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Preharvest Multiple Applications of GABA Improve Quality Traits and Antioxidant Compounds of Pomegranate Fruit during Storage

José Manuel Lorente-Mento [1], Daniel Valero [2], Domingo Martínez-Romero [2], Fátima Badiche [2], María Serrano [1,*] and Fabián Guillén [2]

[1] Department of Applied Biology-CIAGRO, University Miguel Hernández, Carretera Beniel, km 3.2, 03312 Orihuela, Alicante, Spain; jlorente@umh.es

[2] Department of Food Technology-CIAGRO, University Miguel Hernández, Carretera. Beniel, km 3.2, 03312 Orihuela, Alicante, Spain; daniel.valero@umh.es (D.V.); dmromero@umh.es (D.M.-R.); fbadiche@umh.es (F.B.); fabian.guillen@umh.es (F.G.)

* Correspondence: m.serrano@umh.es; Tel.: +34-9-6674-9616

Abstract: GABA (γ-Aminobutyric acid) is an amino acid with diverse effects on plant development, including delayed senescence in horticultural commodities. The main goal of this research was to investigate the impact of GABA applications to pomegranate trees on the 'Mollar de Elche' quality traits and antioxidant compounds during storage for 60 days at 10 °C. In the 2019 experiment, pomegranate trees received foliar sprays of GABA at 10-, 50- or 100-mM concentrations at three stages of fruit development. Pomegranates harvested from GABA-treated trees exhibited enhanced fruit firmness, titratable acidity, the red color of skin and aril, and higher levels of total phenolics and total and specific anthocyanins compared to those obtained from the control group. These quality traits were maintained at higher levels during 60 days of preservation at 10 °C, and the most important effects were observed for 100 mM dose. In a 2020 experiment, GABA was sprayed at a 100 mM dose, and results were confirmed, showing a retard of the ripening process in pomegranates from GABA-treated trees concerning controls for two months of storage and an extension of their shelf life (with optimal quality traits appreciated by consumers) from 30 to 60 days. Moreover, the increase in anthocyanin content due to GABA treatments resulted in redder fruits, which would appeal more to consumers and have improved health benefits.

Keywords: *Punica granatum*; anthocyanins; phenolics; firmness; titratable acidity; total soluble solids; colour

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1. Introduction

Fruit from the plant species pomegranate (*Punica granatum* L.) have high-quality properties, the most important being external visual appearance and aril colour, taste, and flavour, which make this fruit very appreciated by consumers worldwide [1–3]. In addition, pomegranate fruits provide health benefits known since ancient times. However, the scientific evidence proving these beneficial effects has been reported mainly in the last few years and attributed to antioxidant compounds, primarily phenolic compounds, including anthocyanins [4–7]. In Spain, the most important pomegranate cultivar is 'Mollar de Elche', which is highly appreciated by its balanced sugars-acids ratio and its small-sized seeds with a soft and thin coat, which is imperceptible when eating. Thus, this is a cultivar with excellent flavour and taste. However, 'Mollar de Elche' skin and aril colour is not as red as in 'Wonderful' and other worldwide-known cultivars due to their low concentration of anthocyanins [1,8–11]. Then, for the 'Mollar de Elche' cultivar is difficult to achieve good prices in international markets, in which redder cultivars are valued. To solve this problem, several studies have been performed aimed at increasing skin and aril coloration. For

example, the application of deficit water treatment to pomegranate trees during the linear phase of on-tree fruit growth (in summer) led to increased aril anthocyanin content [12], as well as treatments with salicylates [13], oxalic acid [2] and methyl jasmonate [14] during critical points of on-tree pomegranate fruit development. These treatments would enhance the economic value of 'Mollar de Elche' pomegranate in markets worldwide apart from its health benefits because it is well known that anthocyanin pigments are responsible for aril colour also for their health-beneficial properties [4,5,7]. On the other hand, the quality of this fruit species decreases during preservation, primarily due to fruit weight loss and softening and to decreases in aril acid content, which leads to reduced fruit acceptability by loss in freshness and juiciness and taste changes [1,13–16].

γ-Aminobutyric acid (GABA), an amino acid that is not involved in protein synthesis, has several effects on plant development, the first ones reported being the induction of plant resistance to abiotic [17,18] and biotic stresses [18–20]. In addition, previous reports have shown that GABA postharvest treatments delay the senescence of horticultural commodities and increase fruit chilling tolerance, with additional effects on fruit quality preservation after storage at low temperatures [17,21–23]. For example, dipping treatment with GABA (in the range of 2.5–10 mM) decreased chilling injury (CI) symptoms and led to enhanced concentrations of bioactive compounds (mainly phenolics, flavonoids and anthocyanins) on cornelian fruit [21]. Accordingly, 200 µM GABA postharvest treatments of mango fruits led to higher firmness levels and increased concentrations of ascorbic acid and phenolic compounds along preservation at 15 °C [24]. In blood orange fruit, reduced weight, firmness, and acidity losses during cold storage, and lower CI symptoms, have also been reported for GABA dipping treatments, these effects being dose-dependent [25,26].

The literature regarding the treatments with GABA of pomegranate fruits is scarce. Recently, Nazoori et al. [27] have reported that GABA dipping treatments at 5, 10 and 15 mM (for 15 min) maintained firmness levels, total phenolic concentration and antioxidant activity and diminished CI along preservation at 4 °C. Accordingly, edible coating based on carnauba wax, with the addition of GABA, at 5 or 10 mM, delayed the appearance of CI symptoms and preserved quality traits in 'Malas Saveh' cultivar [28]. On the other hand, GABA treatments of the pomegranate tree, applied by foliar spray during fruit development on-tree, have been reported to enhance the yield of pomegranate crop, apart from some quality attributes of fruit, such as skin and aril colour [29]. However, our current understanding, there are no previously published reports exploring the potential impacts of pomegranate trees' GABA treatments on the evolution of pomegranate quality attributes during preservation, which was the major objective of the present research. Especial emphasis was given to the effects of these treatments on anthocyanin content to increase the commercial value of this poor-coloured 'Mollar de Elche' cultivar after two mounts of preservation at 10 °C, with additional benefits on enhancing its antioxidant properties and healthful effects.

