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Changes in Polyphenols and Expression Levels of Related Genes in 'Duke' Blueberries Stored under High CO_2 Levels

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ABSTRACT: Blueberries are highly perishable fruits, and consequently, storage under high CO_2 and low O_2 levels is recommended to preserve the highly appreciated polyphenols. However, high CO_2 levels might be detrimental for certain cultivars. The aim of this study was to investigate the impact of storage conditions on various quality parameters, including polyphenol composition in 'Duke' berries. Results show that storage under 18 kPa CO_2, coupled with 3 kPa O_2, resulted in accelerated softening of berries, which was accompanied by lower levels compared to other conditions of hexosides and arabinosides of malvidin, petunidin, cyanidine, and delphinidin. However, this storage condition had no negative impact on chlorogenic acid levels. Expression data of key polyphenol-biosynthesis genes showed higher expression levels of all investigated genes at harvest time compared to all storage conditions. Of particular importance is the expression level of chalcone synthase (*VcCHS*), which is severely affected by storage at 18 kPa CO_2.

KEYWORDS: *blueberry, Vaccinium corymbosum, polyphenols, high CO_2 storage*

INTRODUCTION

Highbush blueberry (*Vaccinium corymbosum*) is an economically important fruit crop in Europe and North America. This is due to high levels of polyphenolics (i.e., flavonoids) in these fruits, which have positive effects on human health; highbush blueberry is one of the best with regard to their anthocyanins content.[1] In this respect, several major health benefits are linked to the consumption of blueberries such as reduced risk for cardiovascular[2] and neurodegenerative[3] diseases, prevention of cancer, and reverse cognitive and behavioral deficits related to stroke and aging.[4]

The polyphenol composition of highbush blueberry is complex as it includes anthocyanins, flavonols, and hydroxycinnamic acid derivatives.[5] In addition, large qualitative and quantitative differences exist between different blueberry varieties. While Burdulis et al.[6] concluded that malvidin is the dominant anthocyanidin in blueberries, Rodríguez-Mateos et al.[7] reported that delphinidin and malvidin are the predominant anthocyanidins. However, Huang et al.[8] attributed the strong antioxidant activities of blueberries to the high levels of proanthocyanidins and anthocyanidins in fruits.

Changes in the phenolic profile of blueberries during fruit growth and development, as well as after harvest and storage, were also investigated: Castrejón et al.[9] found that levels of anthocyanins of all investigated varieties increased during successive maturation and harvest stages, whereas the amount of flavonols and hydroxycinnamic acids decreased during fruit ripening. Thus, it was concluded that the antioxidant activity of blueberries decreased during ripening. Furthermore, Harb et al.[10] reported that the concentration of water-soluble antioxidants in blueberries decreased with prolonged storage. Similarly, Remberg et al.[11] found that the total antioxidant capacity decreased considerably during refrigerated storage of the 'Bluecrop', 'Hardyblue', 'Patriot', 'Putte', and 'Aron' varieties. The study by Connor et al.[12] showed that the immature harvested blueberries 'Elliott' exhibited an increase in the antioxidant activity, total phenolic and anthocyanin content during the first 3 weeks of storage. Also, Kalt et al.[13] observed that there was no ascorbate loss in highbush blueberries during 8 days of storage at various temperatures, with a significant increase in anthocyanin content measured after 8 days shelf life at 20 °C.

To date, the impact of decreasing O_2 and/or increasing CO_2 on polyphenols in controlled atmosphere (CA) storage is still unclear. Holcroft and Kader[14] stated that treatments with CO_2 inhibited the increase in anthocyanin concentration by affecting its biosynthesis, degradation, or both. Moreover, it was reported

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that CO_2 enriched atmospheres, which are used widely to reduce the incidence of decay of strawberries, resulted in the degradation of fruit internal color, which is manifested in a remarkable decrease in the anthocyanin content.[15] In another study, it was found that arils of pomegranates, which were stored in air enriched with 10 kPa CO_2, had a lower anthocyanin concentration than air-stored fruit.[16] In addition, Harb et al.[10] reported a dramatic loss in ascorbic acid and lower antioxidative capacity of water-soluble antioxidants in blueberries stored under CA conditions. In another study that addressed superatmospheric O_2 treatments with 'Duke' blueberries, researchers found significant increases in the antioxidant levels of blueberries that were stored at 60−100% O_2.[17]

Despite the above-mentioned findings, detailed studies that address quantitative and qualitative changes of polyphenols during storage of blueberries, as well as changes in the expression levels of selected genes that have roles in the biosynthesis of polyphenols, are limited. Accordingly, this study aims to quantify changes in polyphenols in blueberries in addition to the relative quantification of expression levels of some related genes. The findings of this study provide new insights into the postharvest biology of blueberries which may have implications on storage technologies for these fruits.

