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Pre-harvest application of sodium alginate functionalized with melatonin enhances secondary metabolism in strawberry fruit

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ABSTRACT

The application of priming agents is a promising strategy to enhance the nutritional content of fruits and overall fruit quality. The current study aimed to assess the effect of the pre-harvest application of various priming agents [melatonin (Mel), sodium alginate (NaA), sodium alginate/melatonin conjugate (Mel-NaA), and putrescine dihydrochloride (Put)] on fruit quality attributes and secondary metabolite profile of a strawberry cultivar (*Fragaria* x *ananassa Duchesne* cv. 'Felicity Q3'). The priming agents were directly applied on fruit at three successive developmental stages, namely large green (LG), small white (SW) and large white (LW). The use of Mel-NaA and Put showed promising results in improving fruit quality indicators (i.e. firmness, color), while Mel-NaA and putrescine-treated fruit were characterized by increased total flavonoid content. HPLC-DAD-ESI-MS/MS data showed variable regulation of flavan-3-ols, hydroxycinnamic acids, and conjugates contents by the different treatments, while ellagitannins and ellagic acid derivatives were significantly enhanced following Mel-NaA pre-treatment. Priming treatments did not result in the differential regulation of volatile organic compounds (VOCs) in comparison with controls, suggesting that primed fruit retain their aroma quality with no aroma profile 'penalty'. In addition, molecular analysis revealed that fruit pre-treatment with the priming agents resulted in variable transcriptional regulation of known strawberry allergenic proteins, with the Mel-NaA treatment showing no significant effect. This 'green' approach holds promise for advancing our understanding of the effects of NaA as a smart delivery mechanism of chemical priming agents and its potential impact on the sustainable improvement of the physicochemical attributes of strawberries during the pre-harvest stage.

1. Introduction

Strawberry (*Fragaria* x *ananassa*) is one of the most important fleshy fruit commercially, mainly due to its desirable aroma, juicy texture, bright red color, and sweetness [37]. It is a significant source of vitamins and health-promoting bioactive compounds with high antioxidant capacity (reviewed in [37,44]) and has shown considerable increase in production over the recent years, with China and the United States being the leading producers (UN Food and Agriculture Organization, Corporate Statistical Database, [65]).

Agrochemicals are usually incorporated into the cultivation practices to increase crop production, enhance fruit quality or extend shelf-life of strawberry fruits. However, concerns regarding the environmental impact and human health of such chemicals has shifted the interest into the discovery of sustainable alternatives [11]. Interestingly, the exogenous application of chemical priming agents, both at the pre- and

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post-harvest level, is being considered as a highly-promising technological approach towards alleviation of plant stress conditions and enhanced yield efficiency [19,57]. Priming agents are natural metabolites, synthetic compounds and/or nanomaterials that are generally known to enhance cell tolerance and amelioration of stress-induced plant growth inhibition [57] that can subsequently lead to higher yield and improved fruit quality attributes [3]. Notably, the use of nanocarriers (including biopolymers and nanoparticles) as smart delivery vehicles for chemical agents towards improved growth and stress protection in plants is nowadays being extensively explored (reviewed in [19]).

Precisely, nutritional and organoleptic quality of fruits could be improved by exogenous application of hormones, which play a vital role in physiological, biochemical and molecular control of ripening [50]. Melatonin (N-acetyl-5-methoxytryptamine, Mel), is a well-conserved hormone that acts as a growth regulator, being present both in animals and plants [4]. Mel is a small indolamine with amphiphilic structure that has a high antioxidant capacity. Application of agents like melatonin have been proven to significantly improve plant responses against abiotic stresses. A key trait of melatonin that makes it an ideal molecule for priming is its ability to easily diffuse through the cell membrane and into the cytoplasm, thus leading to fast activation when plant cells are exposed to stressful conditions [75]. For instance, the application of higher concentrations of Mel to strawberries through injection at the green stage accelerated ripening, which was accompanied by a higher activity of PAL enzyme, increased accumulation of total phenolics and anthocyanins, as well as increased scavenging capacity [39]. Okatan et al., [48] found that the pre-harvest foliar application of Mel on different strawberry cultivars positively affected fruit yield, quality (fructose and titratable acidity), and antioxidants (phenolics and ascorbic acid). However, the mechanistic effect of Mel at pre-harvest level in strawberry fruit has been scarcely examined.

As fruits ripen, the presence of reactive oxygen species (ROS) leads to oxidative stress and cell membrane disruption, contributing to fruit ripening [27]. Polyamines (PAs) are essential components of the antioxidative system and play a crucial role in safeguarding membranes from oxidative injury caused by ROS [29,58]. Furthermore, PAs are key elements in numerous biological processes and stress responses in plants, including plant growth, morphogenesis, and fruit development [16], while they are also widely applied to prolong the shelf-life of perishable horticultural crops [49]. Exogenous application of putrescine (Put) on strawberry either alone [30] or in combination with chitosan [6], has been reported to maintain strawberry quality attributes leading to extended shelf-life. However, such studies do not provide supporting evidence regarding the mechanistic action of polyamines on fruit physiology.

Natural polysaccharides and oligosaccharides have also been reported to prolong the shelf-life and to enhance fruit quality attributes. Such polysaccharides could be used as moisture and gas semi-permeable edible barriers, thus affecting the fruit internal atmosphere [8]. Alginate is a natural polysaccharide derived from brown marine algae (Phaeophyceae), with strong affinity for water and is a biopolymer that has a coating function [1]. NaA is the most common salt of alginate [74] and is prepared by the neutralization of purified alginic acid with appropriate pH control agents (FDA, TITLE 21). Recent studies have presented compelling evidence supporting the use of NaA for preserving fruit firmness and prolonging the shelf life of strawberries [2,7], without however any study on the effect of alginate salts when applied at pre-harvest level.

While there is currently limited research on the pre-harvest application of NaA on strawberries, given the valuable carrier properties attributed to alginate, it becomes imperative to explore its potential as a biodegradable biopolymer for smart and sustainable delivery of priming agents, such as Mel, to enhance the quality of strawberry fruits. We hypothesized that the exogenous pre-harvest application of priming agents (Mel, NaA, their conjugated form Mel-NaA, and Put) on strawberry fruit undergoing ripening on-bush under non-stressful conditions results in improved physicochemical properties and fruit quality attributes. To this end, our study aimed to assess the effect of the exogenous pre-harvest application of the aforementioned priming treatments on the fruit quality attributes, polyphenolic profile, and gene expression levels of an early-harvested strawberry cultivar.

2. Material and methods

2.1. Plant material and treatment application

Strawberry plants (*Fragaria* x *ananassa* Duchesne cv. ‘Felicity Q3’, a cultivar that is amenable to early production in the Mediterranean basin during winter period), were planted in clay soil. To avoid water, raised beds of 1 m width and 0.6 m space between the beds (total 1.6 m) were used in an experimental farmer’s field in Ayios Ioannis Malountas village (35°04’48"N 33°10’40"E, at 313.45 m altitude) at Nicosia district, Cyprus. The raised beds were mulched with silver plastic, with 12 plants planted per linear meter, spaced in three rows per raised bed, whereas each plant was spaced 0.30 m from each other. Plants were covered with low plastic tunnels (0.9 m) with aeration holes and fertilized with 50 kg/ha of 16–8–24–6Ca+TE (ULTRASOL SQM) using double drip irrigation on each raised bed (1.6 L/h spaced every 0.22 m). Experiment was set up as a completely randomized design with five treatments (described below). Prior to treatment application, 100 fruits per treatment were labelled at the large green (LG) stage (Fig. 1); fruit of similar developmental stage were selected in order to be synchronized during ripening. Experimental applications were targeted exclusively onto labelled fruit with different treatments being applied to fruits growing in different plants, and included the following five treatments: (T0) Water-sprayed (control), (T1) NaA (0.5 % w/v), (T2) Mel (100 μM), (T3) Mel-NaA (100μM-0.5 % w/v) and (T4) Put (1 mM). All plants were root-watered according to standard cultivation practices. Treatments were applied at weekly intervals starting on 15 December, corresponding to the following successive developmental stages: green receptacle with enlarged achenes (LG), small white receptacle and green achenes (SW), and large white receptacle with brown achenes (LW), as described by Symons et al. [62]. To ensure fruit full coverage and high binding of the priming agents, 0.1 % w/v Tween-20 surfactant was added in each solution, freshly prepared for every time point.