2. Materials and Methods

2.1. Plant Material and GABA Treatments

Trees of pomegranate (*Punica granatum* L.) 'Mollar de Elche' grown on a farm settled at Elche (Alicante, Spain) were used for the experiments in the 2019 and 2020 growing cycles. Trees were 12 years old in 2019 and 13 years old in 2020, planted at 6 × 5 m and trained following a single stem pattern. Nitrogen and potassium, at 160 kg ha^{-1}, and phosphorus, at 80 kg ha^{-1}, were supplied as fertilizers. Irrigation was applied in agreement with agronomic practices for this crop species in the South-East of Spain. In the 2019 experiment, GABA treatments were applied, at 10, 50 or 100 mM, by sprinkling 3 L of GABA solutions (freshly prepared) to the tree canopy with an atomizer. Treatments were repeated at 15 days intervals, from 30 to 120 days after full blossom. The equatorial diameter of the fruit at the first treatment was 5–6 cm.

GABA (Sigma-Aldrich, Madrid, Spain) freshly prepared solutions in tap water before application and contained 0.5% Tween 20. Tap water with Tween 20 at 0.5% was sprayed

to control trees. Three replicates were used for each treatment, composed of three trees. Fruits were harvested at the maturation stage in accordance with commercial practices, based on fruit equatorial diameter, skin colour and appropriate content of total soluble solids (ca. 14 °Brix) for this cultivar. Thus, fruits with these commercial criteria were harvested from all the experimental trees on 15 October 2019 and 8 October 2020 for storage experiments. Fifteen pomegranates were gathered randomly from each experimental group and delivered to the laboratory in two hours. Then, three lots (or replicates) of five fruits were performed randomly and stored at 10 °C for 0, 30 and 60 days. After that, fruit quality properties and bioactive compounds were measured. GABA treatments at 100 mM yielded the best results according to the highest maintenance of pomegranate fruit quality traits from harvest to the end of storage for 60 days observed for 100 mM GABA treatment. Then, this concentration was chosen to be applied in the 2020 experiment, performed in the same commercial field, and as previously described.

2.2. Fruit Quality Parameter Measures

Weight loss was measured independently in each fruit. Each fruit was weighed before and after the preservation period, and the loss of fruit weight was quantified (in percentage) based on the weight at harvest (day 0). Skin colour was also measured in individual fruits using a colorimeter, CRC-400-Konica-Minolta model (Minolta Camera Company, Tokyo, Japan). The colour was expressed in CIELab-coordinates. Three colour measures were performed on equidistant points of the fruit perimeter. The firmness of the fruit was measured with a Texture Analyser (TA.XT plus-model, from Stable Microsystems Company, Godalming, UK). The system measured the required force (N) to achieve a 5% deformation of fruit diameter and the covered distance (mm) and force/distance (N mm^{-1}) was utilized as the unit for presenting the results. After that, fruits were cut in two halves, and the colour of the arils was measured on the cut surface at three equidistant points with the colorimeter described above. Then, the arils of the five fruits of each of the three replicates were mixed to obtain a uniform sample from which ca. 50 g were pressed throughout two layers of cotton fabric to get a juice sample. Total soluble solid concentration (TSS) was measured in the juice (in duplicate and at 20 °C) by means of an Atago PR-101 model refractometer (Atago Co., Ltd., Tokyo, Japan). TA (titratable acidity) was measured in the same juice (also in duplicate), by titration, up to pH 8.1, with 0.1 N NaOH by mean of a 785-DMP-Titrino-titrator-model (Metrohm, Herisau, Switzerland), as previously reported [29]. Results for all the above-measured parameters were expressed as the mean ± standard error (SE) of three replicates. Another sample of ca. 50 g of arils for each sample was frozen and grown under liquid-N_2 and kept at −20 °C until total anthocyanins and phenolics and individual anthocyanins were measured.

2.3. Total Phenolic, Total Anthocyanin and Individual Anthocyanin Measures

The extraction of total phenolics was performed by homogenisation of 10 g of arils with 30 mL of an extraction agent containing 80% of water and 20% of methanol and NaF at 2 mM (to limit the activity of polyphenol oxidase and avoid degradation of phenolics), for 30 s, by using an Ultraturrax homogenizer (T18-basic model, from IKA company, Berlin, Germany). Then, the extracts were submitted to a centrifugation process for 10 min at 10,000× *g* and at 4 °C. Total phenolic content was measured, in duplicate, in the supernatant according to García-Pastor et al. [14]. In short, 50 µL of the extract was added to 2.5 mL of the reagent Folin-Ciocalteau, previously diluted in water (1:10). The sample was left at 20 °C for 2 min and thereafter, 2 mL of Na_2CO_3 were incorporated, and this mixture was vigorously shaken and left, for 20 min, into a bath of water at a temperature of 50 °C. The absorbance was recorded at 760 nm in a Shimadzu spectrophotometer (UV-Vis-1900i model, Duisburg, Germany). Total phenolic concentration was calculated according to a calibration curve previously obtained with gallic acid as standard. Results (mean ± SE) are reported as mg of gallic acid equivalents per 100 g of fresh weight (FW). To extract total anthocyanins, 10 g of arils were homogenized as above in 30 mL of a mixture of 24% water,

