive compounds improving fruit functional value. Interestingly, a recent report on the direct pre-harvest application of NaA/Mel conjugates on strawberry fruit at three successive developmental stages, namely large green (LG), small white (SW) and large white (LW), revealed similar trends in increased contents in ellagitannins, flavan-3-ols and ellagic acid and their conjugates in fully-ripe harvested fruit (Georgiadou et al., 2025). The present study highlights the promising potential involving novel priming agents like NaA/Mel in enhancing the nutritional value of raspberry fruits through the targeted increase in the content of health promoting polyphenolic compounds.

3.6. Endogenous melatonin content

Plants treated with Mel and NaA/Mel showed that harvested fruits contained higher levels of melatonin compared with the hydro-primed control, supporting their successful donation (**Supplementary Figure 3**). These results further support the stability of melatonin within plant structure, suggesting its potential integration into different metabolic processes (Antoniou et al., 2017). Elevated levels of melatonin content

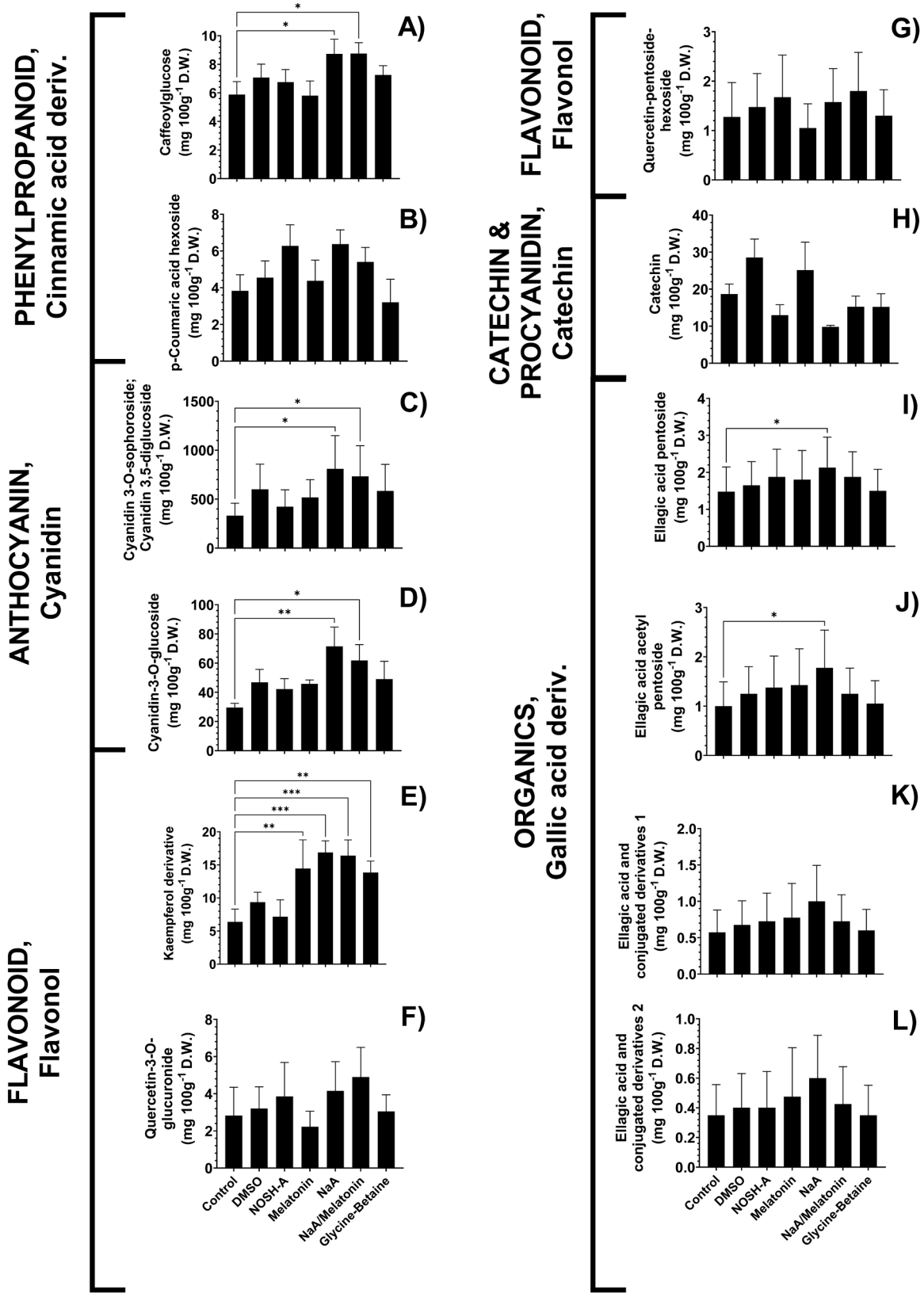

Fig. 4. Content of individual classes (phenylpropanoid, anthocyanin, flavonoid, catechin and procyanidine, organics,) of metabolomics data in raspberry fruits (cv. 'Vica Abril') after the pre-harvest application of DMSO, NOSH-A, melatonin, NaA, NaA/melatonin and glycine-betaine. ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