MATERIALS AND METHODS

Plant Material, Storage Conditions, and Determination of Quality Parameters. Blueberries (cultivar: Duke) were obtained from a private orchard located at Lake of Constance region, Germany. Directly after picking, fruits were stored at 1 °C (±0.3 °C) under the following CA-storage conditions (kPa O_2 + kPa CO_2): 21 + 0.03 (air-storage = control), 3 + 6, 3 + 12, 3 + 18, 10 + 6, 10 + 12, and 10 + 18. Storage containers were 26.4 L hermitic jars that were continuously monitored by gas analyzers. After 4 and 8 weeks of storage, portions of the stored blueberries were assessed for firmness, total soluble solids, and titratable acidity. For each treatment and sampling date, there were three biological replicates, each with 250 g (125 fruits) of berries. All measurements were conducted on the same sampling day after 2 h conditioning period at room temperature. Firmness of 20 fruits for each replicate was measured using a nondestructive device (FirmTech2, BioWorks, Wamego, KS) designed for soft fruits. Readings obtained are expressed in g mm^{-1}. The same fruits used for firmness measurements plus another 30 fruits (a total of 50 fruit per each biological replicate) were used for total soluble solids (TSS) and titratable acidity (TA) measurements. TSS of the juice was determined using a digital refractometer (PR-1; Atago Co. Ltd., Tokyo, Japan) and expressed as °Brix. TA was determined through titration of 10 mL juice plus 50 mL distilled water with 0.1 N NaOH to pH 8.1 using a fully automatic titration robot (Metrohm 702 SM Titrino, Filderstadt, Germany). TA was expressed in meq

Extraction and Determination of Individual Polyphenols Using LC−MS. Twenty fruits per biological replicate (60 fruits for each treatment with three biological replicates) were obtained after a storage period of 8 weeks to be rapidly frozen in liquid nitrogen. The samples were freeze-dried to powder, and 100 mg of the lyophilized tissue (from each biological replicate) was added to 500 μL of absolute methanol and 250 μL of the internal standard solution (50 mg biochanin A in 250 mL absolute methanol). After vortexing for 1 min and sonicating for 5 min, the mixed solutions were centrifuged at 13 200 rpm for 10 min. Supernatants were collected in fresh tubes, and pellets were extracted, vortexed, sonicated, and centrifuged once again as mentioned above. Supernatants were pooled and placed in a Speed-Vac for 2 h. The dried residue from each replicate was dissolved in 35 μL of water, sonicated for 10 min, and finally centrifuged at 13 200 rpm for 10 min. For each replicate, 20 μL of the clear supernatant was placed in a HPLC vial for final analysis using an LC−MS instrument (Agilent HPLC 1100; MS: Bruker Daltonics esquire3000plus, Santa Clara, CA) which was equipped with a Phenomenex column (Luna 3u C18 (2) 100A″, 150 mm × 2.0 mm (Part-Nr. 00F-4251-B0); Aschaffenburg, Germany). The analysis conditions were as follows: column temperature, 28 °C; injection volume, 5 μL; flow rate, 0.2 $mL.min^{-1}$. Solvents were as follows: A, 0.1% formic acid in water; B, 0.1% formic acid in methanol; gradient 0−30 min, 0−50% B; 30−35 min, 50−100% B; 35−50 min, 100% B; 50−55 min, 100−0% B; 55−65 min, 0% B; detection wavelength, 280 nm. MS details are as follows: dry gas, nitrogen at 330 °C; flow rate of 10 L min^{-1}; capillary, −4000 V; end plate offset, −500 V. The electrospray ionization voltage of the capillary was set to −4000 V and the end plate to −500 V. The full scan mass spectra were measured in a scan range from m/z 100 to 800. Tandem mass spectrometry was carried out using helium as collision gas (3.5−6 mbar) with the collision voltage set at 1 V. Spectra were acquired in both positive and negative ionization modes. Data analysis was performed using the DataAnalysis 5.1 software (Bruker Daltonics, Bremen, Germany). Relative concentrations of polyphenols were calculated on the basis of the values of the internal standard (Biochanin A; Sigma-Aldrich, Seelze, Germany).

Gene Expression Analysis. Samples were taken from berries that were stored for 8 weeks at the designated conditions. The gene expression analyses were conducted according to Harb et al.[18] with minor modifications. Ten berries per each biological replicate were randomly selected after 2 h conditioning period at room temperature, shock-frozen in liquid nitrogen, and ground to powder. RNA extraction was conducted according to the protocol of Chang[19] with modifications. Eight grams of powdered tissue were added to 20 mL prewarmed (65.0 °C) CTAB-based extraction buffer and incubated for 15 min at 65.0 °C. After centrifugation at 7740g for 10 min at room temperature, 15 mL from the clear supernatant was added to an equal volume of chloroform/isoamylalcohol (24:1), vortexed, and centrifuged at 7740g for 20 min at room temperature. This step was repeated twice, and RNA was precipitated overnight at 4.0 °C using 1 volume of LiCl (2.5 M). After pellets were washed, RNA was suspended in water. Quality and quantity of extracted RNA were assessed using gel-electrophoresis and the NanoDrop, respectively. The extracted RNAs were purified using RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA) and subsequently subjected to DNase treatment (RNase-Free DNase Set; Qiagen, Valencia, CA).