25% methanol and 1% formic acid. Then, the extracts were submitted to centrifugation at 10,000× *g* at a temperature of 4 °C for 10 min. The concentration of total anthocyanins was measured in the supernatant in duplicate by reading the absorbance at 520 nm in the same spectrophotometer above. Results are expressed as mg 100 g^{-1} of cyn 3-gluc equivalents in an FW basis, according to the formula (Ab × D × V × 100 × 449.2)/(W × 23,900), where Ab is the absorbance, D and V are dilution factor and extract volume, respectively, 449.2 is the cyn 3-gluc molecular weight, W is the sample weight, and 23,900 is the coefficient of molar absorption (L cm^{-1} mol^{-1}) of this anthocyanin. These extracts were also used to quantify individual anthocyanins after filtering through a polyvinylidene fluoride filter (Millipore, Millex HV13 model from Bedford Industries, Burlington, MA, USA) of 0.45 µm. Twenty µL of the filtered extracts were injected into an HPLC system (Agilent (Santa Clara, CA, USA), 1200-Infinity series) equipped with a C18-security guard cartridge (4.0 × 3.0 mm) from Phenomenex (Macclesfield, UK) and a Luna C18 column (25 cm of length and 0.46 cm of i.d., with a particle size of 5 µm, also from Phenomenex). Two mobile phases were used: A, water-formic acid at 95–5% v-v and B, acetonitrile, running at a 1 mL min^{-1} flow rate with the gradient conditions previously described [14]. Chromatograms at 520 nm were recorded, and anthocyanin quantification was carried out according to calibration curves performed with pelargonidin 3-*O*-glucoside (pelg-3-gluc), cyanidin 3,5-*O*-di-glucoside (cyn 3,5-digluc) and cyanidin 3-*O*-glucoside (cyn-3-gluc), purchased from Sigma-Aldrich (Darmstadt, Germany). The quantification of delphinidin 3-*O*-glucoside (delph-3-gluc) was performed using cyn 3-gluc equivalents. The results, presented as mg 100 g^{-1}, were obtained from the mean ± SE of three biological replicates.

2.4. Statistical Analysis

For the 2019 experiment, treatment as the factor was used to perform an ANOVA analysis of variance, for which version 20 of the SPSS software was used (SPSS Inc., Chicago, IL, USA). Tukey's test was employed to compare the means and identify any significant differences between treatments at a significance level of $p < 0.05$. A Student's *t*-test was conducted to ascertain the significance of differences between the control and treated fruit at $p < 0.05$ for the 2020 experiment. The mean ± SE (standard error) of three replicates was used to express the data for both experiments.

3. Results

3.1. The Influence of GABA Treatments on the Physicochemical Quality Parameters of Fruit

Weight loss percentages increased from day 0 to day 60 of storage in pomegranate fruits, reaching 12.25 ± 0.55 and 13.07 ± 1.06% at day 60 in control fruits for 2019 and 2020 experiments, respectively. However, GABA treatments led to significantly lower weight losses than controls, with the best effects being found for 100 mM dose, with reductions of 44 and 30% after 60 days of storage in 2019 and 2020 experiments, respectively (Figure 1A,B). Fruit firmness at the time of harvest was increased significantly ($p < 0.05$) by 50- and 100-mM GABA treatments in the 2019 experiment. Values decreased sharply during storage, although it is worth noting that fruit firmness was significantly ($p < 0.05$) higher in fruit from GABA-treated trees than in fruit from control ones, the effects being dose-dependent (Figure 2A). Accordingly, fruit firmness was found to be significantly ($p < 0.05$) higher, either at harvest or during storage, in pomegranate from 100 mM GABA-treated trees than in controls in the 2020 experiment, with final vales of 20.35 ± 0.83 and 14.04 ± 0.81 N mm^{-1}, respectively (Figure 2B).

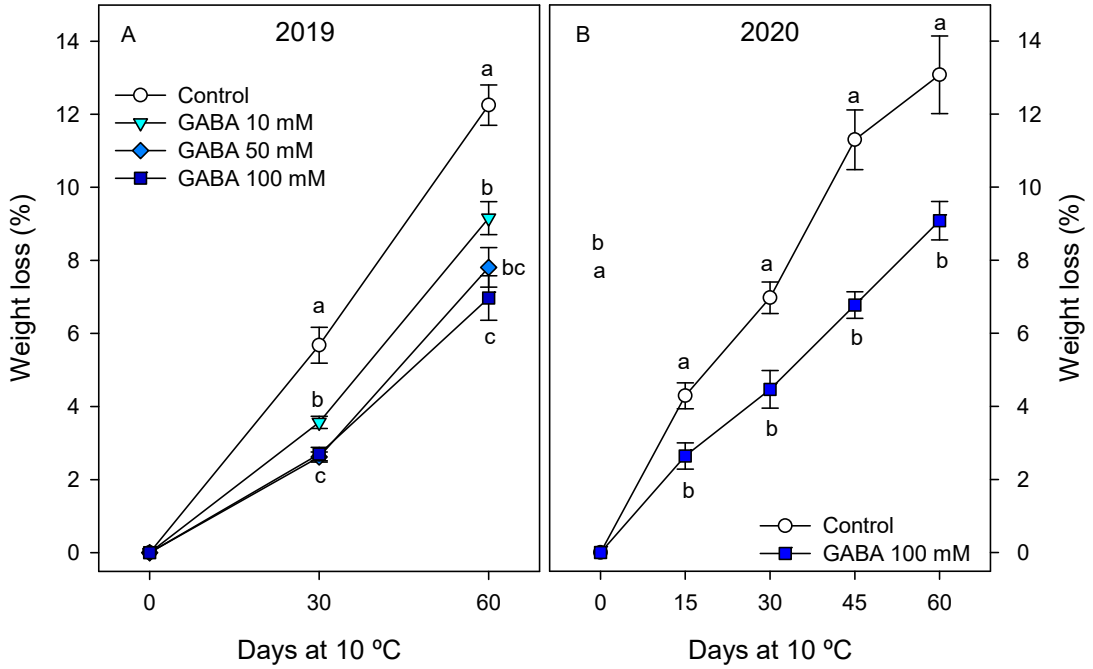

Figure 1. Weight loss of pomegranate fruits from control and 10, 50 and 100 mM γ-aminobutyric acid (GABA) treated trees along preservation at 10 °C in 2019 (**A**). From control and 100 mM GABA-treated trees in 2020 (**B**). Data are provided as the mean ± standard error. Different letters denote significant differences ($p < 0.05$) between treatments for each sampling date.