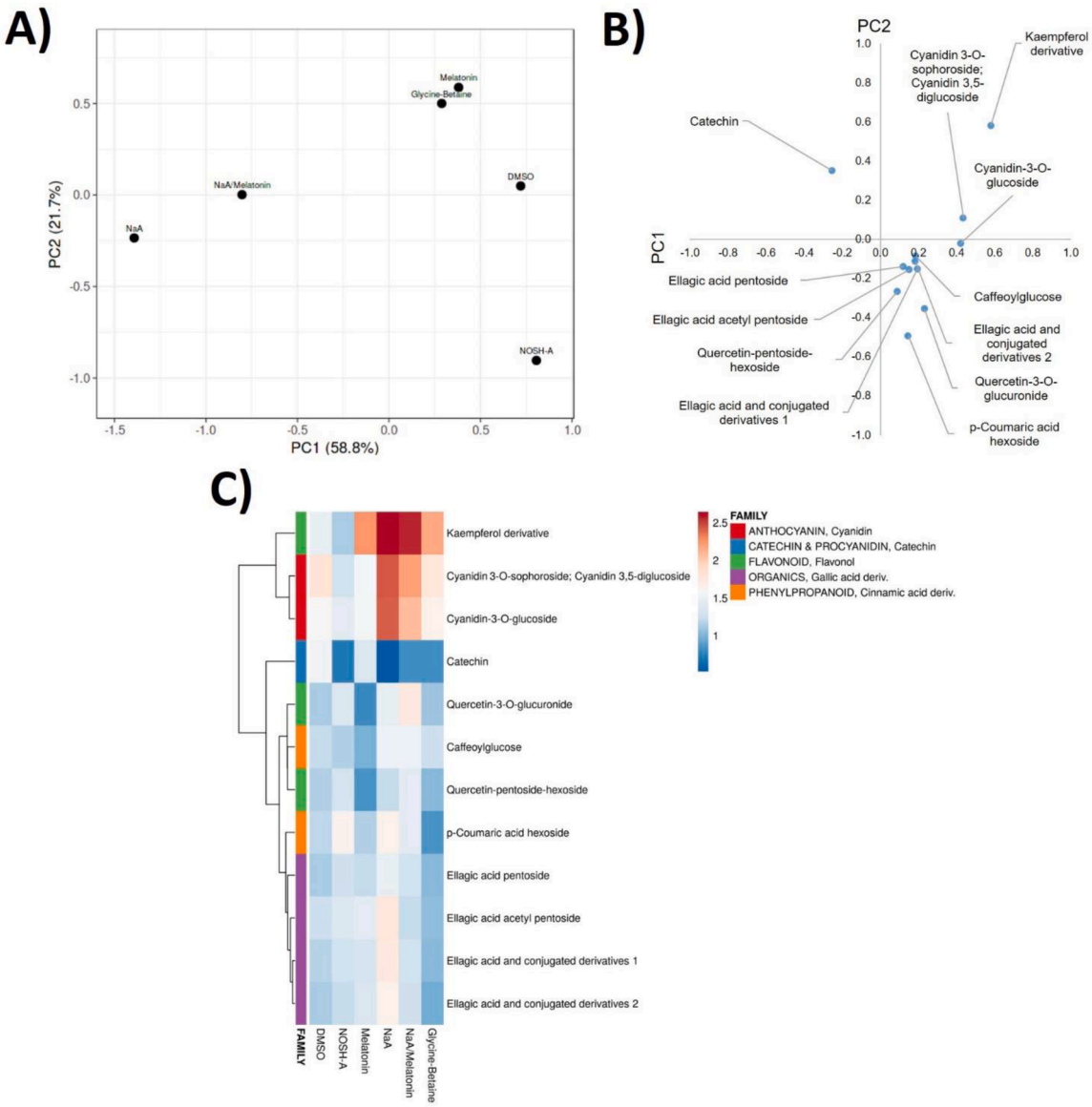

Fig. 5. A) Principal component analysis (PCA), B) xy-scatter plot graph of metabolites and C) heatmap representing the fold changes of metabolomics data of raspberry fruits (cv. '*Vica abril*') after the pre-harvest application of DMSO, NOSH-A, melatonin, NaA, NaA/melatonin and glycine-betaine. The data were normalized to control.

may also indicate the induction of the transcript levels of key enzymes involved in melatonin biosynthesis following exogenous melatonin application, leading to *de novo* synthesis (Priti et al., 2024).

3.7. Volatile compounds in raspberry fruits

The analysis of VOCs in raspberry fruits identified a total of 98 distinct compounds (**Supplementary Table 1**). Among all the samples analysed, the most abundant compounds detected included β-ionone, ethyl acetate, ethanol, α-ionone, 2-hexenal, n-hexanal, 3-hexenal, and acetaldehyde. These compounds were consistently present across all the samples, highlighting their prominence in the volatile profile of raspberry fruits.

Volatile compounds are typically associated with scents detectable by the human nose, often contributing to pleasant aromas and flavours. However, volatile compounds in plants also have diverse ecological and functional roles: they attract pollinating insects, signal the ripeness of fruits for seed dispersal, and alleviate the effects of abiotic stress (Aprea et al., 2015). Most of the compounds that have been identified in this study have been reported in previous studies of volatile compounds in raspberries (Aprea et al., 2015; Gu et al., 2022; Zhang et al., 2021).

The PCA score plot illustrates the distribution of the replicate treatments based on their principal component scores (**Supplementary Figure 4A**). The first two principal components, PC1 and PC2, accounted for 43.7 % of the total variance in VOCs. One of the NOSH-A replicate samples showed to be a clear outlier while the scores for all other treated samples were mixed up, showing no clear separation between the treatments. If any effect was visible this would be the slight separation of the control treatment from all other treatments along PC2. However, further analyses using PLS did not reveal any consistent differences (data not shown).

VOCs with an average contribution greater than 0.5 % across all treatments were considered as main components contributing to the raspberry aroma and their characteristic fragrance is represented in the clustering analyses of Fig. 6. Compounds, such as acetone and acetaldehyde are typically associated with oxidation products. Acetaldehyde is connected with the fermentation, fruit ripening and senescence, while acetone has been associated with fermentative degradation of sugars