The qPCR analyses were conducted using a LightCycler 480II from Roche Diagnostics Deutschland GmbH (Mannheim, Germany) and SensiMix kit from Bioline GmbH (Luckenwalde, Germany). Taqman RT-PCR reagents from Applied Biosystems (Foster City, CA) were used to synthesize cDNA (2 μg DNA-digested RNA from each sample using random hexamer primers). Sequences from the NCBI database and also from the available literature, in particular Zifkin et al.,[20] served to design the following gene-specific primers using Primer3 software: *VcCHS*-F, 5′-cttgactgaggaaatcttgaagg-3′; *VcCHS*-R, 5′-agcctctttgcc-caatttg-3′ (GenBank: JN654702.1); *VcF3′H*-F, 5′-cgagattcgatg-cgtttctgagtg-3′; *VcF3′H*-R, 5′-gatttcggtatcggtgagcttcc-3′; *VcDFR*-F, 5′-cactgagtttaaggggattcctaagg-3′; *VcDFR*-R, 5′-cccttctccctacaagtgtcaatgg-3′; *VcANS*-F, 5′-cttcatcctccacaacatggt-3′; *VcANS*-R, 5′-gctcttgta-cttcccattgctc-3′ (GenBank: JN654701.1); *VcGAPDH*-F, 5′-ggttatcaatgataggtttggca-3′; *VcGAPDH*-R, 5′-cagtccttgcttgatggacc-3′ (GenBank: AY123769.1). *VcGAPDH* served as a reference gene. For each treatment and sampling date, there were two biological replicates, each with three technical replicates. The quantification of the expression levels of the targeted genes were calculated relative to the transcript abundance of the reference gene employing relative quantification with efficiency correction.[21]

Statistical Analysis. The experimental design was completely randomized with a single factor, namely the storage conditions. The CoStat statistical package (CoHort Software, Monterey, CA) was used for the analysis of variance (ANOVA). Comparisons of means were conducted using Student−Newman−Keuls test at $P \leq 0.05$. Moreover, the standrad error (SE) values were calculated and included (n = 3 for all parameters, except gene expression analyses; for those analyses n = 2 (two biological replicates, each with three technical replicates).

Table 1. Influence of Controlled Atmosphere Storage Condition (kPa O_2 + kPa CO_2) on Fruit Firmness (g × mm^{-1}), Total Soluble Solids (TSS, °Brix), and Titratable Acidity (meq) of Blueberries (cv. Duke) after Harvest (HT) and after Four and Eight Weeks Storage at 1 °C[a]

	firmness (g × mm^{-1}) (197.4 ± 10.1 at HT)		total soluble solids (°Brix) (9.0 ± 0.3 at HT)		titratable acidity (meq) (18.6 ± 1.0 at HT)	
storage condition	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
air-storage	215.0 ± 9.8 a	179.9 ± 9.2 c	9.2 ± 0.3	8.7 ± 0.0	18.2 ± 0.7	16.3 ± 0.1 b
03 + 06	228.5 ± 7.0 a	262.5 ± 3.5 a	9.2 ± 0.3	8.7 ± 0.0	19.4 ± 1.2	20.0 ± 0.3 a
03 + 12	190.6 ± 5.4 b	162.6 ± 8.3 c	8.9 ± 0.1	8.3 ± 0.0	19.2 ± 0.5	18.4 ± 0.8 a
03 + 18	153.1 ± 5.8 c	123.7 ± 1.1 d	9.05 ± 0.1	8.4 ± 0.2	18.6 ± 0.2	17.4 ± 0.8 a
10 + 06	224.2 ± 8.0 a	230.8 ± 14.8 b	9.6 ± 0.6	8.8 ± 0.1	19.5 ± 0.9	17.1 ± 0.4 a
10 + 12	188.6 ± 7.0 b	214.2 ± 12.4 b	9.2 ± 0.1	8.6 ± 0.1	18.4 ± 0.1	19.4 ± 0.0 a
10 + 18	168.9 ± 11.7 bc	136.6 ± 11.2 d	9.2 ± 0.0	8.3 ± 0.1	19.2 ± 0.0	18.0 ± 0.6 a
ANOVA	***	***	ns	ns	ns	**

[a]Mean separations were conducted using Student−Newman−Keuls test (n = 3). ± are SE values. Values followed by the same letter do not differ significantly in the column at $p \leq 0.05$.