Strawberry fruits, were harvested at commercial maturity stage 1 week after the last application. Representative fruits with uniform color were assessed based on the absence of pest/disease damage such as *Botrytis* infection. Based on this selection, only 45 fruit per treatment were collected for further analysis. One lot (three 5-fruit sublots corresponding to three biological replications) was flash frozen and kept at −80 °C until needed for biological, enzymatic or molecular analyses as described below. The second lot (three 10-fruit sublots corresponding to three biological replications) was immediately used for fruit quality assessment.

2.2. Fruit quality attributes

Fruit weight, volume, color parameters and firmness were determined according to Hadjipieri et al. [24]. Color parameters were monitored, using a reflection colorimeter (CR-400, Konica Minolta, Osaka, Japan), while flesh firmness (FF) was measured using a texture analyzer (TA.XT plus, Stable Micro Systems, Surrey, U.K.).

Soluble solid content (SSC) and titratable acidity (TA) were measured in fruit juice isolated using a professional juicer according to Hadjipieri et al. [24]. SSC was quantified using a refractometer (Atago, PR-32α, Japan), while TA was determined with the use of an automatic multiple positions titrator (862 Compact Titrosampler, Metrohm AG, Switzerland).

Fig. 1. Strawberry developmental stages and dates of the pre-harvest applications. 15/12/22: 1st spray application, 22/12/22: 2nd spray application, 29/12/22: 3rd spray application, 09/01/23: Harvest day. Abbreviations: Large green (LG), Small white (SW), Large white (LW), Red (R).

2.3. Total soluble sugars (TSS), glucose, fructose, sucrose content

Sugars [total soluble sugars (TSS), glucose, fructose, and sucrose] were extracted according to Hadjipieri et al. [23], with slight modifications. Triplicate strawberry samples (0.3 g) per treatment were extracted with 10 mL of 80 % v/v ethanol and sugars were determined spectrophotometrically as described elsewhere [13,28].

2.4. Determination of total phenolic content

Total phenolic content was extracted from the samples following the procedure of Shehata et al. [59] with slight modifications: 2 mL of 50 % (v/v) methanol was added to 0.05 g of ground frozen strawberry fruit and vortexed, and mixtures were placed at −20 °C for 48 h. Samples were then centrifuged at 16000 × *g* at 4 °C for 10 min (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. [17], with absorbance being measured at 765 nm (TECAN, Infinite 200® PRO). Results were expressed as gallic acid equivalents (GAE; mg 100 g^{-1} FW) and analysis were conducted in triplicate per treatment.

2.5. Determination of reduced ascorbic acid

The extraction for the reduced ascorbic acid was performed as described by Habibzadeh et al. [22] with some modifications. In detail, 0.2 g were vortexed with 1.5 mL 2 % (w/v) metaphosphoric acid and then centrifuged for 1 min at 16000 × g at 4°C (Eppendorf Centrifuge 5415 R). The reduced ascorbic acid was estimated by the method of Georgiadou et al. [17] with modifications. Briefly, 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 absorbance was monitored at 520 nm (TECAN, Infinite 200® PRO). Ascorbic acid (AsA) content was quantified using a standard curve and expressed on a fresh weight base in mg 100 g^{-1}.

2.6. Determination of total anthocyanin content

Total anthocyanin content was extracted from the samples following the procedure of Bal and Ürün [6] 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 strawberry fruit and vortexed. Mixtures were placed at −20 °C for 24 h, centrifuged at 16000 × *g* at 4 °C for 10 min (Eppendorf Centrifuge 5415 R), and supernatants stored at −20 °C.

Total anthocyanin content was quantified by the pH-differential assay, according to Georgiadou et al. [17], with absorbances measured at 510 and 700 nm (TECAN, Infinite 200® PRO). Anthocyanin concentration was calculated as cyanidin-3-O-glucoside (CY3) equivalents and expressed on a fresh weight base as mg 100 g-1.

2.7. Determination of total flavonoid content

Total flavonoid content was quantified from the samples following the procedure of Meyers et al. [43] with some modifications. Ten mL of acetone was added to 1 g of ground frozen strawberry fruit and vortexed, and mixtures were placed at −20 °C for 48 h. Samples were then centrifuged at 16000 × *g* at 4 °C for 10 min (Eppendorf Centrifuge 5415 R), and supernatants stored at −20 °C. Total flavonoid content was estimated by the method of Chang et al. [12] 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 aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm (TECAN, Infinite 200® PRO). The results were expressed on a fresh weight base as mg 100 g-1 quercetin equivalents.

2.8. Quantification of MDA and H_2O_2 content

The assessment of lipid peroxidation was carried out by quantifying the malondialdehyde (MDA) content produced through the thiobarbituric acid (TBA) reaction, as outlined by Filippou et al. [15].

The quantification of hydrogen peroxide (H_2O_2) content was performed through the reaction of H_2O_2 with potassium iodide (KI), by measuring the oxidation of iodide (I^-) to iodine (I) according to Loreto and Velikova [34].

2.9. Quantification of endogenous melatonin and polyamine content

Melatonin extraction assay was conducted following the guidelines of the Melatonin ELISA Kit (Enzo Life Sciences, Farmingdale, NY, USA). In summary, fruit samples weighing 0.5 g were finely ground into powder using liquid nitrogen and homogenized in 125 μL of 1 × stabilizer solution from the kit. Subsequently, 750 μL of cold ethyl acetate was added, and the mixture was subjected to vortexing. Following 5 min incubation on ice, the solution was centrifuged at 1000 × g at 4 °C for 10 min. The resultant organic layer was transferred to a new glass tube and subjected to overnight drying. The resulting pellet was reconstituted in 200 μL of 1 × stabilizer for melatonin quantification following the manufacturer's specified protocols. The levels of free polyamines were quantified by high-performance liquid chromatography (HPLC) separation of dansyl derivatives, as elsewhere described [40]. Analyses were performed in three biological replicates per point of analysis.

2.10. *(Poly)phenolic compound analysis by HPLC-DAD-ESI-MS/MS*

The analysis was achieved as previously reported by Salazar-Orbea et al. [54] and Buendía et al. [9] with modifications. Freeze-dried samples (100 mg) were extracted with 1 mL of MeOH/ H_2O/HOAc (70:29:1, v/v/v). The samples were vortexed for 1 min and then sonicated for 30 min at room temperature. They were then centrifuged for 15 min at 20000 x g at 10 °C (Thermo Scientific ™ Sorvall™ ST 16, Germany). The supernatant was filtered through a 0.22 μm PVDF filter and three replicates were analyzed. Phenolics identification and quantification were performed on an Agilent 1100 HPLC equipment coupled in series to a photodiode array detector (G1315D) and an HCT Ultra Bruker Daltonics ion trap mass spectrometer equipped with an electrospray ionization (ESI) interface HPLC-DAD-ESI-MS/MS (Ion Trap). The chromatographic separation was completed using a Poroshell 120 EC column (3 ×100 mm, 2.7 μm) from Agilent Technologies (Waldbronn, Germany). The mobile phases used were H_2O/HCOOH (Panreac, Barcelona, Spain) 99:1 (v/v) (A) and acetonitrile (J.T. Baker, Deventer, The Netherlands) (B). The gradient was set as indicated: 0 min, 5 % B; 7 min, 18 % B; 17 min, 28 % B; 22 min, 50 % B; 27 min, 90 % B; 29–35 min, 5 % B. The flow rate, the injection volume and the column temperature were 0.5 mL/min, 10 μL, and 25 °C respectively. The UV spectra were recorded in the range of 200–600 nm. The ESI parameters included: nebulizer pressure 65 psi, dry gas flow 11 L/min, and dry gas temperature 350 °C. The capillary voltage was set at 4 kV, and spectra were acquired in the negative ionization mode in the range of m/z 100–1500 and target mass 700. Automatic MS/MS mode was applied with fragmentation amplitude 1 V and 3 no. of parents. Phenolics were classified by their UV spectra, retention time, molecular weight, and MS/MS fragmentation pattern. Their quantification was completed using 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 assess the content of ellagitannins, flavan-3-ols, hydroxycinnamic acids, anthocyanins, ellagic acid conjugates and flavonols respectively. The authentic standard of castalagin was supplied by Prof. Stephan Quideau (ISM, University of Bordeaux, France), while the others standards (catechin, p-coumaric acid, pelargonidin, ellagic acid, and quercetin) were purchased from Sigma-Aldrich.