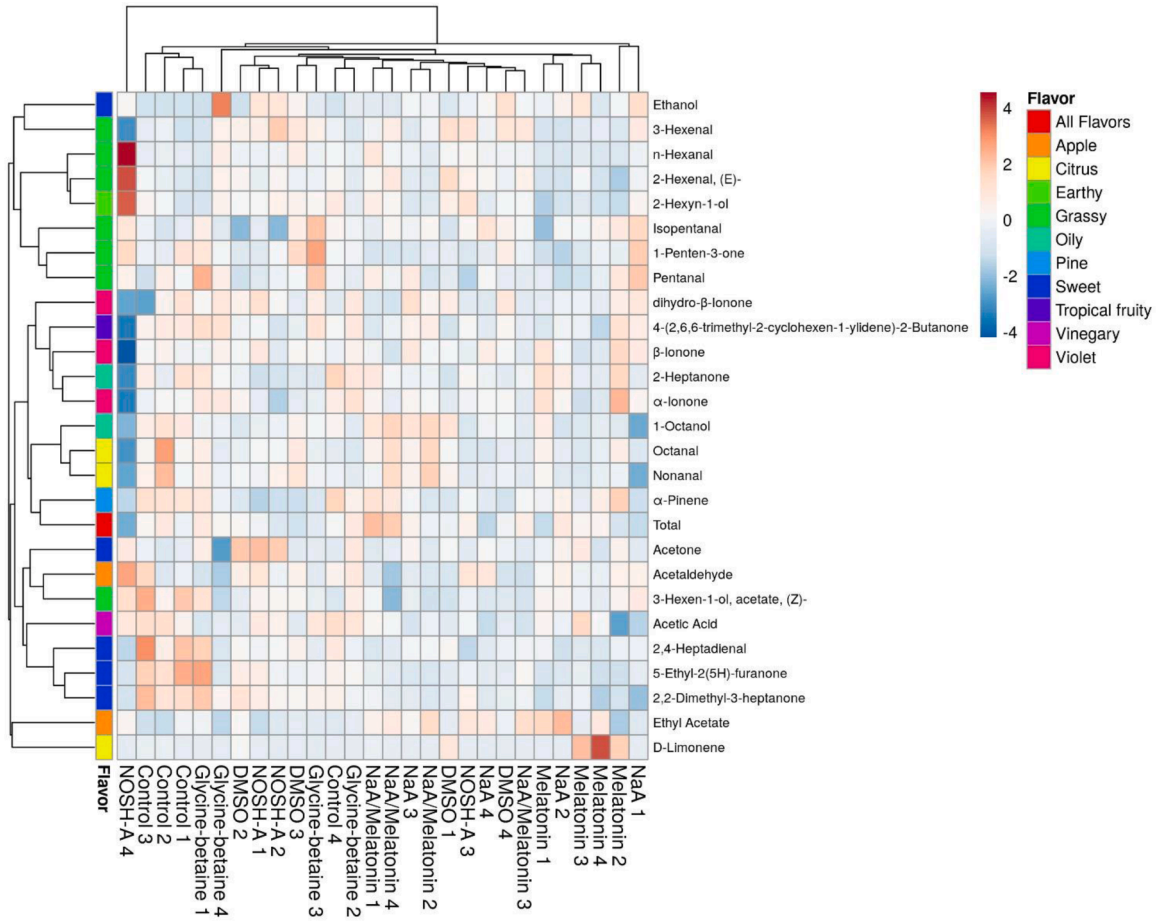

Fig. 6. Heatmap representing the fold changes of VOCs data of raspberry fruits (cv. 'Vica Abril') after the pre-harvest application of DMSO, NOSH-A, melatonin, NaA, NaA/melatonin and glycine-betaine. The data were standardized as described in Section 2.9 Statistical analysis.

and lipids (Strommer and Garabagi, 2009;Chierici et al., 2015; Mus et al., 2020). Moreover, compounds like n-hexanal, (E)-2-hexenal and 3-hexenal represent a distinct chemical sub-group, often associated with grassy notes (Akkad et al., 2023; Su et al., 2020; Yang et al., 2022).

Compounds such as isopentanal, α-pinene, pentanal, 2-heptanone and the α- and β-ionone suggest a contribution from fruity and floral aromas (Amanpour et al., 2019; Aprea et al., 2015; Ma et al., 2022). Ionones (α- and β-) are significant compounds, derived from the same carotenoid-based biosynthetic pathway (Aprea et al., 2015), and both linked to floral notes, which are characteristic of raspberry fruit.

D-limonene was high in three of the four Mel-treated samples corresponding to a citrus-like aroma in raspberry fruits (Ibáñez et al., 2020). An increase in limonene content can enhance the fruity and fresh sensory attributes of the fruit, making it more appealing to consumers (Gu et al., 2022; Paterson et al., 2013). Moreover, this terpene has been associated with various health benefits, such as anti-inflammatory and anti-obesity effects, suggesting that higher limonene content could potentially enhance the health-promoting properties of raspberry fruits (Gu et al., 2022). Arnao et al. (2022) reported that melatonin can increase the content of compounds such as limonene and β-caryophyllene.