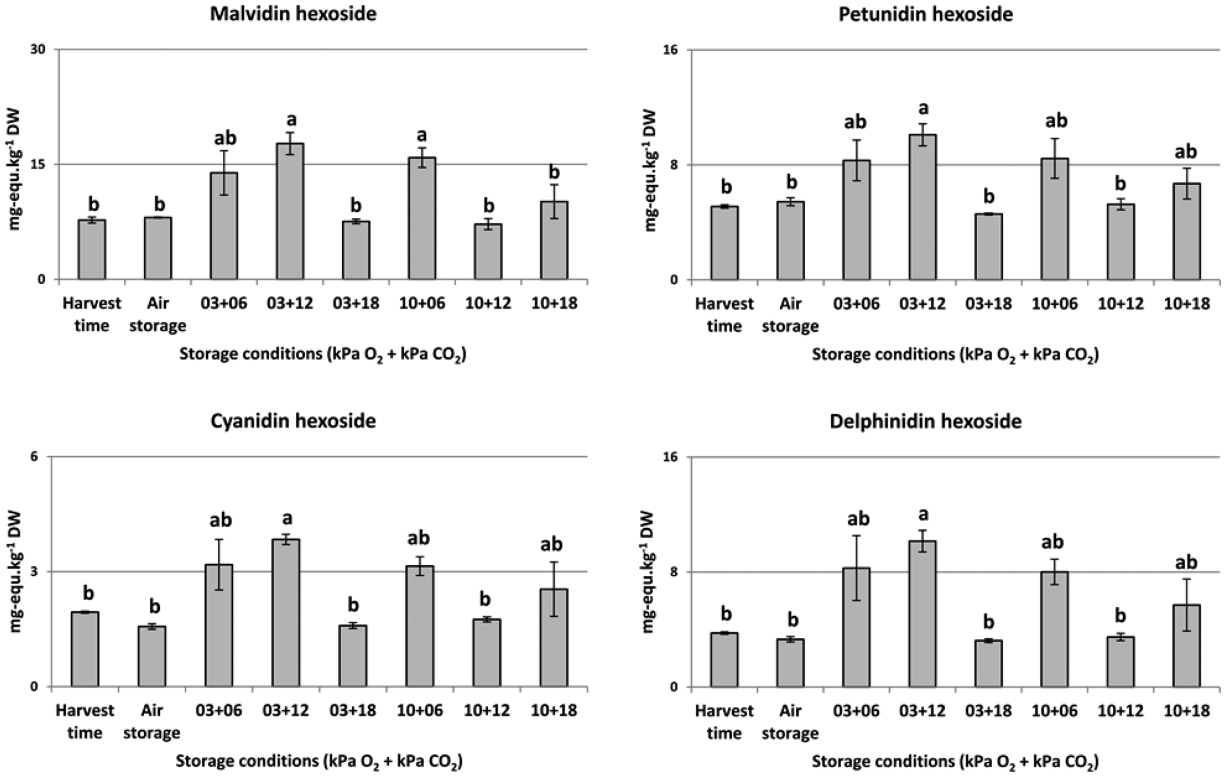

Figure 1. Influence of controlled atmosphere storage condition (kPa O_2 + kPa CO_2) on the relative content (mg-equ kg^{-1} DW) of malvidin hexoside, petunidin hexoside, cyanidin hexoside, and delphinidin hexoside of blueberries (cv. Duke) after 8 weeks storage at 1 °C. Mean separations were conducted using Student−Newman−Keuls test at $p \leq 0.05$ (n = 3). Values followed by the same letter do not differ significantly at $p \leq 0.05$. Error bars are SE values. Contents (Y-axis) are calculated relative to the internal standard (Biochanin A).

RESULTS

Quality Parameters. Storing blueberries for 8 weeks resulted in an accelerated softening of fruits that were stored under high CO_2 partial pressure (18 kPa) (Table 1), but with no significant differences between treatments in respect to total soluble solids contents. However, air-stored berries showed significant decrease in titratable acidity (TA) after 8 weeks of storage. Moreover, the sensory evaluation by the end of the storage trial revealed that berries stored under 3 + 6, 3 + 12, 10 + 6, and 10 + 12 (kPa O_2 + kPa CO_2) storage conditions tasted "good", whereas berries stored under 3 + 18 and 10 + 18 (kPa O_2 + kPa CO_2) had alcoholic off-flavor (data not shown).

Semiquantitative Changes in Polyphenols. The effects of various storage conditions on sets of polyphenols are shown in Figures 1−4. Figure 1 shows changes in the first group of polyphenols (hexosides of malvidin, petunidin, cyanidine, and delphinidin), and it is evident that the storage condition with very high CO_2 partial pressure (18 kPa) and low O_2 prevented the increase in the levels of these polyphenols. Moreover, it was found that blueberries had significantly lower levels of these polyphenols at harvest time as compared to berries that were stored at 3 kPa O_2 with 6 or 12 kPa CO_2. In addition, it is evident that the 3 kPa O_2 + 12 kPa CO_2 storage condition resulted in the highest levels for all compounds of the first group, whereas the levels of these compounds in air-stored