2.11. *VOC profiling*

VOC analysis was adapted from the methodology described by Vandendriessche et al. [66]. For each treatment, three replicate strawberries were sampled. Green parts were removed from the fruit, which were then cut into small pieces. Subsequently the tissue was homogenized with 0.5 mL of 1.0 M NaCl per gram of fruit. The resulting blend was transferred to 15 mL Falcon tubes, snap-frozen in liquid nitrogen, and stored at −80 °C until VOC analysis. Prior to analysis, the samples were thawed overnight at 4 °C. A 5 g aliquot of the juice mixture was transferred to a 20 mL headspace vial (Filter Service, Belgium), flushed with filtered air, and sealed. For solid-phase microextraction (SPME), the vials were incubated for 35 min at 40 °C on a heated tray. Volatiles were then extracted by exposing a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness; Supelco Inc., USA) SPME fiber to the vial's headspace for 30 min at 40 °C. The extracted aroma compounds were analyzed using an Agilent 7890 A gas chromatograph (GC) coupled with an Agilent 5975 C Network Mass Selective Detector (MS) (Agilent Technologies, USA) and a Gerstel Multi-Purpose Sampler MPS 2 (Germany). Following extraction, the compounds were thermally desorbed into the injector, which was heated to 250 °C and equipped with a SPME liner (0.75 mm i.d.; Supelco Inc., USA). Splitless injection was conducted for 0.5 min at a flow rate of 50 mL/min, with a 10-min fiber thermal conditioning. Separation of volatiles was achieved using a 30 m x 250 μm x 0.250 μm HP-5MS column (Agilent Technologies), with helium as the carrier gas at a flow rate of 1.2 mL/min. The GC temperature program was as follows: an initial hold at 35°C for 5 min, followed by a ramp to 150 °C at 4 °C/min, then a second ramp to 240 °C at 50 °C/min, with a final hold at 240 °C for 5 min. The total run time was 40.55 min. The mass spectrometer scanned the m/z range from 30 to 350, with the ion source and quadrupole set to 230 °C and 150 °C, respectively. Chromatographic and spectral data were processed using MassHunter Workstation (Unknowns Analysis v10.1, Agilent Technologies). Identification of volatile compounds was carried out using the NIST 2020 database (NIST20, NIST, USA), with the compounds listed in Supplementary Table 2.

2.12. *RNA isolation and quantitative real-time PCR (RT-qPCR)*

Total RNA was extracted for all treatments in triplicate, using 100 mg strawberry fruit material, as reported in [25]. Total RNA was then treated with recombinant DNase I (RNase-free) (Cat. No. 2270 A, Takara Bio Inc.), in order to remove gDNA. For first-strand cDNA synthesis, 0.5 μg of total RNA from each sample was transcribed into cDNA using the PrimeScript™ RT reagent Kit (Perfect Real Time) following the manufacturer's instructions (Takara Bio, Japan). Expression levels were analyzed in a Biorad IQ5 real-time PCR cycler (Biorad, USA). In total, three biological replicates were performed for each treatment. RT-qPCR was carried out in a final volume of 10 μL, containing 4 μL 5-fold diluted first-strand cDNA, 0.5 μl each gene-specific primer (10 pmol/μL) and 5 μL 2x master mix (KAPA SYBR® FAST qPCR Kit, Kapa-Biosystems). Reaction conditions were initial denaturation 95 °C for 5 min, followed by 40 cycles (95 °C for 30 s, annealing temperature (Ta °C) for 30 s, and 72 °C for 30 s) and a final elongation stage at 72 °C for 5 min. The amplification cycle was followed by a melting curve run, with 61 cycles with 0.5 °C increments between 65 and 95 °C. The primer information related to allergen-related genes and melatonin biosynthetic genes (50 and 58 °C) are shown in Supplementary Table 3. Strawberry *GAPDH* and *ACTIN* were used as housekeeping reference genes. Relative quantification and statistical analysis of gene expression levels using the pairwise fixed reallocation randomization test were carried out using the REST-XL software according to Pfaffl et al. [52]. Heatmaps were created using ClustVis 2.0 according to Metsalu and Vilo [42].

2.13. *Statistical analysis*

For quality attributes, statistical analysis was performed with SPSS v24.0 (SPSS Inc., Chicago, IL, United States), using one-way ANOVA analysis and then Duncan's multiple range test at significance level 5 % ($p \leq 0.05$). For biochemical analysis, one-way ANOVA analysis was performed, followed by Tukey-HSD post hoc test ($P \leq 0.05$). Figures were prepared using GraphPad version 9.4.0 (GraphPad Software, San Diego, CA, USA). For phytochemical analysis, both statistical analysis (one-way ANOVA analysis was conducted including Tukey-HSD post hoc test ($p \leq 0.05$) and plot designs were conducted using GraphPad version 10.1.0 (GraphPad Software, San Diego, CA, USA). For the VOC-data, multivariate statistical analysis, PCA, PLS and VIP analysis, was done using JMP-Pro (v17, SAS Institute Inc., Cary, NC).

3. Results

3.1. The effect of priming agents on quality attributes

As far as the color parameters are concerned, a brighter color, corresponding to higher L values, was recorded in sodium alginate-treated strawberries compared with the other treatments (Supplementary Table 1). Less intense red colour, evidenced by the lower a* values, was recorded in strawberries treated with sodium alginate, both alone and functionalized by melatonin. Strawberries treated with the conjugate Mel-sodium alginate had lower b*, C and h values compared with the other treatments, but higher a*/b* values compared with fruit treated with sodium alginate and Put (Supplementary Table 1). The pre-harvest application of different priming agents showed differences regarding FF. In particular, putrescine-treated strawberries were characterized by higher firmness values compared with the water-sprayed and melatonin applications. However, there were no significant differences in volume, weight, SSC, TA and RI (Table 1).

While no differences in SSC were determined, variations in total soluble sugars and sucrose were recorded (Fig. 2). In particular, application of Mel-NaA induced the highest concentration in TSS ($p < 0.01$) compared with the NaA treatment. However, no significant differences were observed between Mel and Mel-NaA treatment as well as between Mel and NaA. Moreover, the Put treatment also had a significant increase ($p < 0.05$) in TSS content compared with NaA. Regarding the sucrose content, Mel and Mel-NaA treatments had the highest sucrose content compared with the water-sprayed treatment. Furthermore, even though Mel and Mel-NaA induced similar levels in sucrose content, they both showed a significant ($p < 0.05$) increase of sucrose content compared with NaA. Putrescine treatment did not show any significant effect on the sucrose content (Fig. 2).

