Effect of Carbon Dioxide on Anthocyanins, Phenylalanine Ammonia Lyase and Glucosyltransferase in the Arils of Stored Pomegranates

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ABSTRACT. ‘Wonderful’ Pomegranates (*Punica granatum* L.) were placed in jars ventilated continuously with air or air enriched with 10 or 20 kPa CO₂ at 10 °C for 6 weeks. Samples were taken initially and after 1, 2, 4, and 6 weeks, and postharvest quality attributes were measured. The arils of the pomegranates stored in air were deeper red than the initial controls and than those stored in CO₂-enriched atmospheres. This increased color was associated with increased anthocyanin concentration. Arils from fruit stored in air enriched with 10 kPa CO₂ had a lower anthocyanin concentration than air-stored fruit, and atmospheres enriched with 20 kPa CO₂ had even lower levels, possibly from suppressed anthocyanin biosynthesis. Anthocyanin concentration correlated well with the activity of phenylalanine ammonia lyase but not with glucosyltransferase activity. Moderate CO₂ atmospheres (10 kPa) prolong the storage life and maintain quality of pomegranates, including adequate red color intensity of the arils.

Fruit color is used as an index of maturity and stage of ripeness in many fruit. Changes in the color and anthocyanin content have been studied mainly during development and ripening of fruit, such as strawberries (*Fragaria xananassa* Duch.) (Given et al., 1988) and pomegranates (Gil et al., 1995), but there is little information on postharvest color changes. Controlled-atmosphere (CA) storage, i.e., modification of O₂ and/or CO₂ concentrations, has been used to extend the postharvest life of pomegranates by retarding the spread of certain diseases and lowering the incidence of physiological disorders (Artes et al., 1996; Ben-Arie and Or, 1986; Kupper et al., 1995). However, the effect of CO₂-enriched atmospheres on the stability of anthocyanins is not well known. Apple (*Malus domestica* Borkh.) anthocyanin content was adversely affected by high CO₂ (73%) levels during storage (Lin et al., 1989).

Anthocyanin concentration has been shown to increase in pomegranate (Gil et al., 1995) and lowbush blueberry fruit (*Vaccinium angustifolium* Ait.) (Kalt and McDonald, 1996) during cold storage. The biosynthesis of phenolic compounds and the activity of phenylalanine ammonia lyase (PAL; EC 4.3.1.5), the first enzyme in the phenylpropanoid pathway, has been studied in several fruit. An increase in PAL activity has been correlated with anthocyanin production in strawberries (Given et al., 1988) and grape (*Vitis vinifera* L.) suspension cultures (Kakegawa et al., 1995). PAL activity and anthocyanin concentration in the skin of harvested apples was shown to increase following irradiation with UV and white light (Dong et al., 1995). UDP-glucose:flavonoid 3-O-glucosyltransferase (GT; EC 2.4.1.91), the enzyme catalyzing the final step in the anthocyanin biosynthetic pathway (Stafford, 1990), was also correlated with the anthocyanin increase in ripening strawberries (Given et al., 1988; Cheng et al., 1994).

Our hypothesis was that storage in elevated CO₂ atmospheres would adversely affect color development by reducing anthocyanin biosynthesis in the arils of ‘Wonderful’ pomegranate fruit. We investigated changes in the activity of PAL and GT in relation to

anthocyanin concentration as influenced by high concentrations of CO₂ during storage.

Materials and Methods

‘Wonderful’ pomegranates were purchased from a commercial producer near Fresno, Calif., in October 1996 and transported to Univ. of California, Davis. The fruit were sorted to eliminate defective individuals and obtain a uniform sample. Three replicates of five fruit each were placed in jars and stored at 10 °C to avoid chilling injury (Elyatem and Kader, 1984; Kader et al., 1984). The jars were ventilated with a continuous flow of humidified air or air enriched with 10 kPa or 20 kPa CO₂ at a rate of 150 mL·min⁻¹ using needle valve flow boards. The compositions of the gas mixtures were verified regularly with a gas chromatograph equipped with a thermal conductivity detector (model 111; Carle Instruments, Anaheim, Calif.) or an infrared gas analyzer (model PIR-200R; Horiba Instruments, Irvine, Calif.), and maintained within 10% of the required concentration, e.g., 10 ± 1 kPa.

