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Elevated Carbon Dioxide Increases Contents of Antioxidant Compounds in Field-Grown Strawberries

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The effects of elevated CO2 concentrations on the antioxidant capacity and flavonoid content in strawberry fruit (*Fragaria x ananassa* Duch.) were studied under field conditions. Increased CO_2 (300 and 600 μmol mol^{-1} above ambient) concentrations resulted in increases in ascorbic acid (AsA), glutathione (GSH), and ratios of AsA to dehydroascorbic acid (DHAsA) and GSH to oxidized glutathione (GSSG), and a decrease in DHAsA in strawberry fruit. High anthocyanin and phenolic content were also found in fruit of CO_2 treated plants. Growing strawberry plants under CO_2 enrichment conditions significantly enhanced fruit *p*-coumaroylglucose, dihydroflavonol, quercetin 3-glucoside, quercetin 3-glucuronide, and kaempferol 3-glucoside contents, as well as cyanidin 3-glucoside, pelargonidin 3-glucoside, and pelargonidin 3-glucoside-succinate content. Fruit of strawberry plants grown in the CO_2 enrichment conditions also had high oxygen radical absorbance activity against ROO$^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, OH$^{\bullet}$, and $^1O_2$ radicals.

KEYWORDS: Antioxidant; anthocyanin; flavonoid; flavonol; phenolics; *Fragaria x ananassa*

INTRODUCTION

Carbon dioxide is one of the most limiting factors in photosynthesis. The possibility of improving photosynthesis in crops through CO_2 enrichment has interested agriculturists for many years (*1*). CO_2 enrichment has been shown to increase plant growth, development, and yield of agricultural crops, and this response is a function of CO_2 concentration and duration (*2*−*7*). Elevated CO_2 concentrations enhanced vegetative growth, biomass increment, carbohydrate accumulation, fruit productivity, and quality in strawberry under glass-house conditions (*8*). The concentration of carbon dioxide in the atmosphere is increasing rapidly, but there is little information on how strawberries may respond to elevated carbon dioxide under field conditions.

Strawberries are good sources of natural antioxidants (*9, 10*). In addition to the usual nutrients, such as vitamins and minerals, strawberries are also rich in anthocyanins, flavonoids, and phenolic acids (*10, 11*). Strawberries have shown a remarkably high scavenging activity toward chemically generated radicals, thus making them effective in inhibiting oxidation of human low-density lipoproteins (*10*). Our previous studies (*12, 13*) have shown that strawberries have high oxygen radical absorbance activity against peroxyl radicals (ROO$^{\bullet}$), superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH$^{\bullet}$), and singlet oxygen ($^1O_2$), and their antioxidant activities were different among varieties (*12, 13*). There is a positive correlation between antioxidant activity and total phenolic or anthocyanin content (*12, 13*). No information is available on the effect of CO_2 concentration on flavonoid content and scavenging capacity against active oxygen species of field-grown strawberry fruit. This study was performed to evaluate the effect of elevated carbon dioxide on antioxidant activity and flavonoid content in strawberry fruit.

MATERIALS AND METHODS

Chemicals. Ascorbate, β-carotene, chlorogenic acid, *p*-coumaric acid, 5,5′-dithio-bis-(2-nitrobenzoic acid (DTNB), histidine, hydrogen peroxide (30% w/w), glutathione (oxidized form), glutathione (GSH, reduced form), glutathione reductase (GR), guaiacol, hydroxylamine hydrochloride, kaempferol, *N*,*N*-dimethyl-*p*-nitrosoaniline, α-naphthylamine, R-phycoerythrin (R−PE) from *Porphydium cruentum*, quercetin, sodium nitrite, sodium tungstate dihydrate, sulfanilic acid, xanthine, and xanthine oxide were purchased from Sigma (St. Louis, MO). Ether, sodium hypochlorite, α-tocopherol, and titanium (IV) chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), *N*-ethylmaleimide, trichloroacetic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Salicylic acid was purchased from Fisher (Pittsburgh, PA). 2′, 2′ Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). EDTA (ethylenediaminetetracetic acid, disodium salt, dihydrate-Na_2 EDTA.$2H_2O$) was obtained from Life Technologies (Rockville, MD). All anthocyanins and aglycons were purchased from Indofine Chemical Co., Inc. (Somerville, NJ).