3.2. The effect of priming agents on phenolics

The pre-harvest application of the priming agents had a notable effect on strawberries' total flavonoid content; In particular, total flavonoids showed higher concentration in Mel-NaA and Put ($p < 0.01$), as well as in NaA-treated fruit ($p < 0.05$) compared with water-sprayed ones (Fig. 3**D**). On the other hand, no differences in the contents of total phenolics, reduced ascorbic acid, and total anthocyanins were observed (Fig. 3**A,B,C**). The highest total flavonoid content was observed in fruits treated with Put, compared with the control. Additionally, Mel alone did not affect flavonoid content; however, when NaA was used as a carrier for Mel in the form of functionalized alginate-melatonin treatment (Mel-NaA), a significant increase in total flavonol content was observed (Fig. 3**D**).

3.3. The effect of priming agents on stress markers

None of the treatments applied resulted in significant changes in malondialdehyde (MDA) and H_2O_2 contents, which are commonly used stress markers indicative of potential oxidative stress damage (Supplementary Figure 1), thus suggesting that the priming treatments did not result in cellular damage effects in fruit.

3.4. The effect of priming agents on endogenous melatonin and polyamine contents

No significant differences were observed in free polyamine contents (Fig. 4**A,B,C**). However, the highest melatonin levels in fruit were observed in samples treated with Mel-NaA followed by Put. Interestingly, the application of Mel alone did not increase melatonin content within the fruit compared with the control group. However, when functionalized alginate was used as a delivery system for melatonin, there was a significant enhancement in endogenous melatonin levels in the fruit (Fig. 4**D**).

3.5. The effect of priming agents on individual polyphenolic compounds

The phytochemical analysis revealed that pre-harvest treatments differentially regulated the content of several polyphenolic compounds (ellagitannins, flavan-3-ols, hydroxycinnamic acids, ellagic acid and conjugates) (Fig. 5). There are two isomers of pedunculagin (isomer 1 and 2) with same mass and same structure but different stereochemistry in the hydroxyl at C-1 of glucose. NaA resulted in the lowest amount of Bis-HHDP-glucose (Pedunculagin)_isomer 1 compared with the water-sprayed treatment, whereas the rest of the treated fruit had similar Bis-HHDP-glucose (Pedunculagin)_isomer 1 content to water-sprayed fruit and NaA ones (Fig. 5**A,B**). Regarding the content in Bis-HHDP-glucose (Pedunculagin)_isomer 2, Mel-NaA-treated fruit registered the highest content on this compound compared with Mel, NaA and water-sprayed. However, the Put-treated fruit did not show any differences among the other treatments. Regarding the flavan-3-ols, procyanidin dimer B1 registered the highest concentration in putrescine dihydrochloride-treated fruit ($p < 0.05$) compared with water-sprayed (Fig. 5**C**). In addition, procyanidin dimer B2 registered the highest concentration in Mel-NaA ($p < 0.05$) compared with water-sprayed (Fig. 5**D**). The other treatments did not show differences when compared with the water-sprayed one. Putrescine dihydrochloride and Mel showed the highest contents on p-Coumaroyl hexose isomers 1 & _2 compared with other treatments, yet similar to water-sprayed (Fig. 5**E, F**). On the other hand, NaA and Mel-NaA showed lowest content in isomer _1 than water-sprayed. A similar pattern was observed in the contents of p-Coumaroyl hexose_2, yet with less pronounced differences. Several p-coumaroyl-glucose isomers are feasible; the metabolites found here with the same formula and mass spectra signals were detected in agreement with previous studies [9].

Significant differences were monitored with reference to ellagic acid and its conjugates (Fig. 5**G,H**). Mel-NaA had the highest content on ellagic acid rhamnoside compared with water-sprayed and the rest of the treatments. Furthermore, the same treatment positively impacted on the content of ellagic acid, compared with sodium alginate and Mel. This is also consistent with the effects of treatments on ellagitannins described above, and makes sense from the biosynthetic point of view

Table 1
The effect of pre-harvest application of NaA, Mel, Mel-NaA and Put on fruit quality attributes (Fresh fruit weight (g), volume (mL), Soluble solids content (SSC, °Brix), Titratable acidity (TA, % citric acid) and Flesh firmness, N) of strawberry fruits (cv. 'Felicity Q3').

	Weight (g)	Volume (mL)	SSC (ºBrix)	TA (% citric acid)	RI (SSC/TA)	Force (N)
Water-sprayed	19.39 ± 1.32 a	22.07 ± 1.63 a	9.30 ± 0.23 b	0.99 ± 0.03 a	9.45 ± 0.5 a	2.34 ± 0.1 bc
Sodium alginate (NaA)	17.84 ± 0.93 a	20.90 ± 1.08 a	9.23 ± 0.12 b	1.00 ± 0.04 a	9.28 ± 0.25 a	2.63 ± 0.12 ab
Melatonin (Mel)	18.86 ± 1.12 a	22.33 ± 1.47 a	9.47 ± 0.09 ab	1.04 ± 0.02 a	9.13 ± 0.18 a	2.36 ± 0.1 bc
Sodium alginate/Melatonin (Mel-NaA)	17.51 ± 1.65 a	20.56 ± 1.96 a	9.33 ± 0.03 b	1.05 ± 0.04 a	8.89 ± 0.29 a	2.44 ± 0.11 abc
Putrescine dihydrochloride (Put)	17.43 ± 1.28 a	19.63 ± 1.59 a	9.50 ± 0.12 ab	1.01 ± 0.05 a	9.41 ± 0.4 a	2.72 ± 0.12 a

Each treatment measurements in a row: Water-sprayed= Water + Tween 20, NaA= Sodium Alginate + Tween 20, Mel= Melatonin + Tween 20, Mel-NaA = Melatonin + Sodium Alginate + Tween 20, Put = Put dihydrochloride +Tween 20). Data represent the mean (n = 30 fruit) ± SE. Different letters are significantly different ($p \leq 0.05$) within each column.