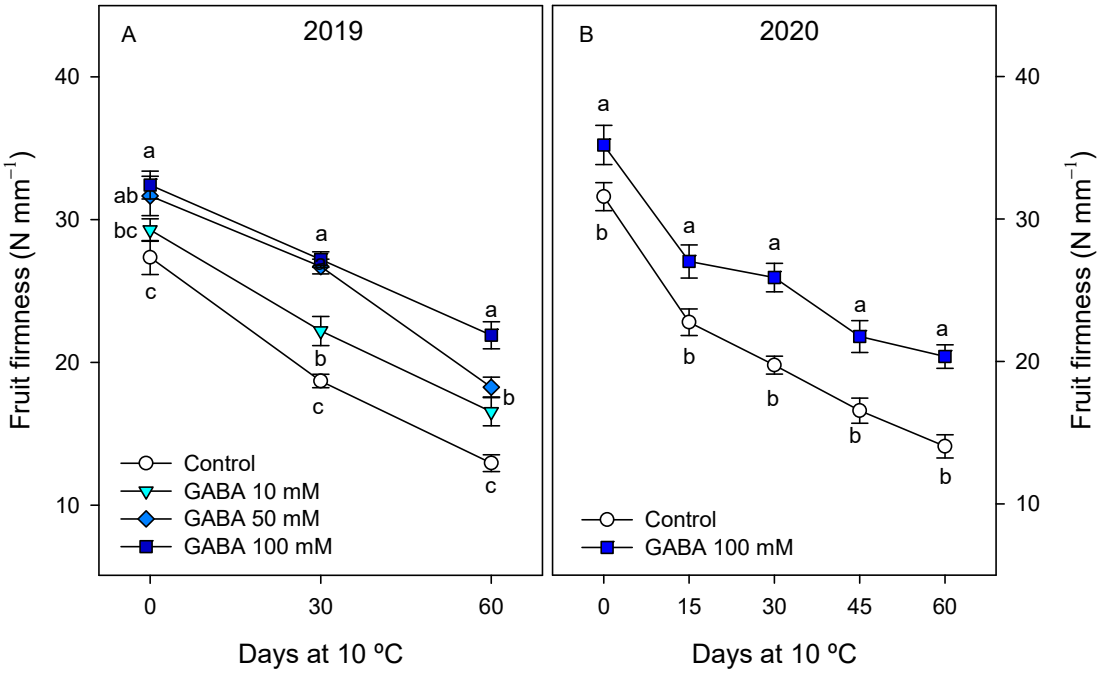

Figure 2. The firmness of pomegranate fruits from control and 10, 50 and 100 mM γ-aminobutyric acid (GABA) treated trees along preservation at 10 °C in 2019 (**A**) and from control and 100 mM GABA-treated trees in 2020 (**B**). Data are provided as the mean ± standard error. Different letters denote significant differences ($p < 0.05$) between treatments for each sampling date.

The CIELab coordinates were measured in skin and arils, and Chroma and hue indexes were calculated. Results showed that the colour a* parameter showed the highest differences between fruit picked from control and treated trees and the highest changes along storage, so results for this parameter are provided. Thus, skin colour expressed

as colour a* parameter was significantly ($p < 0.05$) influenced by GABA treatments since it increased as a consequence of GABA treatments in a dose-dependent way in the 2019 experiment (Table 1). In the 2020 experiment, colour a* at harvest was also significantly ($p < 0.05$) higher in pomegranates from 100 mM GABA-treated trees (15.69 ± 0.51) as compared with controls (11.51 ± 0.77). During storage, skin colour a* increased, either in control or in treated fruits, although higher values were found in GABA-treated fruit than in control ones during the whole storage time, with 30–35% higher values at the last sampling date (Table 1). Aril colour was also increased by GABA treatments, as manifested by significant ($p < 0.05$) higher values of a* parameter in the arils of pomegranates from GABA-treated trees concerning controls in both years of study. Increases in colour a* parameter occurred during storage. However, values were significantly ($p < 0.05$) higher in fruit from GABA-treated trees than in controls, especially for the highest assayed dose (Figure 3A,B).

Table 1. Skin colour (a* parameter), total soluble solids (TSS) and titratable acidity (TA) in pomegranate from control and γ-aminobutyric acid (GABA) treated trees at harvest and after 60 days of storage at 10 °C in 2019 and 2020 experiments.

			2019 Experiment	
Colour a*	Control	GABA 10 mM	GABA 50 mM	GABA 100 mM
Day 0	14.84 ± 0.44 cB	16.99 ± 0.65 bB	17.84 ± 0.67 bB	20.38 ± 0.77 aB
Day 60	17.95 ± 0.51 cA	21.05 ± 0.25 bA	21.95 ± 0.47 bA	24.38 ± 0.56 aA
			2020 Experiment	
Colour a*	Control			GABA 100 mM
Day 0	11.51 ± 0.77 bB			15.69 ± 0.51 aB
Day 60	15.44 ± 0.69 bA			19.92 ± 0.81 aA
			2019 Experiment	
TSS (g 100 g^{-1})	Control	GABA 10 mM	GABA 50 mM	GABA 100 mM
Day 0	15.03 ± 0.04 aA	14.98 ± 0.19 aA	15.01 ± 0.08 aA	15.26 ± 0.12 aA
Day 60	15.17 ± 0.32 aA	15.19 ± 0.08 aA	14.72 ± 0.17 aA	14.90 ± 0.14 aA
			2020 Experiment	
TSS (g 100 g^{-1})	Control			GABA 100 mM
Day 0	15.58 ± 0.15 aA			15.33 ± 0.18 aA
Day 60	15.72 ± 0.10 aA			15.43 ± 0.11 aA
			2019 Experiment	
TA (g 100 g^{-1})	Control	GABA 10 mM	GABA 50 mM	GABA 100 mM
Day 0	0.31 ± 0.01 aA	0.32 ± 0.01 aA	0.32 ± 0.01 aA	0.33 ± 0.01 aA
Day 60	0.23 ± 0.02 bB	0.24 ± 0.01 bB	0.27 ± 0.01 abB	0.31 ± 0.01 aA
			2020 Experiment	
TA (g 100 g^{-1})	Control			GABA 100 mM
Day 0	0.34 ± 0.02 aA			0.35 ± 0.01 aA
Day 60	0.26 ± 0.01 bB			0.32 ± 0.02 aA