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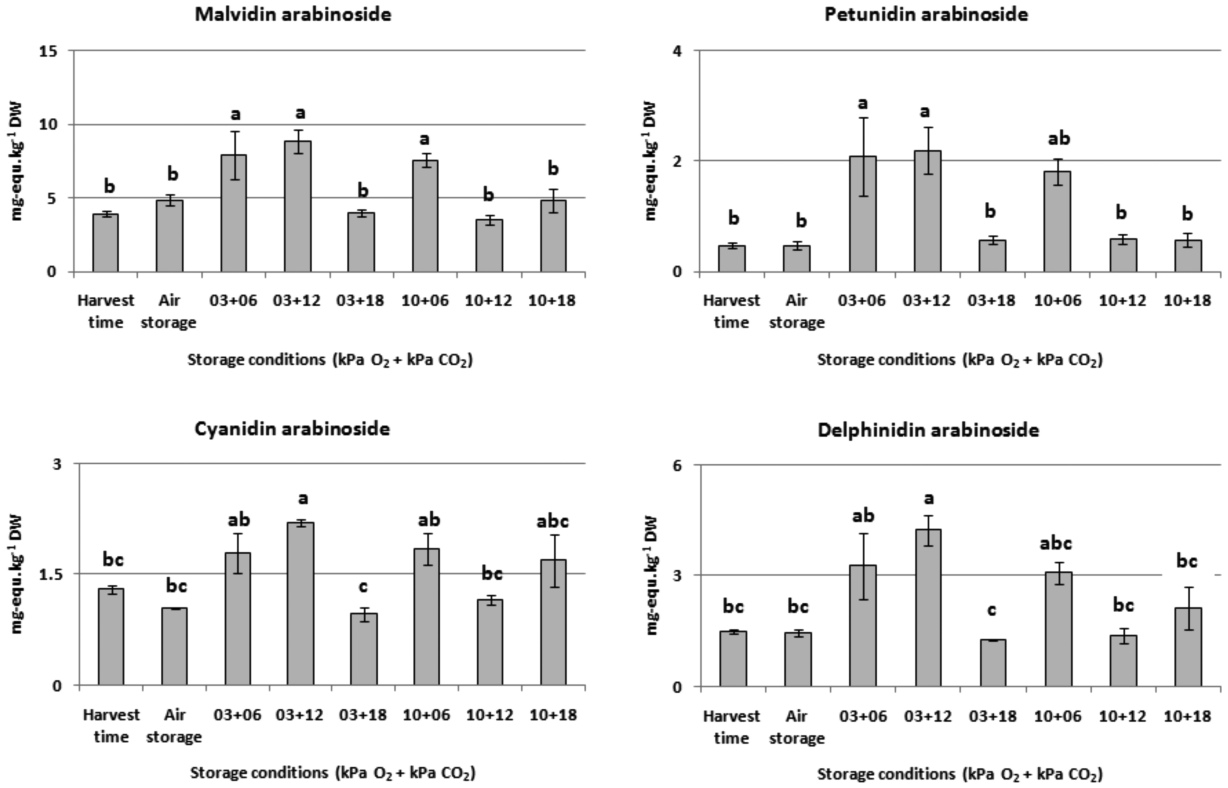

Figure 2. Influence of controlled atmosphere storage condition (kPa O_2 + kPa CO_2) on the content (mg-equ kg^{-1} DW) of malvidin arabinoside, petunidin arabinoside, cyanidin arabinoside, and delphinidin arabinoside of blueberries (cv. Duke) after 8 weeks storage at 1 °C. Mean separations were conducted using Student−Newman−Keuls test at $p \leq 0.05$ ($n = 3$). Values followed by the same letter do not differ significantly at $p \leq 0.05$. Error bars are SE values. Contents (*Y*-axis) are calculated relative to the internal standard (Biochanin A).

berries remained low with no significant differences with regard to harvest time.

The second group of polyphenols (arabinosides of malvidin, petunidin, cyanidine, and delphinidin; Figure 2) shows a similar trend to the first group, but with slight differences. Increasing O_2 partial pressure to 10 kPa led mostly to lower levels of the compounds. With this group of compounds, the influence of the highest CO_2 partial pressure (18 kPa) was more pronounced than with compounds of the first group.

The polyphenols of the third group (Figure 3) behaved differently. The highest CO_2 partial pressure (18 kPa) had less influence on their content. Moreover, it is clear that most storage conditions significantly induced, in comparison to harvest time, the biosynthesis of chlorogenic acid.

As for the compounds of the last group (Figure 4), two trends are clear: catechin (and also epicatechin; data not shown) levels were already high at harvest time, and declined steadily with all storage conditions, except under regular air-storage. The decrease was most noticeable under 3 kPa O_2 + 18 kPa CO_2. The second trend is for the unknown compounds (only unknown 1 is shown; Figure 4). It is clear that the highest partial pressure of CO_2 (18 kPa), irrespective of O_2 level, resulted in highly elevated levels of these compounds.

Expression of Polyphenol-Related Genes. Figure 5 depicts the expression levels of a few genes that encode enzymes involved in the biosynthesis of various polyphenols. The expression levels of chalcone synthase (*VcCHS*) and dihydroflavonol-4-reductase (*VcDFR*) were already high at harvest time, but declined significantly and drastically upon storage under very high CO_2 partial pressure levels. For both *VcCHS* and *VcDFR*, the decrease in the expression levels correlates well with the increasing partial pressure of CO_2, especially when combined with 3 kPa O_2. For both flavonoid 3′-hydroxylase (*VcF3′H*) and anthocyanidin synthase (*VcANS*) genes, berries at harvest time showed the highest expression levels, and differences between storage treatments are minimal with no clear trends between storage conditions.