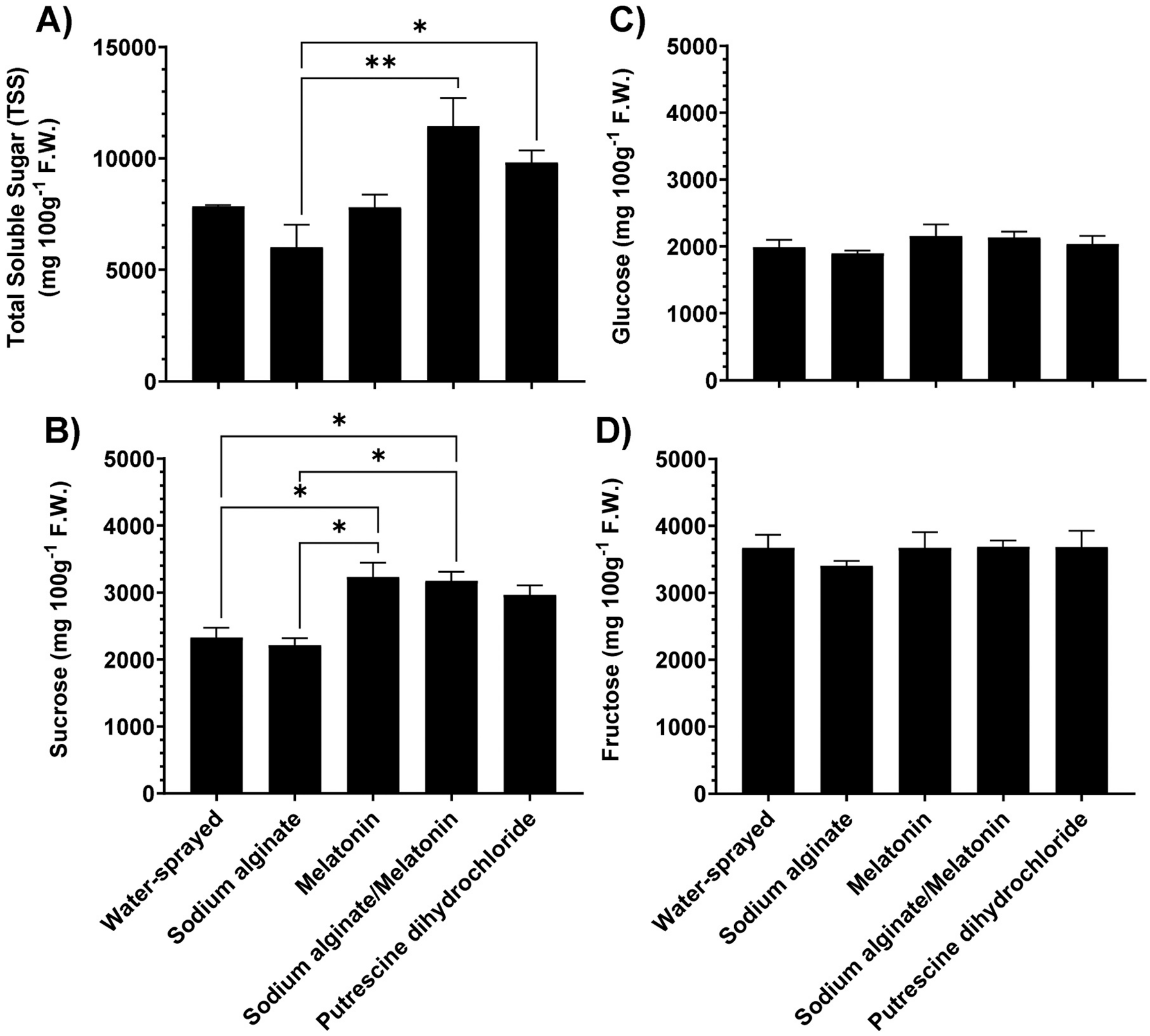

Fig. 2. The effect of pre-harvest application of Mel, NaA and Put on total soluble sugars (TSS) (A), sucrose (B), glucose (C), fructose (D) at fully ripe strawberry fruits (cv. 'Felicity Q3').

and with similar biological implications.

3.6. The effect of priming agents on VOC profile

A total of 118 VOCs were identified and 'quantified' in terms of their relative peak area to account for SPME fiber variations (Supplementary Table 2). The PCA score plot revealed a variation between replicate fruit as large as the variation between the different treatments, showing no clear separation between the treatments (Supplementary Figure 2). The PLS-DA analyses using the VOCs as X-variables and priming treatment as the Y-response was not able to establish robust prediction models for any of the treatments. These results suggest that the VOC profile was not systematically affected by the imposed priming treatments.

3.7. The effect of priming agents on allergenic protein and Mel biosynthesis gene expression

The expression levels of selected genes involved in the biosynthesis of known strawberry allergenic proteins and melatonin (Allergenic proteins: *Fra a2, Fra a3, Fra a1-A, Fra LPT46, Fra A4* Melatonin: *Fra TDC, Fra TSH, Fra SNAT, and Fra ASMT*) were evaluated following treatment with different priming agents (Fig. 6; Supplementary Figure 3; Supplementary Tables 4,**5**). Of the treatments, NaA generally caused moderate changes in gene expression, leading to the up-regulation of *Fra a3* and *Fra TDC*, with a significant fold increase for *Fra a3* (4.891 fold-increase; $p = 0.0010$) (Supplementary Table 4). On the other hand, other genes, including *Fra a2*, *Fra a1-A*, *Fra A4*, and *Fra ASMT*, were predominantly down-regulated ($p < 0.05$), whereas the expression changes for other genes (e.g., *Fra LPT46*, *Fra SNAT*) were not statistically significant in the case of NaA (Fig. 6). In the case of Mel treatments, *Fra a3* was up-regulated (25.670-fold, $p = 0.0375$), while a significant down-regulation effect of melatonin on *Fra TDC* (-16.145-fold) and *Fra ASMT* (-16.011-fold, $p = 0.0010$) was further noted (Supplementary Table 4).

In the case of conjugation of Mel-NaA, significant up-regulation of *Fra a3* (14.774-fold, $p = 0.0010$) was achieved. However, the combination strongly down-regulated genes such as *Fra TDC* (-8.821-fold, $p = 0.0010$) and *Fra ASMT* (-9.553-fold, $p = 0.0010$) (Supplementary Table 4). The application of Put resulted in a pronounced elevation in the expression of *Fra a3* (24.710-fold, $p = 0.0345$) and a moderate increase in the expression of *Fra SNAT* (1.574-fold, not significant). Additionally, it significantly down-regulated *Fra TDC* (-4.976-fold, $p = 0.0010$) and *Fra ASMT* (-3.169-fold, $p = 0.0010$), while the changes for other genes were relatively mild (Supplementary Table 4).

Overall, the statistical analysis confirmed the significance of expression changes, particularly for *Fra a3*, *Fra TDC*, and *Fra ASMT*, in response to specific treatments. Notably, Mel and Put treatments resulted in significant upregulation for *Fra a3* ($p < 0.05$; Supplementary

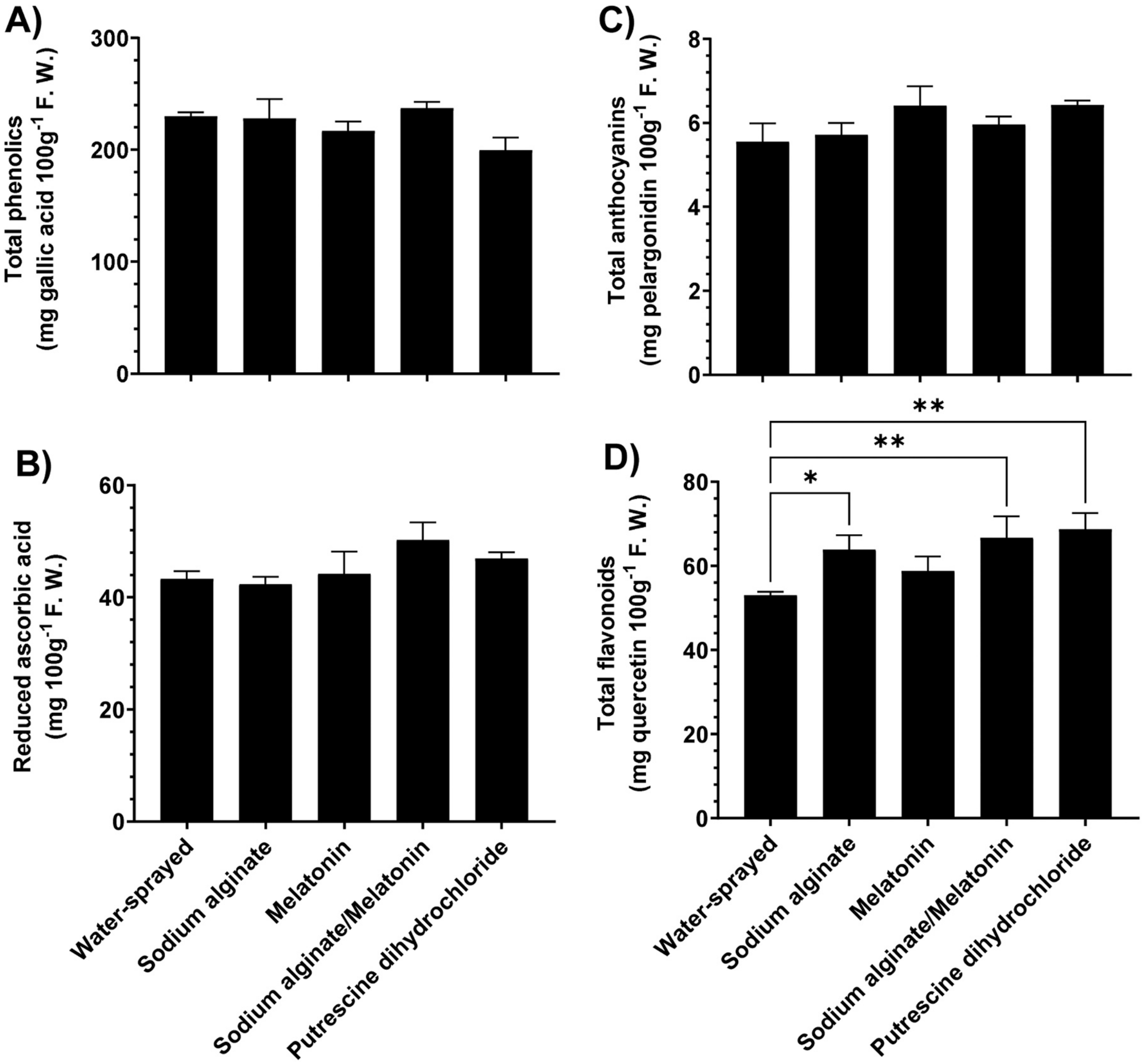

Fig. 3. The effect of pre-harvest application of Mel, NaA, Mel-NaA, and Put on total phenolic (A), reduced ascorbic acid (B), total anthocyanin (C) and total flavonoid (D) contents of strawberry fruit (cv. ‘Felicity Q3’).