Plant Materials and Experimental Plans. *Fragaria x ananassa* Duchesne cv. Honeoye obtained as rooted runners from Miller Nurseries, Canandaigua, NY, were planted into field plots at the

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Beltsville Agricultural Research Center in March, 1998. Sixteen plants were transplanted into each of six open-topped clear acrylic chambers, each of which covered 1.1 m^2 of ground. Chambers were 1.8 m in height. A blower pulled air out of each chamber at the base, at a rate of 6 m^3 min^{-1}. Carbon dioxide was introduced into four of the chambers at the inlets of mixing fans positioned above the canopies. Flow rates of CO_2 were such that two chambers had a [CO_2] of 300 $\pm$ 50 μmol mol^{-1} above that of outside air, and two chambers had a [CO_2] of 600 $\pm$ 50 μmol mol^{-1} above that of outside air, while two chambers received no supplemental CO_2. Samples of air from each [CO_2] treatment were pumped sequentially through an absolute infrared analyzer in an adjacent air-conditioned shelter, and [CO_2], air temperatures, and photosynthetic photo flux density (PPFD) were logged every 5 min. The chambers transmitted 90% of the PPFD, and had air temperatures that averaged 1 °C above those of outside air. The mean daytime [CO_2] of ambient air was 353 μmol mol^{-1}, with concentrations at night of 400 to 600 μmol mol^{-1}. The ventilation of the chambers kept the humidity for all of the chambers the same as that of outside air. The duration of the entire experiment was 28 months (from early spring of 1998 continuing through the end of fruiting in June, 2000). Full details of this experimental plan were described in a previous publication (*14*).

Fruit Sample Preparation. Strawberry fruits were harvested at the commercially ripe stage in 1999 and 2000. The ripeness of fruit was determined by color. Firm, red-ripe fruits free from defects or decay were selected and lyophilized. The lyophilized samples were pulverized with a cold mortar and pestle in a cold double-distilled water and then centrifuged at 14 000*g* for 20 min at 4 °C. The supernatants were transferred to vials, stored at −80 °C, and then used for analyses.

Determination of Ascorbate (AsA) and Dehydroascorbate (DHAsA). AsA and DHAsA were determined using the methods of Arakawa et al. (*15*) and Nakagawara and Sagisaka (*16*). Total AsA (AsA plus DHAsA) was determined through a reduction of DHAsA to AsA by dithiothreitol. The AsA assay mixture contained 0.1 mL of the fruit extract, 0.5 mL of absolute ethanol, 0.6 M trichloroacetic acid, 3 mM bathophenanthroline, 8 mM H_3PO_4, 2 mM *N*-ethylmaleimide, and 0.17 mM $FeCl_3$. The final total volume was 1.5 mL, and the solution was allowed to stand at 30 °C for 90 min, for the Fe^{2+}-bathophenanthroline complex to develop. The absorbance of the colored solution was read at 534 nm. The total AsA assay mixture contained 0.1 mL of the fruit extract, 0.15 mL of 3.89 mM dithiothreitol, and 0.35 mL of absolute ethanol in a total volume of 0.6 mL. Then, the reaction mixture was left standing at room temperature for 10 min. After reduction of DHAsA to AsA, 0.15 mL of 0.24% *N*-ethylmaleimide in ethanol and 0.15 mL of 20% trichloroacetic acid were added. The color was developed by adding the following reagents in this sequence; 0.15 mL of 0.4% (v/v) H_3PO_4/ethanol, 0.3 mL of 0.5% (w/v) bathophenanthroline/ethanol and 0.15 mL of 0.03% (w/v) $FeCl_3$/ethanol. The final volume was 1.5 mL, and after incubation at 30 °C for 90 min, the absorbance at 534 nm was recorded. DHAsA concentrations were estimated from the difference of "total AsA" and "AsA" concentrations. A standard curve in the range of 0−10 μmol AsA or DHAsA was used.

Measurement of GSH and Oxidized Glutathione (GSSG). GSH and GSSG were assayed using the method described by Castillo and Greppin (*17*). Total glutathione equivalents were determined by reacting 0.1 mL fruit extracts with 60 mM KH_2PO_4/2.5 mM EDTA buffer (pH 7.5), 0.6 mM DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid)] in 200 mM Tris-HCl, pH 8.0, 1 unit of glutathione reductase (GR, from spinach, EC 1.6.4.2) and 0.2 mM NADPH. The reaction was followed as the rate of change in absorbance at 412 nm with a Shimadzu UV-160A spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD), and the total glutathione content was calculated from a standard curve. GSSG was determined after removal of GSH from the sample juice. GSH was determined from the reaction mixture by mixing 0.1 mL of fruit extract with 60 mM KH_2PO_4/2.5 mM EDTA buffer (pH 7.5), 0.6 mM DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid)] in 200 mM Tris-HCl, pH 8.0. The mixture was incubated at 30 °C for 10 min, and the reaction was followed as the rate of change in absorbance at 412 nm. GSSG was determined by subtraction of GSH from total glutathione.