Fruit samples were analyzed initially and after 1, 2, 4, and 6 weeks of storage. Each fruit was analyzed for external skin color (four equatorial points) and internal integument color (five measurements per fruit) using Commission Internationale de l’Éclairage L*, a*, b* color space coordinates obtained with a colorimeter (model CR-300; Minolta, Ramsey, N.J.). A 20-g sample of arils from each of the five fruit was mixed, frozen in liquid nitrogen, and stored at –80 °C for enzyme analysis. A similar sample of arils was squeezed and the juice was analyzed for soluble solids (SS), pH, titratable acidity (TA), and absorbance. SS was measured using a refractometer (Abbe model 10450; American Optical, Buffalo, N.Y.). Samples of 4 g of juice were diluted with 20 mL of distilled water, and pH and TA were measured using an automatic titrator fitted with a pH meter and an autoburette (PHM85 Precision, ABU80; Radiometer, Copenhagen, Denmark). TA was expressed as percent citric acid. Juice absorbance was recorded on a spectrophotometer (UV-1601; Shimadzu Scientific Instruments, Columbia, Md.) at 446 nm (browning compounds) and at 510 nm (anthocyanins) after a 1:5 dilution with citric acid buffer (pH 3.4), and absolute results were presented (Table 1).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF ANTHOCYANINS. An undiluted juice sample from each replicate

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was centrifuged in an eppendorf tube (2 min at 15 600 g_n), filtered through a 0.45- μ m filter, and transferred to -20 °C until analyzed. A sample of 20 μ L of juice was analyzed using an HPLC system (Hewlett Packard 1050 pump) coupled with a photodiode array detector (DAD) (series 1040M, series II) and an autosampler (series 1050), operated by HP ChemStation software (Hewlett Packard, Palo Alto, Calif.). A reverse-phase C_{18} nucleosil column (150 $\times$ 4.6 mm; particle size 5 μ m) (MetaChem Technologies Inc., Torrance, Calif.) with a guard column containing the same stationary phase (Safeguard holder 5001-CS) was maintained at 40 °C. The mobile phase was acidified water (1.32 M formic acid) (A) and acidified methanol (1.32 M formic acid) (B) in a linear gradient, where A:B ratio changed from 85:15 to 65:35 in 15 min and held for 2 min before returning to the initial conditions. The flow rate was 1 mL·min⁻¹ and detection was at 510 nm.

ANTHOCYANIN IDENTIFICATION AND QUANTIFICATION. Identification was based on retention times, UV spectra, and comparison with published results (Du et al., 1975; Gil et al., 1995). Individual anthocyanins were quantified by comparison with an external standard of cyanidin 3-glucoside (Apin Chemicals Ltd., Abingdon, Oxon, U.K.). The total anthocyanin concentration was calculated from the sum of the six individual anthocyanins detected in each chromatogram and concentrations were expressed as μ g·L⁻¹ of juice.

ENZYME EXTRACTION. A 20-g sample of frozen arils was thawed for 30 min and pressed in a juicer, taking care not to crush the seeds. The juice and pressed arils were stirred in 100 mL extraction buffer (Lister et al., 1996) for 3 min. The solution was filtered through cheesecloth and, after squeezing, the seeds were weighed and subtracted from the initial mass. The procedure of Lister et al. (1996) was followed to obtain the protein pellet, which was redissolved in 2 mL of dialysis buffer and dialyzed for 24 h to obtain a partially purified extract. The extract was frozen in liquid nitrogen and stored at -80 °C. The total protein content was determined by the Bradford microassay (Bradford, 1976).

ENZYME ASSAYS. PAL activity was assayed according to Lister

et al. (1996). PAL assays were carried out in triplicate, and the tubes were incubated for 1 h at 30 °C. GT activity was assayed according to Lister et al. (1996), except that UDP-glucose was used and quercetin-3-glucose was produced. Duplicate GT assays were incubated for 30 min at 30 °C. The product (quercetin-3-glucose) was quantified by HPLC using the reverse-phase C_{18} nucleosil column previously used in the anthocyanin analysis, but maintained at 30 °C. The mobile phase was acidified water (0.66 M formic acid) (A) and acidified methanol (0.66 M formic acid) (B) in a linear gradient, where A:B ratio changed from 50:50 to 15:85 in the first 5 min, followed by an isocratic mixture for 1 min before returning to initial conditions. The flow rate was 1 mL·min⁻¹ and the injection volume was 5 μ L. Quercetin-3-glucose was detected at 350 nm and quantified against a standard. Enzyme activity was expressed as pkat·g⁻¹ fresh mass and as pkat·mg⁻¹ protein, where 1 kat is the amount of enzyme required for the formation of substrate at a rate of 1 mol·s⁻¹ under assay conditions.