Data are provided as the mean ± standard error of three replicates of five fruits. For each sampling date, different lowercase letters denote significant differences ($p < 0.05$) among treatments, and different capital letters show significant differences ($p < 0.05$) at harvest or after 60 days of storage for each treatment.

TSS in pomegranate arils ranged between 15 and 15.5 g 100 g^{-1}, and no significant differences were observed due to GABA treatments or storage time in the 2019 or 2020 years (Table 1). However, significant differences were observed in TA attributed to GABA treatments during storage time. Thus, TA values at harvest were similar in fruit from control and GABA-treated trees, and they decreased significantly ($p < 0.05$) in pomegranate arils during the whole storage period. However, these decreases were retarded in fruits from GABA-treated trees. Thus, the arils of pomegranates from treated trees showed

significant ($p < 0.05$) higher TA values at day 60 of storage, especially for the highest dose (Table 1).

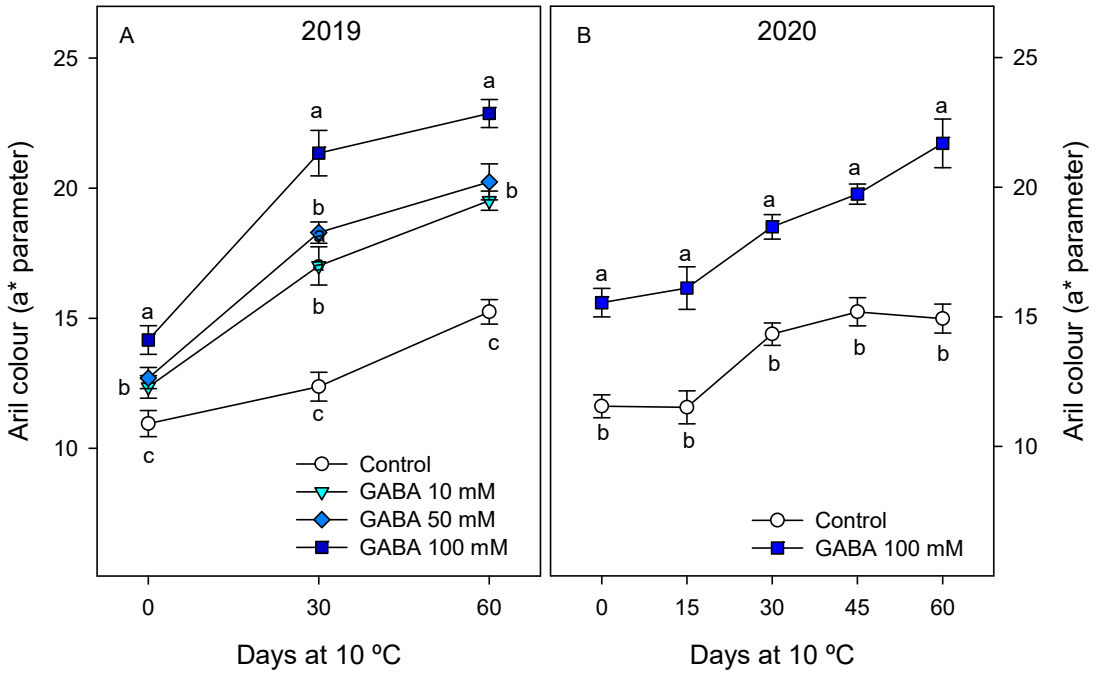

Figure 3. Aril colour a* parameter in pomegranate fruits from control and 10, 50 and 100 mM γ-aminobutyric acid (GABA) treated trees along preservation at 10 °C in 2019 (**A**). From control and 100 mM GABA-treated trees in 2020 (**B**). Data are provided as the mean ± standard error. For each sampling date, different letters denote significant differences ($p < 0.05$) between treatments.

3.2. Effects of GABA Treatments on Bioactive Compounds

Total anthocyanin concentration in arils at harvest was significantly ($p < 0.05$) enhanced by GABA treatments, the increase being higher as was the dose of applied GABA in the 2019 experiment, with values of 10.79 ± 0.96 and 17.35 ± 0.75 mg 100 g^{-1} for fruit picked from control and 100 mM GABA-treated trees, respectively (Figure 4A). Furthermore, an increasing trend was observed in aril anthocyanin concentration during storage, either in fruit from treated trees or in controls, with values always being higher due to preharvest GABA treatments (Figure 4A). These effects of GABA treatment were confirmed in a 2020 experiment when pomegranate trees were treated with 100 mM GABA significantly ($p < 0.05$) higher total anthocyanin concentration in the last ones during the whole storage time (Figure 4B). Four individual anthocyanins were detected and quantified in pomegranate arils at harvest, and all of them were found at significant ($p < 0.05$) higher concentrations in arils of pomegranates from GABA-treated trees, the highest increases being found for the major one, cyn 3-gluc, when 100 mM GABA dose was applied (Figure 5A,B).