Table 4), while Mel and Mel-NaA resulted in significant downregulation for *Fra TDC* and *Fra ASMT* ($p < 0.001$; Supplementary Table 4). As noted above as well, Mel and Put showed significant upregulation of *Fra a3*, with Mel exhibiting the highest expression increase for *Fra a3* (25.670-fold). In contrast, NaA and Mel-NaA treatments demonstrated moderate expression changes across most genes (Fig. 6).

4. Discussion

Enhancing fruit quality attributes by employing sustainable practices is one of the highest priorities for horticultural products. Exogenous application of plant growth regulators has been widely investigated in sustainable agricultural production and among them, natural compounds (e.g., Mel, Put) delivered by nanocarriers (e.g., alginate) are a promising approach to be used as priming agents in strawberry cultivation to enhance fruit quality and polyphenol profile [45]. Such approaches have been mainly studied at the post-harvest level. Revelant studies [36,35] demonstrated that functionalizing chitosan nanoparticles with glycine betaine and arginine chitosan can serve as an effective carrier for delivering such compounds as a fruit coating treatment at postharvest level that led to enhanced plum fruit quality attributes but also to extended shelf-life during cold storage conditions. The aim of this study was to investigate the efficacy of such nanocarriers and evaluate their potential as a sustainable approach to improve strawberry fruit quality at pre-harvest level.

Visual quality is one of the most important parameters consumers assess when buying strawberry fruits; therefore, color value was the first quality attribute to be taken into account. NaA- treated strawberries showed higher L* values which indicate brighter fruit and hence more visually attractive [55]. Strawberries treated with Mel-NaA were characterised by lower b*,C and h and higher a*/b* values. Lower hue angle (h) and chroma (C) values are linked with more intense red color and lower color saturation, respectively [67]. Such sensory changes may increase the attractiveness to consumers. Even though chromaticity parameters linked to red color have been correlated with the anthocyanin content [67]; it is not the case in results reported herein. The co-occurrence of organic acids, pH and the occurrence of co-pigments can affect the final strawberry color perceived although the anthocyanin content can be similar.

Another important attribute for strawberries is flesh firmness as it affects the resistance to handling, storage life and the overall marketability of the strawberry and is influenced by both the cultivar and other factors (ripening stage, fruit size, temperature, etc.). Increased fruit firmness is connected to reduced post-harvest losses and extended shelf-life. In our study, fruit treated with Put were firmer than those subjected to other priming treatments. This comes in accordance with the findings

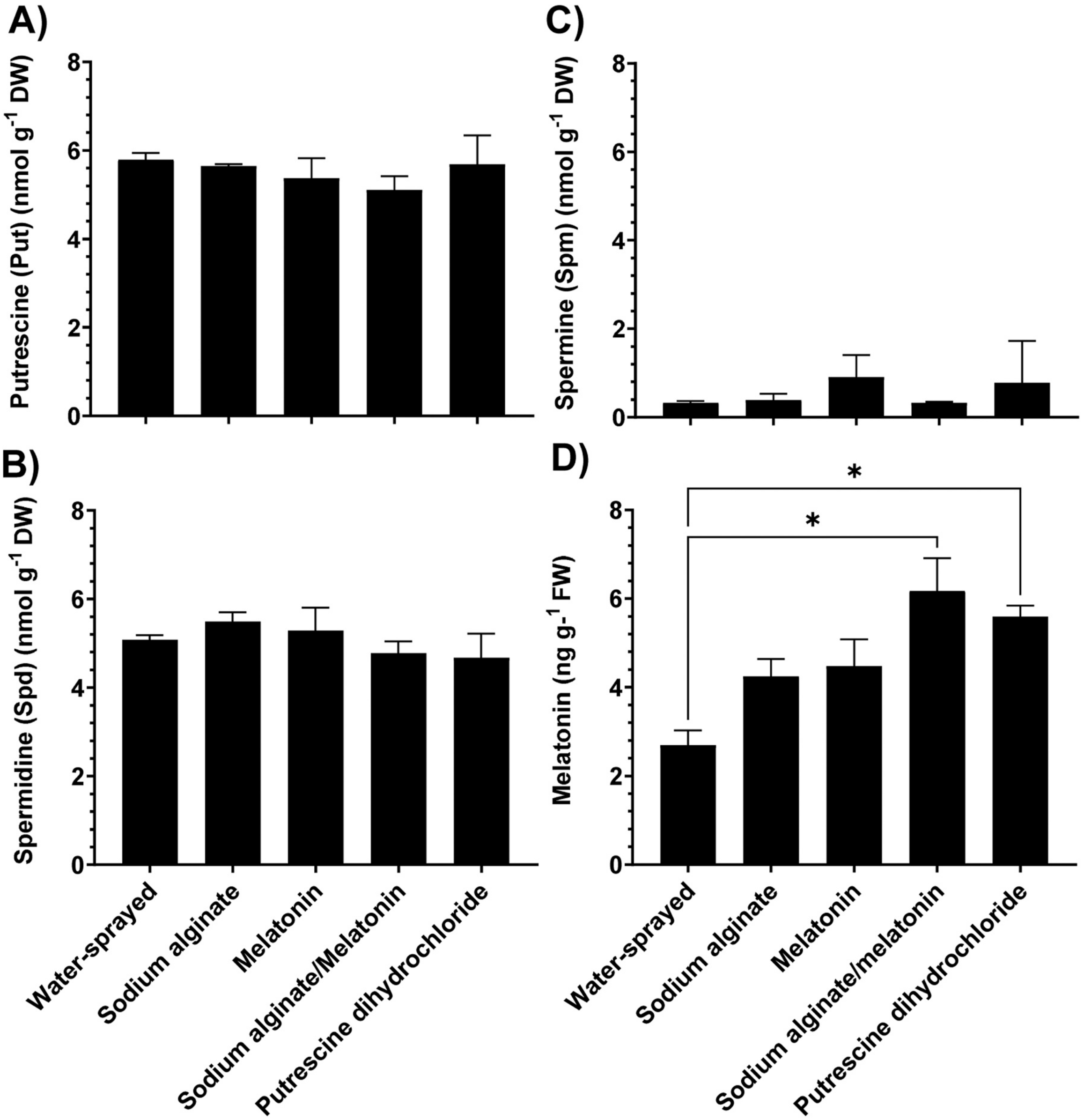

Fig. 4. The effect of pre-harvest application of Mel, NaA, Mel-NaA and Put on endogenous content of putrescine (A), spermidine (B), spermine (C) and melatonin (D) of strawberry fruits at fully ripe stage (cv. 'Felicity Q3').

of studies in other fruit crops, where pre-harvest Put application maintained the fruit firmness of pear fruit [60]. The exogenous application of Put on strawberry fruit has been reported to delay fruit ripening, which corresponded with changes in multiple physiological parameters such as firmness, anthocyanin, sugar and polyamine contents, among others [21]. To conclude that putrescine treatment has a beneficial effect on strawberry firmness retention, further studies are needed that will have a considerable bigger pool of fruits examined and will additionally consider the fruit size and weight effect as smaller fruit tend to be denser and firmer.