DISCUSSION

A CO_2 enriched storage atmosphere is highly recommended to control decay in various fruits, including strawberry and blueberry.[17] Blanch et al.[22] attributed this antifungal efficacy of high CO_2 atmosphere to the induction of the biosynthesis of catechin, which is believed to reduce fungal decay. Despite this positive effect, our previous studies[23] showed that CO_2 partial pressure (above 12 kPa) led to an accelerated softening of fruits that renders them to be unmarketable. In the current study, several polyphenols (Figures 1 and 2) showed significant increases following storage under nonstressful CO_2 partial pressure (6−12 kPa), in particular when coupled with low O_2 partial pressure (3 kPa). Similar trends were reported by Kalt et al.,[24] who found that anthocyanins continued to be synthesized during storage. Schotsmans et al.[25] also found that blueberries showed the highest antioxidant activity after 6 days at 20 °C (shelf life), which indicates that the enzymatic machinery for flavonoids biosynthesis is still active during storage. Our results reveal also that medium to high (6 or 12 kPa) CO_2 partial pressure as well as low O_2 (3 kPa) partial pressure preserved the capacity of cells to synthesize flavonoids. This was clear with the major polyphenols, in particular malvidin hexoside, which is considered one of the major polyphenols in blueberry.[6] In contrast, Beaudry[26] set the lower O_2 limits for the storage of

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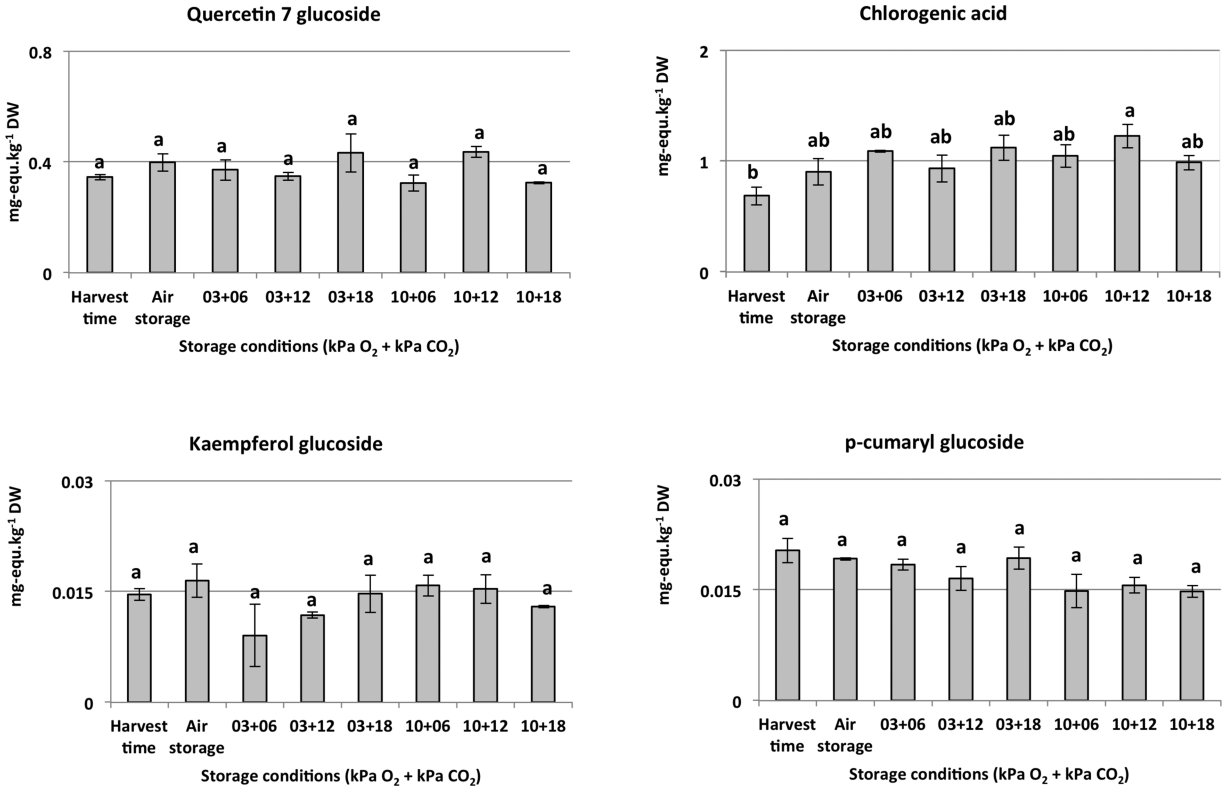

Figure 3. Influence of controlled atmosphere storage condition (kPa O_2 + kPa CO_2) on the content (mg-equ kg^{-1} DW) of quercetin-7-glucoside, chlorogenic acid, kaempferol glucoside, and p-coumaryl glucoside of blueberries (cv. Duke) after 8 weeks storage at 1 °C. Mean separations were conducted using Student−Newman−Keuls test at $p \leq 0.05$ ($n = 3$). Values followed by the same letter do not differ significantly at $p \leq 0.05$. Error bars are SE values. Contents (*Y*-axis) are calculated relative to the internal standard (Biochanin A).