The content of total phenolic at harvest was 49.48 ± 1.77 mg 100 g^{-1} in arils of fruit picked from control trees in the 2019 experiment and significantly higher ($p < 0.05$), 57.95 ± 0.86, 61.08 ± 1.19 and 66.13 ± 2.52 mg 100 g^{-1}, in those in fruit picked from trees treated with GABA at 10, 50 and 100 mM doses, respectively. The concentration of total phenolic increased during storage in arils of fruit from control and treated trees, especially during the second month of storage, with values being significantly higher ($p < 0.05$) in GABA-treated ones as compared with controls from day 0 to day 60 of storage (Figure 6A). Accordingly, in the 2020 experiment, a similar increasing trend was observed in phenolic concentration in pomegranate arils of fruit picked from control and 100 mM GABA-treated trees, with significant ($p < 0.05$) higher values in the last ones during the 60 days storage period (Figure 6B).

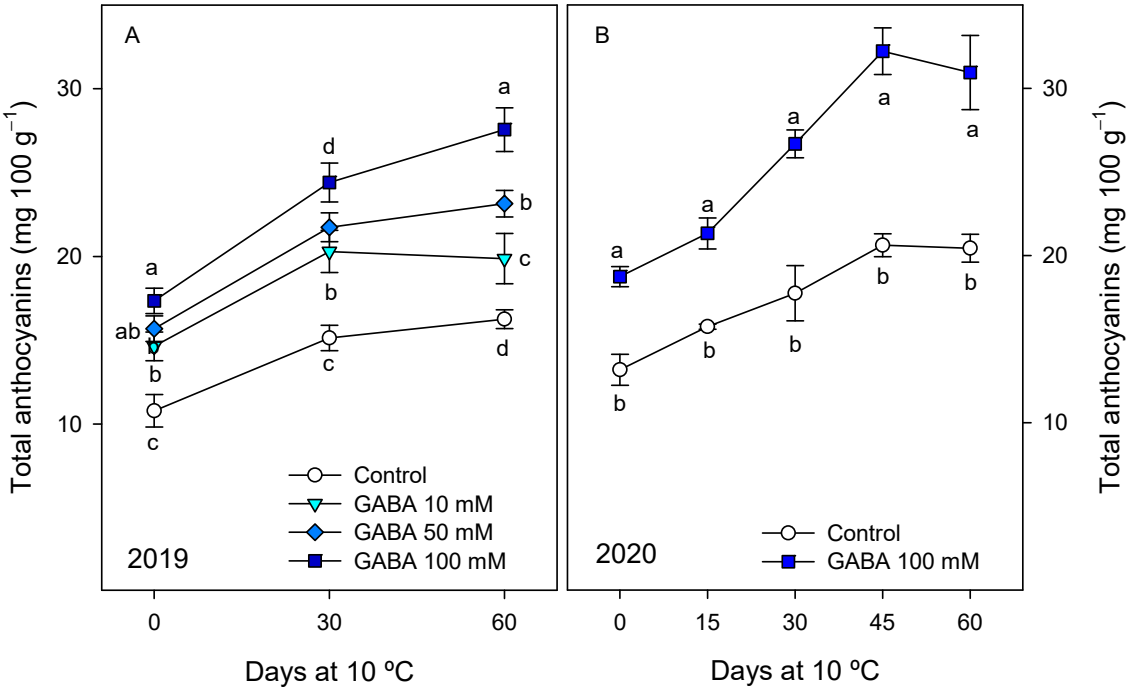

Figure 4. The concentration of total anthocyanin in arils of pomegranate fruits from control and 10, 50 and 100 mM γ-aminobutyric acid (GABA) treated trees along preservation at 10 °C in 2019 (**A**) and from control and 100 mM GABA-treated trees in 2020 (**B**). Data are provided as the mean ± standard error. For each sampling date, different letters denote significant differences ($p < 0.05$) between treatments.

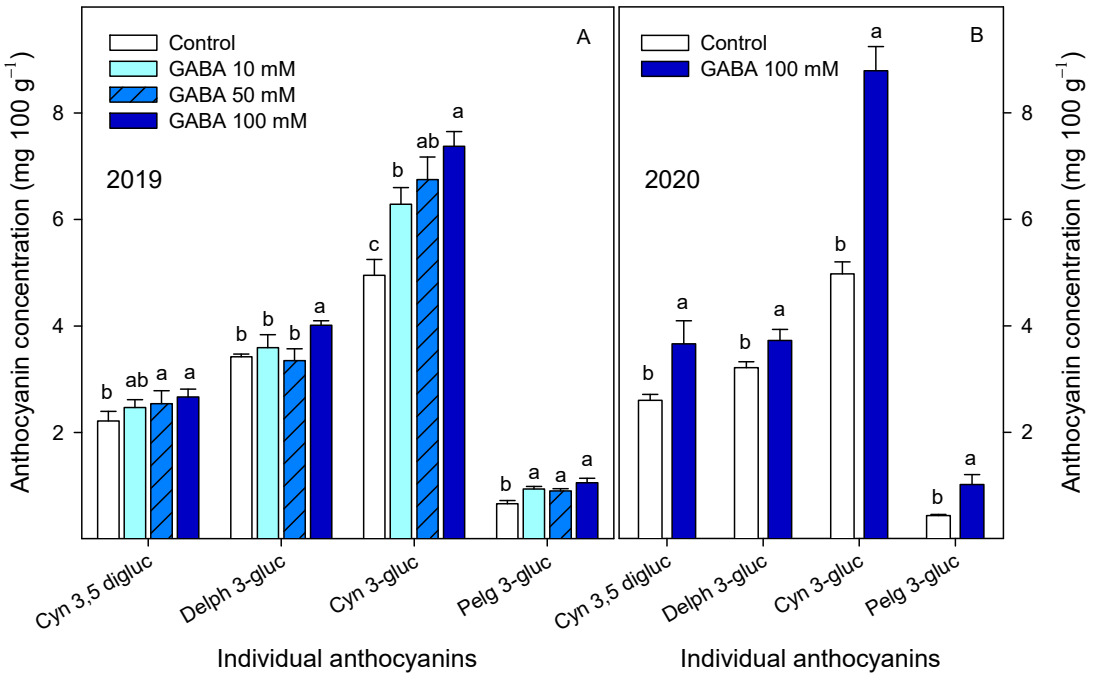

Figure 5. The concentration of individual anthocyanin in arils of pomegranate fruits from control and 10, 50 and 100 mM γ-aminobutyric acid (GABA) treated trees at harvest in 2019 (**A**) and from control and 100 mM GABA-treated trees in 2020 (**B**). Data are provided as the mean ± standard error. For each sampling date, different letters denote significant differences ($p < 0.05$) between treatments.