Sweeter fruits are usually more desirable to consumers and taste is highly depended on sugar content. Pre-harvest treatments Mel-NaN on strawberries had an overall significant effect on the total soluble sugar (TSS) and sucrose. Melatonin could regulate carbohydrate metabolism and support the plant's osmoregulatory response, as critical function during stressful conditions [5]. Several reports have shown that melatonin application can influence sugar metabolism by altering the expression of sugar transporter genes and enzymes involved in carbohydrate metabolism and TSS accumulation in different horticultural crops like apple [73], pear [32], and peach [77].

Ascorbic acid, the well-known Vitamin C, is a water-soluble micronutrient that is not synthesised by the human body and is obtained through the diet, primarily from fruits and vegetables. Amongst the fruits, strawberries are considered an excellent source of *L*-ascorbic acid [47]. Previous studies have reported that the exogenous application of melatonin has been shown to increase ascorbic acid content in apple leaves [69], while Okatan et al. [48] reported higher levels of ascorbic acid in four strawberry cultivars after pre-harvest exogenous melatonin application. In addition, Li et al. [31] showed that the use of edible coatings, including alginate, maintained the increased ascorbic acid content of strawberry fruit under storage conditions. However, although Mel-NaA treatment resulted in an increased content of ascorbic acid, it was not statistically significant compared with the other treatments.

Secondary metabolites, particularly polyphenols are responsible for increased quality of vegetables and fruits, including color, flavor, firmness, and bitterness, contributing at the same time to the antioxidant capacity and plant defense mechanisms [61]. Anthocyanins are pigments found in many vegetables, fruits and flowers, with a health

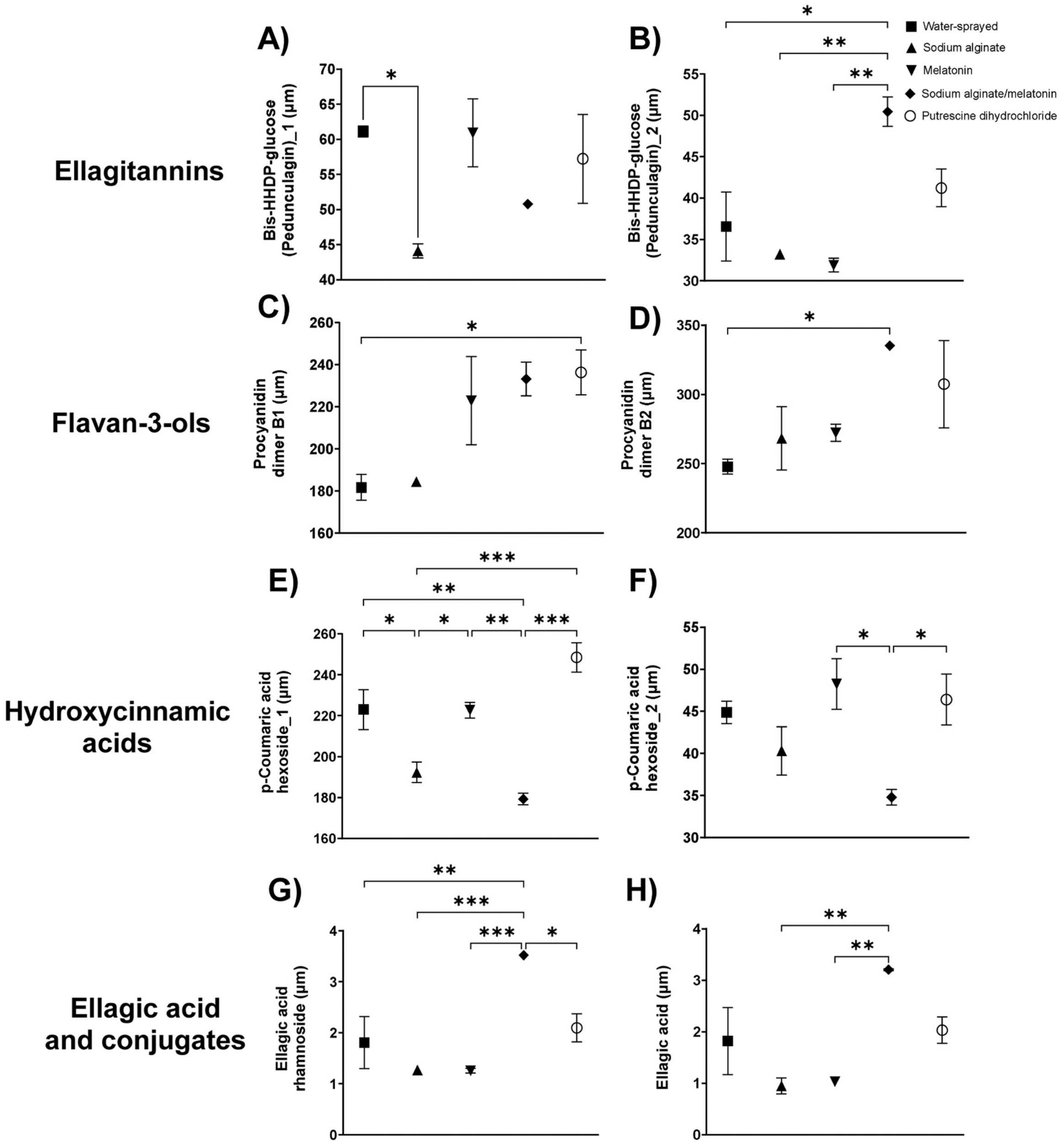

Fig. 5. The effect of pre-harvest application of Mel, NaA and Put on polyphenolic compounds of classes Ellagitannins (A, B), Flavan-3-ols (C, D), Hydroxycinnamic acids (E, F) and Ellagic acid and conjugates (G, H) of an early-harvested strawberry cultivar (Fragaria x ananassa cv. 'Felicity Q3').

beneficial role to plant and human health. Strawberries are a rich source of anthocyanins and numerous reports have focused on the quantification of their content [37]. In the current study, fruit treated with Put did not reveal a meaningful increase of total anthocyanin content compared with the other priming strategies (NaA alone or in combination with Mel). To our knowledge, no studies have been found to directly connect the increased anthocyanin content to pre-harvest application of Put, while its postharvest application has been reported to maintain the total anthocyanins in high levels under storage conditions [6].

Melatonin, spermidine, and putrescine can upregulate transcript levels of antioxidant enzymes in plants [68,76] and improve the quality of fruit, elevating at the same time the number of beneficial substances, like sucrose, polyphenols, and antioxidant compounds [70,72]. In the present study, melatonin alone or conjugated with sodium alginate were found to regulate polyphenol metabolism, as they increased the concentration of an important number of polyphenols, such as ellagitannins, flavan-3-ols, hydroxycinnamic acid, ellagic acid and their conjugates. Particularly, Mel-NaA-treated fruit significantly impacted Bis-HHDP-glucose (Pedunculagin)_isomer 2 content compared to other treatments. Pedunculagin has two isomers (isomer 1 and 2) with same mass and same structure but different stereochemistry in the hydroxyl at C-1 of glucose which were both present in treated fruits of these study. These type of ellagitannins have been associated with the defense of strawberries against plant pathogens [20]. Xu et al. [71] reported results that are in accordance with current findings, as they indicated an increase in the content of total phenols, flavonoids, and proanthocyanidins of grape berries, after the exogenous application of melatonin. In specific, they found that melatonin treatment significantly increases the contents of non-flavonoid compounds such as coumaric acid [71]. Ellagitannins and ellagic acid derivatives were significantly enhanced with some of the priming treatments applied (Mel-NaA). These could impact the strawberry taste, as these polyphenols can impart astringent or mouth feeling sensations, and some of these are part of the characteristic strawberry fruit flavor [56]. In addition, these phenolics can