STATISTICS. CoStat Statistical Software (CoHort, Minneapolis, Minn.) was used to perform analysis of variance and obtain LSD (5%) values of each of the main effects.

Results and Discussion

EXTERNAL APPEARANCE. The chroma (C^* value) of the skin of pomegranates was maintained better when the fruit were stored in air enriched with CO₂ than in air alone (Table 1). The loss in intensity of color in air was noticeable after 4 weeks of storage. Air stored pomegranates were subject to microbial decay after 4 to 6 weeks of storage, while CO₂ inhibited the growth of decay organisms (data not shown).

INTERNAL APPEARANCE. The L^* value of the internal integuments of the fruit decreased with time, indicating that browning had developed. The integuments were darker ($P < 0.05$) after 6 weeks of storage in 20 kPa CO₂, indicating CO₂ injury (Table 1). The chroma and hue angle did not change during storage (data not shown).

The visual appearance of the arils was darker red after 6 weeks

Table 1. Changes in external chroma (C^*) value, integument L^* value, absorbance of juice at 510 nm and at 446 nm, and the ratio of total monoglucoside to diglucoside anthocyanin derivatives (ratio) of 'Wonderful' pomegranates initially and after 1, 2, 4, or 6 weeks at 10 °C in air or CO₂-enriched air. Each value represents the mean of 15 fruit $\pm$ SD.

Storage duration and atmosphere	Color		Absorbance		Ratio (mono:di)
	External C^*	Integument L^*	510 nm	446 nm	
Initial	44.8 $\pm$ 1.4	78.7 $\pm$ 1.2	2.858 $\pm$ 0.472	1.193 $\pm$ 0.189	1.23
After 1 week					
Air	44.4 $\pm$ 1.1	80.9 $\pm$ 0.5	3.592 $\pm$ 0.230	1.508 $\pm$ 0.109	1.09
10 kPa CO ₂	44.5 $\pm$ 1.0	82.3 $\pm$ 1.8	3.369 $\pm$ 0.750	1.409 $\pm$ 0.301	1.03
20 kPa CO ₂	46.4 $\pm$ 0.8	81.4 $\pm$ 0.8	3.492 $\pm$ 0.870	1.499 $\pm$ 0.310	1.24
After 2 weeks					
Air	45.4 $\pm$ 1.9	80.4 $\pm$ 1.4	3.487 $\pm$ 0.440	1.457 $\pm$ 0.173	1.66
10 kPa CO ₂	44.3 $\pm$ 0.4	80.5 $\pm$ 0.5	3.677 $\pm$ 0.564	1.548 $\pm$ 0.231	1.23
20 kPa CO ₂	45.4 $\pm$ 0.9	80.7 $\pm$ 0.1	3.261 $\pm$ 0.055	1.452 $\pm$ 0.045	1.04
After 4 weeks					
Air	41.0 $\pm$ 1.5	79.9 $\pm$ 2.0	5.056 $\pm$ 0.541	2.135 $\pm$ 0.229	2.32
10 kPa CO ₂	44.3 $\pm$ 0.8	79.0 $\pm$ 2.0	4.063 $\pm$ 0.437	1.733 $\pm$ 0.185	1.36
20 kPa CO ₂	44.9 $\pm$ 1.2	77.0 $\pm$ 1.5	3.873 $\pm$ 0.350	1.726 $\pm$ 0.155	1.06
After 6 weeks					
Air	39.0 $\pm$ 1.3	77.7 $\pm$ 1.3	6.431 $\pm$ 0.396	2.762 $\pm$ 0.182	3.05
10 kPa CO ₂	42.9 $\pm$ 1.1	77.5 $\pm$ 0.7	4.616 $\pm$ 0.848	1.931 $\pm$ 0.333	1.42
20 kPa CO ₂	43.8 $\pm$ 0.4	73.4 $\pm$ 2.8	3.005 $\pm$ 0.910	1.412 $\pm$ 0.360	1.04
LSD (5%) weeks	1.1	1.4	0.548	0.213	
LSD (5%) CO ₂	0.9	1.1	0.425	0.165	

Table 2. Changes in pH, titratable acidity (TA), and soluble solids (SS) initially and after 1, 2, 4, or 6 weeks at 10 °C in air or CO₂-enriched air. Values are means of 15 fruit ± SD.