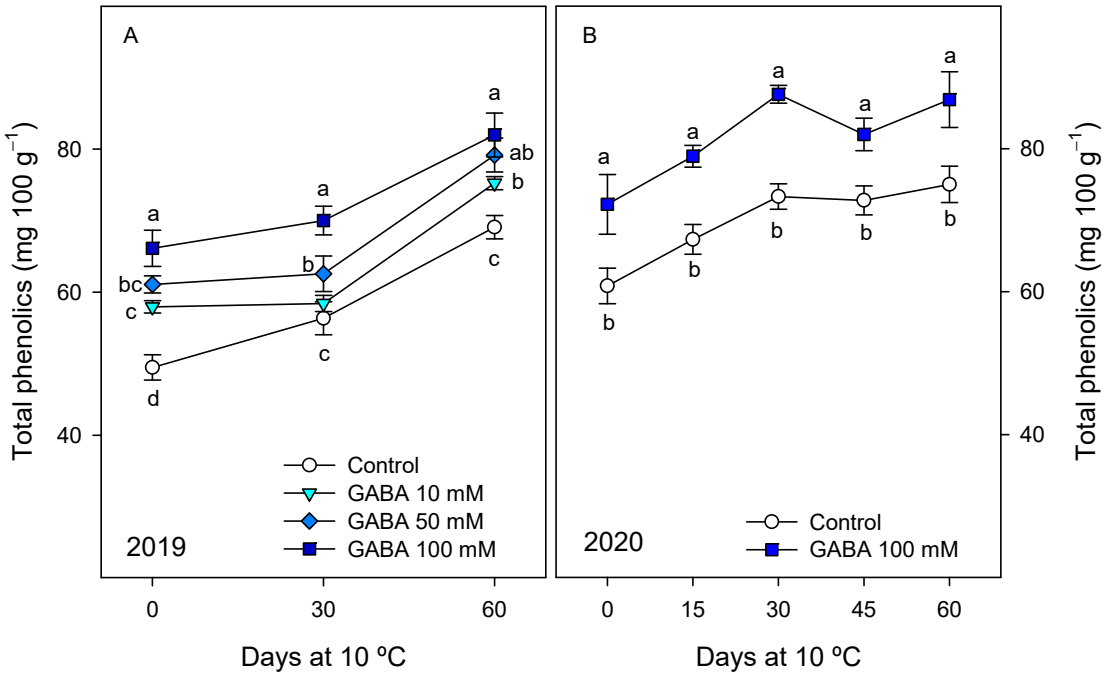

Figure 6. The concentration of total phenolic in arils of pomegranate fruits from control and 10, 50 and 100 mM γ-aminobutyric acid (GABA) treated trees along preservation at 10 °C in 2019 (**A**), and from control and 100 mM GABA-treated trees in 2020 (**B**). Data are provided as the mean ± standard error. For each sampling date, different letters denote significant differences ($p < 0.05$) between treatments.

4. Discussion

The primary factors influencing the quality of pomegranate fruit include the colour of the peel and arils, fruit firmness, and TSS and TA concentration in arils [1,7,15]. Results showed higher values of fruit firmness and higher red colour in both skin and arils (manifested by enhanced colour a*) at harvest in fruit from GABA-treated trees as compared with controls. Thus, results clearly show that preharvest applications of GABA increased quality attributes, especially when applied at 100 mM dose. However, TSS content and TA levels, which are also important indicators of the pomegranate on-tree maturation process [1,2,8], were not influenced by GABA applications independently of the applied concentration. So then, an apparent effect of GABA treatment on fruit maturation was not observed.

On the other hand, these treatments improved crop yield, especially for 50 and 100 mM doses, due to an increase in the number of fruit per tree harvested [29]. In addition, decreases in fruit firmness and acidity levels and increases in weight loss and red colour of skin and arils occurred during storage, either in control as in treated fruits, which are common changes observed during the postharvest maturation process in 'Mollar de Elche' and in other cultivars of this fruit species [13,30,31]. However, it is worth noting that preharvest GABA treatments reduced weight losses, while firmness, TA, colour a* as well as total concentrations of phenolic and anthocyanin compounds were maintained at higher levels, and no changes were observed in TSS. TSS and TA are important quality traits since they affect sweetness and sourness. They are due to fructose and glucose, as major sugars, and to malic acid, followed by succinic and citric acids, as major organic acids in the 'Mollar de Elche' cultivar [32]. Thus, maintaining TSS and TA close to values at harvest after 60 days of storage, because of 100 mM GABA treatments, would lead to fruit with the freshness, juiciness and taste consumers demand [1,13,14,33,34]. Therefore, according to the evolution of all the measured quality parameters during storage, it could be inferred that control pomegranate could be stored at 10 °C with high-quality parameters for consumption for 30 days in 2019 and 2020. However, this period could be extended up to 60 days in fruit from 100 mM GABA-treated trees, showing an effect of preharvest GABA treatments on delaying the ripening process of pomegranate during storage.