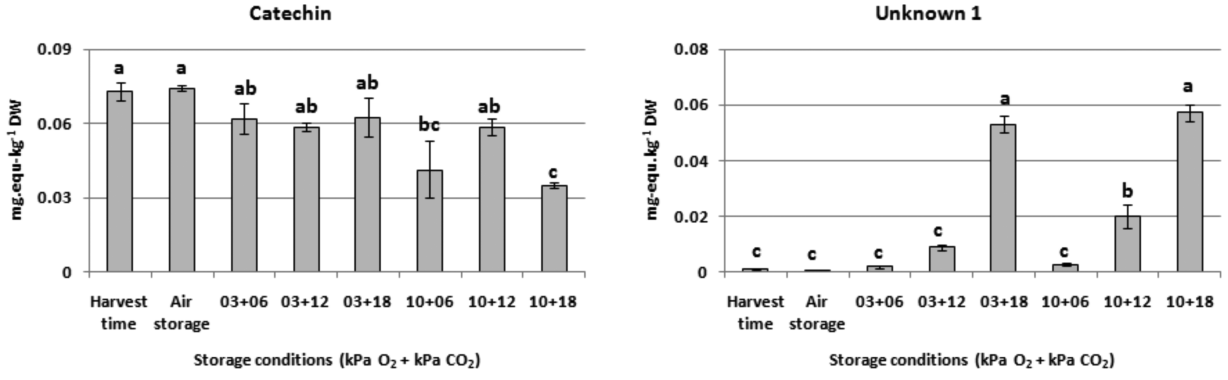

Figure 4. Influence of controlled atmosphere storage condition (kPa O_2 + kPa CO_2) on the content (mg-equ kg^{-1} DW) of catechin, and unknown 1 of blueberries (cv. Duke) after 8 weeks storage at 1 °C. Mean separations were conducted using Student−Newman−Keuls test at $p \leq 0.05$ ($n = 3$). Values followed by the same letter do not differ significantly at $p \leq 0.05$. Error bars are SE values. Contents (*Y*-axis) are calculated relative to the internal standard (Biochanin A).

blueberry (cvs 'Bluecrop' and 'Elliot') at 6, 7, and 11 kPa O_2 for the 5, 20, and 40 kPa CO_2 treatments, respectively. Taking into account that 'Duke' blueberries were used in our study, and that 3 kPa O_2 caused no negative effect, it is possible to predict that genotypes differ in their tolerance.

Referring to the above-mentioned impact of medium to high CO_2 partial pressure and low O_2 partial pressure on the biosynthesis of flavonoids, it is hard to elucidate the possible mechanism for such impact. Earlier studies showed that elevating CO_2 partial pressures in storage resulted usually in the prevention of significant increases in the levels of polyphenols. Since studies addressing blueberry, a nonclimacteric fruit, are rare, studies with other nonclimacteric fruits (e.g., strawberry and table grapes) will be used for further comparison and interpretation of the current results. With strawberry, it was found that air-storage, as well as storage under low CO_2 concentrations, resulted in higher levels of anthocyanin and intense red coloration compared to strawberries stored under elevated CO_2 concentrations.[14,15] In addition, it was reported that arils of pomegranates stored under 10 kPa CO_2 had lower anthocyanin concentration than air-stored fruit.[16] Moreover, various studies reported significant reductions in organic acids, mainly malic acid, upon storage at high CO_2 atmospheres.[14,27] Regarding anthocyanins, Holcroft and Kader[28] assumed that the slight changes in pH may affect the biosynthesis of anthocyanins. In this sense, it is worth

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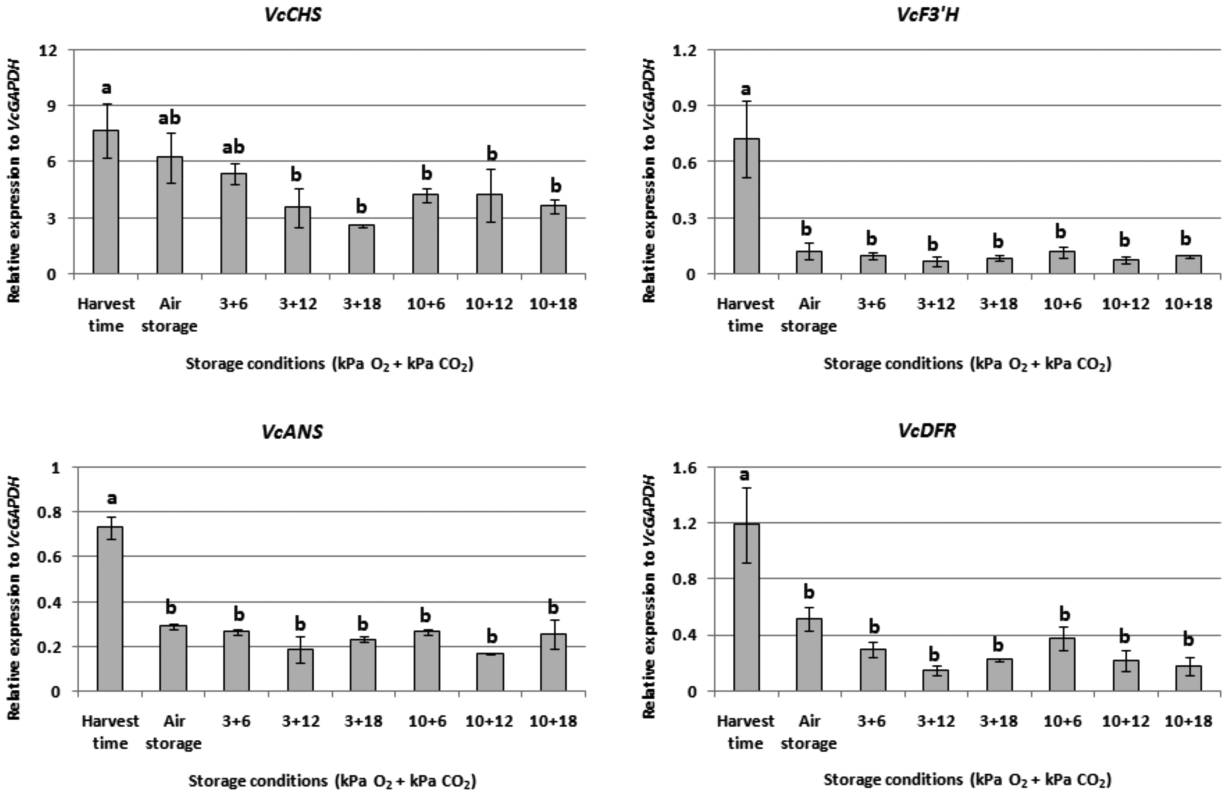