Storage duration and atmosphere	pH	TA (% m/v)	SS (% m/v)
Initial	3.54 ± 0.03	1.03 ± 0.05	19.2 ± 0.3
After 1 week			
Air	3.45 ± 0.04	1.05 ± 0.11	19.5 ± 0.0
10 kPa CO ₂	3.43 ± 0.11	1.11 ± 0.19	19.2 ± 0.6
20 kPa CO ₂	3.43 ± 0.18	1.17 ± 0.31	19.4 ± 0.1
After 2 weeks			
Air	3.56 ± 0.05	0.91 ± 0.06	18.7 ± 0.4
10 kPa CO ₂	3.47 ± 0.12	1.07 ± 0.18	19.2 ± 0.2
20 kPa CO ₂	3.47 ± 0.08	0.99 ± 0.03	18.9 ± 0.1
After 4 weeks			
Air	3.56 ± 0.08	0.89 ± 0.06	19.1 ± 0.2
10 kPa CO ₂	3.55 ± 0.04	0.94 ± 0.11	18.7 ± 0.2
20 kPa CO ₂	3.49 ± 0.04	1.02 ± 0.08	18.7 ± 0.4
After 6 weeks			
Air	3.49 ± 0.06	0.98 ± 0.11	19.6 ± 0.4
10 kPa CO ₂	3.45 ± 0.07	1.07 ± 0.14	19.2 ± 0.3
20 kPa CO ₂	3.48 ± 0.09	1.03 ± 0.20	19.2 ± 0.3

of storage in air compared with CO₂-enriched treatments, but there was no observable browning of the arils in any of the treatments. The absorbance of the juice at 510 nm increased during storage for fruit stored in air and 10 kPa CO₂ (Table 1). Conversely, juice from fruit stored in 20 kPa CO₂ did not change in absorbance over the storage period. Since changes in absorbance at 446 nm paralleled those at 510 nm for all treatments (Table 1), we concluded that this was a result of the increase in anthocyanin concentration and not phenolic polymerization products (whose absorption maximum is 446 nm).

pH, TA, AND SS. Carbon dioxide had no effect on pH or TA of pomegranate juice (Table 2), in contrast with strawberry (Gil et al., 1997; Ke et al., 1991) and lettuce (Siriphanich and Kader, 1986). This may be a result of the higher buffering capacity of pomegranate juice, which has a TA of ≈1.0% compared to 0.7% in strawberry (Gil et al., 1997). SS content was not affected by CO₂ or storage time (Table 2).

ANTHOCYANIN CONCENTRATION. The total anthocyanin concentration of the arils increased during storage in air and 10 kPa CO₂, although this increase was only significantly different from the initial value after 4 weeks of storage ($P < 0.05$) (Fig. 1). After 6 weeks, fruit held in air had a greater concentration of anthocyanin (352.0 µg·mL⁻¹) than fruit stored in 10 kPa CO₂ (283.0 µg·mL⁻¹) ($P < 0.05$). The anthocyanin concentration of pomegranates stored in 20 kPa CO₂ (170.2 µg·mL⁻¹) for 6 weeks was lower than the initial concentration (206.2 µg·mL⁻¹). Even though fresh mass was not monitored, we believe the increase in anthocyanin concentration cannot be attributed to water loss during storage.

The 'Wonderful' pomegranates used in this experiment had a higher concentration of anthocyanins at harvest than Tunisian cultivars (Gil et al., 1996), but slightly less than the intensely colored Spanish cultivar Mollar (Gil et al., 1996). The major pigments in 'Wonderful' pomegranate juice were cyanidin (Cy) 3-glucoside, Cy 3,5-diglucoside, delphinidin (Dp) 3,5-diglucosides and Dp 3-glucoside. Pelargonidin (Pg) derivatives were present in small amounts (≤7 µg·mL⁻¹ monoglucoside and ≤3.5 µg·mL⁻¹ diglucoside) (Fig. 2).

In addition to total quantitative changes, the anthocyanin profiles changed in response to storage in the different atmospheres. A comparison of individual anthocyanins showed a similar trend

for all monoglucoside derivatives (Fig. 2). Dp, Cy, and Pg 3-glucosides from pomegranates held in air and 10 kPa CO₂ increased during storage. This increase was higher in air and accounted for the greater anthocyanin concentration in these fruit. The diglucosides were always present in lower concentrations than the monoglucosides in these treatments (Fig. 2). The ratio of monoglucosides to diglucosides is a useful parameter to compare the effects of CO₂. This ratio increased in air-stored fruit because of an increase in monoglucoside concentration (Table 1). In fruit stored in 10 kPa CO₂, the increase was slight because mono- and diglucosides derivatives accumulated (Table 1; Fig. 2). In contrast, the ratio decreased after 2 weeks of storage in 20 kPa CO₂. The 20-kPa CO₂ treatment decreased levels of all anthocyanins, especially

Fig. 1. Total anthocyanin concentration (µg·mL⁻¹) of 'Wonderful' pomegranate juice initially and after 1, 2, 4, or 6 weeks at 10 °C in air or CO₂-enriched air.