Oxygen Radical Absorbance Capacity (ORAC) Assay. ORAC assays for fruit extract were carried out following procedures modified from a method previously described by Cao et al. (*18*). The reaction mixture contained 1.7 mL of 75 mM phosphate buffer (pH 7.0), 100 μl of R-PE (3.4 μmol mol^{-1}), 100 μL of 320 mM AAPH, and 100 μL of sample. Phosphate buffer was used as a blank, and 1 μM Trolox (a water-soluble α-tocopherol analogue) was used as a standard during each run. The final volume of 2 mL was used in a 10-mm wide fluorometer cuvette. R-PE, phosphate buffer, and samples were preincubated at 37 °C for 15 min. The reaction was started by the addition of AAPH. Fluorescence was measured and recorded every 5 min at the emission of 570 nm and excitation of 540 nm, using a Shimadzu RF-Mini 150 Recording Fluorometer (Shimadzu Scientific Instruments, Columbia, MD), until the fluorescence of the last reading decreased to less than 5% of the first reading. This usually took approximately 70 min. One blank, one standard, and a maximum of 10 samples were analyzed at the same time. Each sample was repeated three times. The ORAC value refers to the net protection area under the quenching curve of R-PE in the presence of an antioxidant. The final results (ORAC value) were calculated and expressed using Trolox equivalents per gram dry weight basis (*18*).

Superoxide Radical ($O_2^{\bullet-}$) Assay. The assay for $O_2^{\bullet-}$ was done using the methods of Elstner and Heupel (*19*) with slight modifications. The $O_2^{\bullet-}$ was generated by xanthine/xanthine-oxidase systems (*20*). Nitrite formation from hydroxylammonium chloride was determined at 530 nm in the spectrophotometer. The reaction mixture contained 1.0 mL of 65 mM Na-phosphate buffer (pH 7.8), 0.1 mL of 7.5 mM xanthine, 0.1 mL of 10 mM hydroxylammonium chloride, 0.1 mL of fruit extract, and 0.4 mL of double-distilled H_2O. The reaction was started by addition of 0.3 mL of xanthine oxidase (containing 60 μg of protein). The total reaction volume was 2.0 mL and was incubated at 25 °C for 20 min. Then, 0.5 mL was removed from the above reaction mixture, 0.5 mL of 19 mM sulfanilic acid and 0.5 mL of 1.0% α-naphthylamine were added, and the mixture was shaken for 5 min. After standing at room temperature for 20 min, the optical density of the mixture was determined at 530 nm against blanks that had been prepared similarly but without fruit extract. The final results were expressed as percent inhibition of $O_2^{\bullet-}$ production in the presence of fruit extract. The scavenging capacity of α-tocopherol at various concentrations (1 to 10 μg) on superoxide radical ($O_2^{\bullet-}$) was measured and used for determining the $O_2^{\bullet-}$ scavenging capacity of fruit extract. The antioxidant capacity of fruit extract against the $O_2^{\bullet-}$ value was expressed as μmole of α-tocopherol equivalent per gram dry weight.

H_2O_2 Assay. The assay for hydrogen peroxide in fruit extract of strawberry was carried out following procedures previously described by Patterson et al. (*21*). This assay measures the direct reaction of hydrogen peroxide and Ti (IV). Titanium reagent (135 μL of 20% $TiCl_4$ in conc HCl) was added to 100 μL of fruit extract, 0.815 mL Na-phosphate buffer (0.17 M, pH 7.4), 200 μL of NH_4OH (17.0 M) and 100 μL of H_2O_2 (1.35 mM) to give a Ti−H_2O_2 complex (precipitated). The precipitate was dissolved in 3 mL of 1 M H_2SO_4. The reaction was measured at 410 nm against blanks that had been prepared similarly but without fruit extract. The final results were expressed as percent inhibition of H_2O_2 production in the presence of fruit extract. The scavenging capacity of ascorbate at various concentrations (1 to 10 μg) on hydrogen peroxide (H_2O_2) was measured and used for determining the H_2O_2 scavenging capacity of fruit extract. The antioxidant capacity of fruit extract against H_2O_2 was expressed as μmole of ascorbate equivalent per gram dry weight.