There are no available references in the literature regarding the effects of GABA treatments, applied as preharvest treatments, on the changes of fruit quality traits during storage for comparative purposes. However, there are some published reports about the effects of postharvest GABA treatments. For example, fruit quality traits, such as TA and firmness, were preserved at higher values in GABA-treated apple fruit [23] as compared with controls during cold storage, as well as on aonla fruit (*Emblica officinalis*) [35]. Accordingly, weight loss, softening, ascorbic acid losses and colour changes were delayed during storage in carambola GABA-treated fruit, leading to the maintenance of fruit quality [36]. Similar results were obtained in blueberries [37] and mango [24] fruits after postharvest GABA applications, which maintained quality parameters, delayed senescence, and prolonged shelf life, in agreement with the present results. These effects were explained by the effect of GABA on reducing reactive oxygen species levels, due to increased activity of the enzymes with antioxidant activity, namely catalase, glutathione reductase, ascorbate peroxidase, superoxide dismutase and peroxidase. Nevertheless, authority approval would be required for practical pre- or postharvest GABA treatment applications.

It has been reported that pomegranate arils have various antioxidant compounds, mostly ascorbic acid, and phenolic compounds, including anthocyanins, other complex flavonoids, and hydrolysable tannins which are present at higher levels than in other common fruits of the Mediterranean diet. However, different concentrations have been observed based on the cultivar, maturation stage at harvest, cultural techniques, and environmental factors [9,38–41]. Total anthocyanin and phenolic concentrations in pomegranate arils at harvest were increased by GABA treatments, and these effects increased as did GABA doses in the range from 10 to 100 mM and were found at higher levels concerning those of pomegranate from control trees during the storage at 10 °C for 60 days. Regarding the anthocyanin profile, cyn 3-gluc was found at the highest concentration, followed by delph 3-gluc and cyn 3,5-digluc. In contrast, pelg 3-gluc was the minor one, in agreement with previous reports for the 'Mollar de Elche' cultivar [7,13,42–44]. However, the concentrations of each anthocyanin in these previous reports were slightly different from those in the present experiments. These are attributed to the effects of cultural practices and/or environmental conditions on anthocyanin biosynthesis. A similar anthocyanin profile has been reported previously in many other cultivars of pomegranate, although the relative concentration of each one was different depending on the cultivar. In this sense, in the 'Wonderful' cultivar, cyn 3-gluc was the anthocyanin found at the highest concentration [29].

In contrast, delph 3-gluc was the predominant one for some cultivars from Croatia [38], pelg 3,5-digluc in cultivars from Iran [45] and cyn 3,5-digluc in cultivars from Tunisia [46]. The Anthocyanin profile was not influenced by preharvest GABA treatments, independently of the assayed concentration. However, it is worth pointing out that GABA treatments increased the concentrations of all the individual anthocyanins. The highest effect is found for the significant one and 100 mm GABA dose. The increase in total and individual anthocyanin content after preharvest GABA applications led to fruit with a deeper red colour in the arils, as observed in the photographs in the graphical abstract. Thus, fruit from GABA-treated trees would be more appreciated by consumers due to their increased aril red colour [1,8–11].

On the other hand, phenolics, especially anthocyanins, have been reported to be highly correlated with antioxidant activity in pomegranate arils [14,16,34]. They are compounds responsible for the health benefits attributed to the consumption of pomegranate fruit [4–6,47–50]. Therefore, preharvest GABA treatments would lead to pomegranates with enhanced health-beneficial effects.

Previous recent reports have shown that postharvest GABA treatments led to increased phenolic compound concentrations, including anthocyanins, during storage in 'Sahebi' grapes [19], 'Moro' blood oranges [26] and cornelian cherry fruits [21]. These effects were attributed to the increased activity of phenylalanine ammonia-lyase (PAL), the key enzyme pivotal in phenolic biosynthesis, and reduced polyphenol oxidase activity (PPO), the major

enzyme involved in their degradation. Similarly, phenolic, and flavonoid content were increased in blueberries during storage due to postharvest GABA treatments due to the enhanced activity of PAL, cinnamate-4-hydroxylase and 4-coumarate/coenzyme A ligase, which are two pivotal enzymes involved in the phenylpropanoid pathway in plants [37]. In pomegranate fruit, Nazoori et al. [27] reported that GABA dipping treatments before storage maintained higher levels of total phenolics and anthocyanins after 90 days of storage. However, the effects were positive for 5 and 15 mM concentrations but not for 10 mM. However, this is the first report on the effects of GABA treatments of pomegranate trees, applied at key points of on-tree fruit development, on increasing phenolic and anthocyanin concentration at harvest and after prolonged storage provided. On the other hand, the increase in anthocyanin concentration in arils of 'Mollar de Elche' fruit from GABA-treated trees, either at harvest or during storage, would contribute to an increase in its income and commercial value in international markets, which appreciate more red-coloured cultivars, such as the Spanish 'Katirbasi', 'CG8', 'White' or 'Wonderful' [42,51] or other Turkish, Tunisian, Iranian or Croatian cultivars [1,9,10,38,40].

5. Conclusions

In a 2019 experiment, pomegranate trees treatment with GABA at 10, 50 and 100 mM increased the quality attributes of pomegranate fruit at the time of harvest. They were maintained during 60 days of storage at 10 °C. The most important effect is found for 100 mM dose. Similar results were obtained in the 2020 experiment, when a 100 mM GABA dose was chosen to repeat the treatments, showing a delay of the postharvest ripening process in pomegranates from GABA-treated trees concerning controls. Thus, the storage time of pomegranates with optimum quality traits for consumption could be extended from 30 days, for fruit from control trees to 60 days for those from GABA-treated ones. In addition, redder arils with enhanced anthocyanin content were observed because of preharvest multiple GABA applications, which would lead to an increase in the profit and commercial value in international markets of 'Mollar de Elche' pomegranate since fruit with enhanced skin and aril red colour would be more appreciated for consumption. Moreover, GABA applications showed an additional effect on improving antioxidant compounds, mainly total phenolics and total and individual anthocyanins, increasing the health-beneficial effects of pomegranate consumption. Future research is needed to determine if the number of GABA applications could be reduced without compromising its beneficial effects to achieve a cost-effective treatment for practical purposes.

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