Fig. 2. Changes in individual (A) diglucoside and (B) monoglucoside anthocyanin derivatives ($\mu\text{g}\cdot\text{mL}^{-1}$) of 'Wonderful' pomegranate juice initially and after 1, 2, 4, or 6 weeks at 10°C in air or CO_2 -enriched air.

monoglucoside derivatives, as evidenced by a decrease in concentration between 4 and 6 weeks. These results agree with those of Robinson et al. (1966), who reported that diglucosides were more stable than monoglucosides. After 6 weeks, the ratios of mono- to diglucoside derivatives were very different among the three treatments (Table 1).

PAL AND GT ACTIVITIES. The activity of PAL increased with time in arils of fruit stored in air and 10 kPa CO_2 (Fig. 3), reaching a maximum at 4 weeks. The PAL activity of fruit stored in 20 kPa CO_2 decreased during storage. PAL activity was closely correlated with total anthocyanin content ($r = 0.76$) ($P < 0.01$) (Fig. 1). It is not clear if the effects of CO_2 -enriched atmospheres were limited to a reduction in biosynthesis or whether degradation was enhanced resulting in a greater anthocyanin turnover. Other researchers have reported a CO_2 -induced increase in PAL enzyme activity in lettuce (Siriphanich and Kader, 1985) and avocado suspension cells (Prusky et al., 1996).

The activity of GT did not differ among treatments and storage times (Fig. 3), in contrast with apple skin (Ju et al., 1995; Lister et al., 1996) and strawberries (Given et al., 1988), where GT activity paralleled PAL activity. The major difference is that their studies focused on changes during development of the fruit, whereas this paper is limited to changes during storage.

During development and ripening of most fruit, PAL activity and total anthocyanins are generally less well correlated. This is because PAL is the key enzyme for the phenylpropanoid pathway and not specific to anthocyanin biosynthesis. Since anthocyanins are probably the major phenolic compounds being synthesized in mature pomegranates during storage, the anthocyanin content correlated well with PAL.

After 4 weeks, the anthocyanin concentration continued to increase in air compared to 10 kPa CO_2 despite similar PAL

activities. These results suggest that an additional enzyme further down the biosynthetic pathway may be the rate-limiting step, as has been proposed for strawberries (Cheng and Breen, 1991). Expression of the genes involved in anthocyanin biosynthesis in grapes suggests that GT is regulated independently of the other genes and is the major control point in the pathway (Boss et al., 1996). However, the control point in anthocyanin biosynthesis in grapes is later than in snapdragon (*Antirrhinum majus* L.), maize (*Zea mays* L.), or petunia (*Petunia $\times$ hybrida* Hort.). In snapdragon, it has been suggested that flavonoid-3'-hydrolase

Fig. 3. Changes in phenylalanine ammonia lyase (PAL) and glucosyltransferase (GT) activities ($\text{pkat}\cdot\text{mg}^{-1}$ protein basis) of 'Wonderful' pomegranate arils, initially and after 1, 2, 4, or 6 weeks at 10°C in air or CO_2 -enriched air.

is the control point (Martin and Gerats, 1993), while in petunia the major control point appears to be dihydroflavonol 4-reductase (Boss et al., 1996; Quattrocchio et al., 1993). Lister et al. (1996) suggested that the regulatory step in apples is probably the conversion from leucoanthocyanin to anthocyanin by the anthocyanidin synthase complex, but this enzyme has not been isolated (Stafford, 1990).

Despite the fact that the air-stored fruit had a more intense aril color, fruit stored under moderate CO₂ concentrations (10 kPa) had adequate aril color and the additional advantages of maintaining the external appearance, delaying maturity, and controlling decay. Carbon dioxide did not affect anthocyanin stability in pomegranates compared to strawberries (Gil et al., 1997). Factors such as pH and copigmentation are important in maintaining the stability of anthocyanins (Macheix et al., 1990). The greater buffering capacity of the pomegranate juice may help prevent pH changes and so maintain aril pigmentation. Therefore, the effect of CO₂-enriched atmosphere storage on color and anthocyanin concentration depends on fruit species.

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