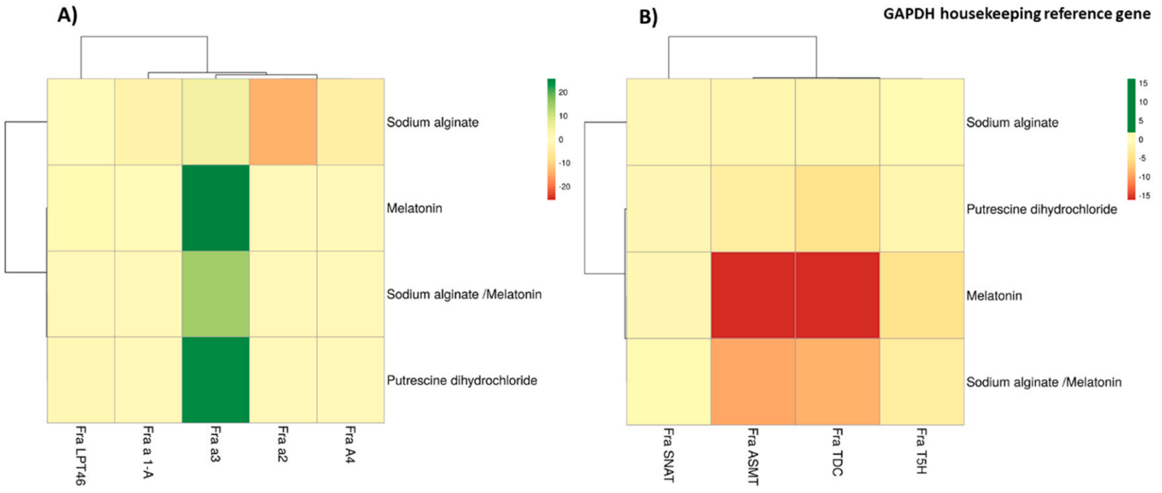

Fig. 6. Heat map of the relative expression levels of A) allergen-related genes (*Fra a2, Fra a3, Fra a 1-A, Fra LPT46* and *Fra A4*) and B) melatonin biosynthetic genes (*Fra TDC, Fra T5H, Fra SNAT* and *Fra ASMT)* on pre-harvest application of Mel, NaA, Mel-NaA and Put of strawberry fruits at fully ripe stage (cv. 'Felicity Q3') (n = 3). Relative mRNA abundance was evaluated by real-time RT-qPCR using three biological repeats. Up-regulation is indicated in green; down-regulation is indicated in red. A scale of color intensity is presented as a legend. Control (water-sprayed) samples and *GAPDH* housekeeping reference gene were used for calibrating gene expression values. Actual relative expression levels are shown in Supplementary Table 4.

have protective effects against the development of plant pathogens (*Pseudomonas*); therefore, the treatments can have beneficial effects during strawberry development [64].

Interestingly, priming treatments did not result in the differential regulation of VOC compounds constituting aroma profile in comparison with water-sprayed control plants, suggesting that primed fruit retain their aroma quality with no aroma profile 'penalty', as seen in other cases such as in grapevine plants sprayed with swelling agent CPPU that resulted in the decreased content of several VOCs such as hexanal, phenyl ethanol, damascenone and linalool [53].

Current findings revealed that strawberry fruit treated with Mel-NaA exhibited the highest levels of endogenous melatonin, suggesting that alginate may be an effective carrier to enhance melatonin's efficacy with lower dose needs. Carriers like alginate enable the controlled, sustained release of priming agents such as melatonin, which can improve the efficiency of these active compounds. These biopolymer-based carriers like alginate and chitosan represent a promising, sustainable approach in agricultural technology, providing an eco-friendly method to deliver priming agents and effectively enhance not only plant stress resilience against different abiotic stress, but also fruit quality and yield [19]. For instance, Gohari et al. [18] reported that using chitosan as a bio-polymer-based nanocarrier for delivery of melatonin significantly boosted melatonin's efficacy in spearmint plants under salinity stress.

An additional point of concern following exogenous application of priming agents by direct spraying on fruit was the potential induction of allergenic proteins. Of the five known allergenic proteins in strawberry that were examined by RT-qPCR, four did not show any increase in transcript levels, in line with a biosafe approach. The exception was *Fra a3* which encodes an allergen of fruits and belong to the *PR10* family [46]. This gene family has been well-correlated and regulated in the case of pathogen infection, exhibiting antimicrobial activities against bacteria, fungi and viruses [41]. By modifying the biosynthesis of flavonoid and anthocyanin pathways, *Fra a3* has also been shown to be involved in stress response and fruit ripening [46]. *Fra a3* was the most strongly up-regulated gene across all treatments, particularly with Mel and Put. In a similar study by Petriccione et al. [51], application of chitosan also increased the transcript level of *Fra a3* at all ripening stages. Such an increase in *Fra a3* transcript levels could indeed suggest a potential increase in allergenic risk to sensitive consumers, raising concerns. However, theoretical population-level health risk remains low unless the protein accumulates in high concentrations in the consumable part of the fruit [26], therefore rendering future protein content analyses as essential. In any case, it is important to note that *Fra a3* expression levels were lower in NaA-Mel treated samples, in comparison with Mel-treated ones, suggesting that delivery of Mel with NaA constitutes a more biosafe approach in terms of potential allergenicity effects than direct agent application.

Tryptophan decarboxylase (TDC) is an enzyme responsible for the catalysis of conversion of L-tryptophan (Trp) to tryptamine, which is a precursor of serotonin and melatonin in plants [63]. A number of reports have clearly reported the positive effects of melatonin in biosynthesis of metabolites and delayed senescence in the case of strawberries [14,33,38]. In this context, the enhanced transcript levels of *Fra TDC* is paramount with respect to melatonin biosynthesis. According to present findings, Mel and Mel-NaA treatments significantly down-regulated *Fra TDC* (-16.145-fold and −8.821-fold, respectively). Similarly, Put down-regulated *Fra TDC*, albeit to a lesser extent (-4.976-fold). *Fra ASMT* (Acetylserotonin O-Methyltransferase) is a critical enzyme in the biosynthesis of melatonin [10]. As in the case of *Fra TDC*, transcript levels of *Fra ASMT* were down-regulated by Mel, and Mel-NaA and Put, as well. Results suggest that these treatments could potentially modulate the melatonin biosynthetic pathway, redirecting metabolic fluxes towards alternative stress-response mechanisms.

5. Conclusions

Current results suggest that alginate, as a biopolymer-based carrier, may function as an effective and robust delivery system for melatonin in strawberry plants with special reference to secondary metabolism linked to fruits' phytochemical content. Ellagitannins and ellagic acid derivatives in particular were enhanced following application of the Mel-NaA conjugate. VOC profiling revealed no significant effect in aroma components following any of the priming treatments in comparison with water-sprayed fruit, suggesting no aroma 'penalty'. Overall, this approach could offer substantial benefits that can be additionally explored at post-harvest level towards fruit preservation and quality enhancement, positioning alginate-based delivery systems as a promising strategy in the development of advanced fruit coating technologies. Global transcriptomic analyses are scheduled in futured experiments in order to further evaluate the potentially positive effect of these priming agents on the quality of strawberries and to improve the understanding of their mode of action. The extent to which these promising priming agents can enhance yield efficiency beyond their beneficial effect on secondary metabolism, needs to be clarified in large scale experiments or trials under semi-commercial conditions.

Author contributions

V.F. designed the experiment. E.C.G., C.J.G., A.M.T., S.G., N.V., A.V., G.G., M.B. and R.A. performed the experiments. V.F. and G.A.M. wrote the paper. M.L.A.T.M.H and F.A.T. edited the manuscript. E.C.G. and V. F. revised the manuscript. All authors discussed and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: George Manganaris reports financial support was provided by Horizon Europe. Vasileios Fotopoulos has patent #WO/2023/099627A1 pending to Cyprus University of Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2025.100515.

Data availability

Data will be made available on request.

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