Subsequently individual compounds were grouped based on their chemical classes to reveal a possible overall shift in the VOC pathways (Fig. 7). The main shift was observed in terms of total aldehydes and terpenoids. Mel-treated samples exhibited the lowest concentrations of aldehydes and the highest concentrations of terpenoids compared with all the other treated and control samples. Aldehydes are generally associated with not only secondary (Gutensohn et al., 2011) but also primary metabolism, as they are involved in lipid peroxidation, carbohydrate metabolism, and amino acid metabolism (O'Brien et al., 2005; Rizzo, 2014). In contrast, terpenoids are primarily linked to secondary metabolism (Chen et al., 2011). This pattern indicates that melatonin may enhance the activity of enzymes involved in secondary metabolic pathways, particularly those related to terpenoid biosynthesis. A recent study by Eghlima et al. (2025) confirmed that melatonin enhances the essential oil yield of *Thymus vulgaris* under water-deficit conditions. This increase in secondary metabolites was attributed to stimulated terpenoid biosynthesis, driven by enhanced meristem activity and the activation of enzymatic pathways. These findings align with the increased concentration of the terpenoid d-limonene observed in the Mel-treated samples of the present study. Similarly, Arnao et al. (2022) reported that exogenous melatonin positively influences the expression of genes involved in terpenoid biosynthesis, thereby contributing to the increased accumulation of terpenes, including limonene, as previously discussed. Interestingly, the direct pre-harvest application of Mel and NaA/Mel on strawberry fruit during different developmental stages prior to fully ripe stage did not lead to a significantly altered aroma profile in comparison with hydro-primed control samples (Georgiadou et al., 2025), highlighting the importance of the timing of application for potential aroma effects.

In spite of the observed shift from aldehydes to terpenoids in Mel treated fruit (Fig. 7), largely induced by the change in d-limonene, given the lack of a consistent sample clustering (Fig. 6 and **Supplementary Figure 4A**) no clear discrimination was observed between priming treatments.