Figure 5. Influence of controlled atmosphere storage condition (kPa O_2 + kPa CO_2) on the expression levels of chalcone synthase (*VcCHS*), flavonoid 3′-hydroxylase (*VcF3′H*), anthocyanidin synthase (*VcANS*), and dihydroflavonol-4-reductase (*VcDFR*) of blueberries (cv. Duke) normalized to *VcGAPDH* (reference gene) after 8 weeks storage at 1 °C. Mean separations were conducted using Student−Newman−Keuls test at $p \leq 0.05$ ($n = 2$). Values followed by the same letter do not differ significantly at $p \leq 0.05$. Error bars are SE values.

mentioning that pH is an important factor in maintaining the stability of anthocyanins,[29] and lowering pH values improved the stability of strawberry color more than any other factor.[30] Concerning the slightly negative impact of 10 kPa O_2, compared to 3 kPa, it is most probable that the high O_2 partial pressure promoted the oxidation of polyphenols. Odriozola-Serrano et al.[31] reported that high O_2 concentrations around stored strawberries promoted greater losses of phenolic acids and vitamin C compared to low O_2 levels. Moreover, it was evident that oxygenation had a detrimental effect on bioactive phenolic compounds of red wine.[32]

In addition to the above-mentioned reductions in flavonoids under the highest CO_2 levels, changes in chlorogenic acid levels deserve special attention. This is due to the fact that chlorogenic acid is the main representative of the chlorogenic acid pathway and the major hydroxycinnamic acid in blueberries.[5,17] In the current study, high CO_2 coupled with either low or high O_2 levels had no negative impact on chlorogenic acid contents. In contrast, these conditions led to significant increases in chlorogenic levels during storage. Similar trends were also observed with loquat fruits, either during fruit ripening[33] or under CA-storage.[34] With pears, the chlorogenic acid content was the lowest by air-stored fruits, compared to fruits stored in CO_2 enriched environment.[35] In another study with 'Red d'Anjou' pear, 1-MCP treatment inhibited the transcription of key flavonoid biosynthetic genes with the concomitant decrease of chalcone synthase activity, which resulted in the diversion of the carbon flux from flavonoid pathway to chlorogenic acid pathway.[36]

Our results, as well as results from the above-mentioned studies, clearly indicate that the biosynthesis of chlorogenic acid through the chlorogenic acid pathway is independent from other polyphenols (Figures 1 and 2) that are synthesized by the flavonoid pathway. Accordingly, we hypothesize that high CO_2 may have negative impact on the expression of certain enzymes (e.g., chalcone synthase (*VcCHS*)) that are involved in flavonoid biosynthesis. In the current study, the expression level of *VcCHS* by freshly harvest blueberries is significantly higher than that for blueberries that were stored for 8 weeks under 18 kPa CO_2 condition (Figure 5); comparing storage treatments alone (without the harvest time) reveals that the expression level by air-stored blueberries is also significantly higher than those stored under 18 kPa CO_2 (data not shown). With grapes, a nonclimacteric fruit, storage under air at 0 °C increased phenylalanine ammonia-lyase, chalcone synthase and stilbene synthase mRNA levels in the skin, whereas the accumulation of these transcripts was lower in the skin of grapes that were stored for 3 days under high CO_2 level.[37] Taking into account that chalcone synthase is the first committed enzyme in flavonoid biosynthesis that catalyzes the formation of naringenin chalcone from 4-coumaroyl-CoA and malonyl-CoA to naringenin chalcone, its expression is crucial for the biosynthesis of a large set of polyphenols (e.g., malvidin hexoside). Although the start points for the control of anthocyanin differ between plant species,[38−40] we assume that the reduction in the expression of *VcCHS* is significant and may contribute highly to lower levels of polyphenols that are synthesized through the flavonoid pathway. In this respect, Gil et al.[15] reported that elevated CO_2 atmospheres led to bleaching of the strawberry internal flesh.

In conclusion, it is evident that storing 'Duke' blueberries under 18 kPa CO_2 significantly retarded, in comparison to

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other conditions with lower CO_2 partial pressures, the biosynthesis and accumulation of polyphenols from the flavonoid pathway. Such an effect was not recorded on polyphenols from the chlorogenic pathway. Further investigations are planned to assess changes at metabolome and transcriptome levels to elucidate the causes of the rapid loss of polyphenols upon storage under high CO_2 partial pressure. In addition, the interaction between the low levels of polyphenols and the rapid softening of blueberries, which were stored under 18 kPa CO_2, deserves further investigations.

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