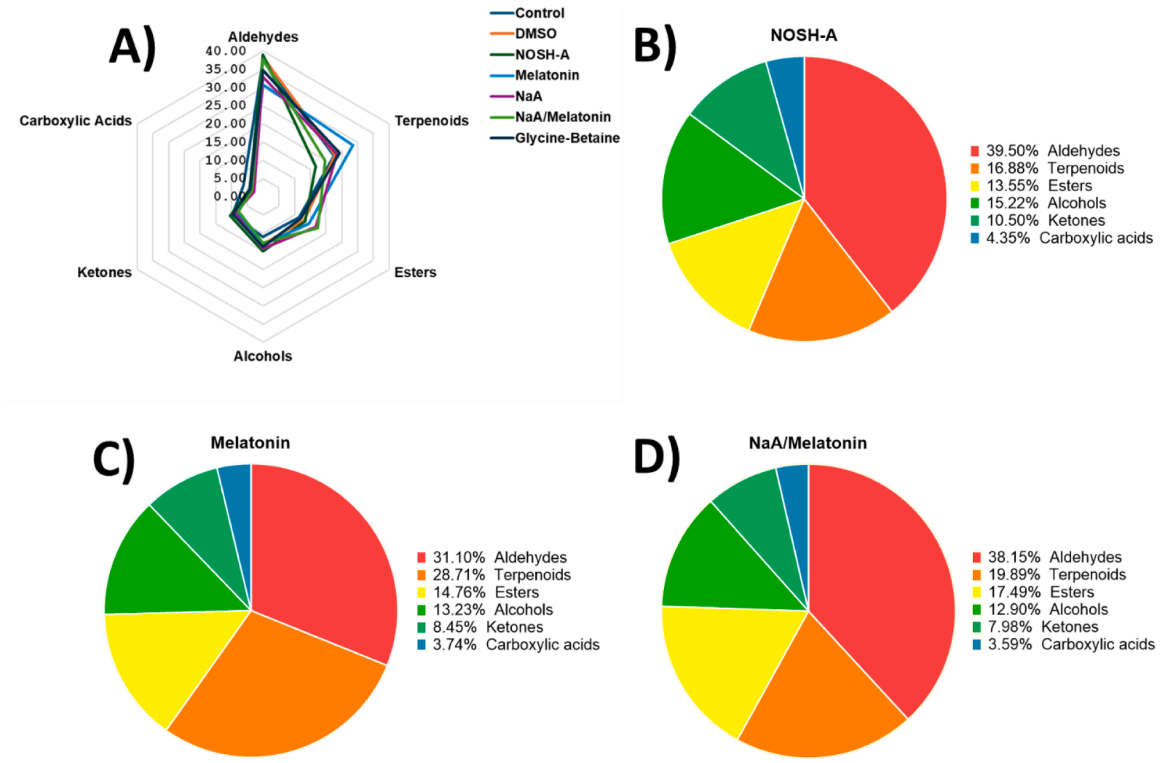

Fig. 7. A) Radar chart of the higher average percentage (%) of chemical groups of VOCs across the different treatments and pie chart of the B) NOSH-A, C) melatonin and D) NaA/melatonin treatments with the higher percentage (%) of chemical groups of VOCs of raspberry fruits (cv. '*Vica abril*').

4. Conclusions

The present study provides new knowledge on the impact of selected priming agents on vegetative growth, physiological performance, yield efficiency and fruit primary and secondary metabolites of a primocane raspberry cultivar. While priming did not affect vegetative growth and physiological parameters, significant improvements were observed in yield and/or fruit quality attributes in a priming agent-dependent manner. Notable transient improvements in early harvests were observed in melatonin-primed plants, suggesting time-dependent yield improvement which is particularly important in periods of low fruit availability. Additionally, pre-treatment with NaA and NaA/Mel along the developmental period led to alterations on fruit sugar composition and polyphenolic content, with special reference to enhanced contents in kaempferol derivatives, total flavonoids, and anthocyanins, compounds with potent antioxidant properties. NOSH-A treatment demonstrated particularly promising increases in ascorbic acid and sucrose contents, while GB treatment resulted in an increase in total anthocyanins. Beside some changes in d-limonene content the observed improvements in yield and/or fruit quality attributes were not at the expense of any drastic consistent changes in aroma composition. Overall, this study indicates that chemical priming with Mel, alone or in combination with sodium alginate, can be further exploited as a sustainable strategy to enhance fruit productivity and nutritional value of raspberry fruit, without compromising plant growth. Further investigation of the mechanistic action of these compounds is needed to identify the exact biochemical pathways involved and optimize application protocols across variable environmental conditions.

Supporting information

Supporting information may be found in the online version of this article.

CRediT authorship contribution statement

Nicolas Valanides: Methodology, Investigation. **Egli C. Georgiadou:** Writing – original draft, Supervision, Methodology, Formal analysis, Data curation. **Eleni D. Myrtsi:** Writing – original draft, Methodology, Formal analysis. **Carlos Javier Garcia Hernandez Gil:** Methodology, Formal analysis, Data curation. **Anna Maria Taliadorou:** Methodology, Investigation. **Sofia Torrado:** Methodology, Investigation. **Maarten L.A.T.M. Hertog:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Francisco Tomás-Barberán:** Writing – review & editing, Validation, Data curation. **Vasileios Fotopoulos:** Writing – original draft, Visualization, Project administration, Methodology, Conceptualization. **George A. Manganaris:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

V.F. is a coinventor of patent WO/2015/123,273 dealing with the use of NOSH-A in plants, and a coinventor of patent #WO/2023/099627A1 pending dealing with the use of sodium alginate conjugates with priming agents in plants. The remaining authors declare no conflicts of interest.

Acknowledgements

This study has received funding from the European's Union Horizon Europe programme with acronym PRIMESOFT, entitled 'Development of innovative priming technologies safeguarding yield security in soft fruit crops through a cutting-edge interdisciplinary approach' [Grant Agreement No 101079119].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2025.114465.

Data availability

Data will be made available on request.

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