Hydroxyl Radical ($OH^{\bullet}$) Assay. The assay for $OH^{\bullet}$ was done using the methods of Richmond et al. (*20*) with slight modifications. The $OH^{\bullet}$ in aqueous media is generated through the Fenton reaction. The reaction mixture contained 0.24 M K-phosphate buffer (pH 7.4), 1.0 mM salicyclic acid, 0.3 mM $FeSO_4$/EDTA (4 mM), 0.8 mM H_2O_2, and 100 μL of fruit juice. The total reaction volume was 5.0 mL and was incubated at 25 °C for 90 min. Then, 120 μL of 6 M HCl was added, followed by extraction in 4 mL of chilled ether. Ether was evaporated to dryness in a water bath at 40 °C, and the residue was dissolved in 1 mL of cold double-distilled water, to which the following was added: 0.5 mL of 10% (w/v) trichloroacetic acid in 0.5 M HCl, 1 mL of 10% (w/v) sodium tungstate, 1 mL of 0.5% (w/v) $NaNO_2$. After standing for 5 min, absorbance at 510 nm was read immediately after adding 2 mL of 0.5 M KOH. Relative scavenging efficiency

Table 1. Effect of Elevated Carbon Dioxide Treatments on Ascorbic Acid (AsA), Dehydroascorbic Acid (DHAsA), Glutathione (GSH), and Oxidized Glutathione (GSSG) Content in Fruit of Strawberry[a]

CO_2 treatment (μmol mol^{-1})	(μmol/g dry weight)		(nmol/g dry weight)		ratio	
	AsA	DHAsA	GSH	GSSG	AsA/DHAsA	GSH/GSSG
ambient (350)	32.3 ± 1.3	6.0 ± 1.2	745.2 ± 7.3	160.0 ± 2.4	5.38	4.66
ambient +300	35.6 ± 0.4	3.8 ± 0.3	768.0 ± 8.8	164.9 ± 2.1	9.37	4.66
ambient +600	36.6 ± 0.4	3.1 ± 0.2	2019.9 ± 22.9	210.6 ± 18.2	11.8	9.59
$LSD_{0.05}$	0.42	0.35	18.7	46.8	1.84	1.58
significance[b]	sig	sig	sig	ns	sig	sig

[a] Data expressed as mean ± SEM ($n = 12$). [b] sig = significant, ns = nonsignificant at $p \leq 0.05$.

(% inhibition of hydroxylation) of fruit extract was estimated from the difference in absorbance (OD) with and without addition of the fruit extract. The scavenging capacity of chlorogenic acid at various concentrations (1 to 10 μg) on hydroxyl radical ($OH^{\bullet}$) was measured and used for determining the $OH^{\bullet}$ scavenging capacity of fruit extract. The antioxidant capacity of fruit extract against $OH^{\bullet}$ value was expressed as μmole of chlorogenic acid equivalent per gram dry weight.

Singlet Oxygen ($^1O_2$) Assay. The production of $^1O_2$ by sodium hypochloride and H_2O_2 was quantity by using a spectrophotometric method according to Chakraborty and Tripathy (*22*) with minor modifications in which N, N, dimethyl-*p* nitrosoaniline was used as a selective scavenger of $^1O_2$ and histidine as trap for $^1O_2$ acceptor. The bleaching of *N*, *N*, dimethyl-*p*-nitrosoaniline, as induced by the reaction of $^1O_2$ with histidine, was monitored spectrophotometrically at 440 nm. The assay mixture contained 45 mM Na-phosphate buffer (pH 7.1), 10 mM histidine, 10 mM NaOCl, 10 mM H_2 O_2, 50 mM of *N*, *N*, dimethyl-*p*- nitrosoaniline, and 0.1 mL of fruit extract. The total reaction volume was 2.0 mL and was incubated at 30 °C for 40 min. The extent of $^1O_2$ production was determined by measuring the decrease in absorbance of *N*, *N*, dimethyl-*p*-nitrosoaniline at 440 nm. Relative scavenging efficiency (% inhibition production of $^1O_2$) of fruit extract was estimated from the difference in absorbance of *N*, *N*, dimethyl-*p*-nitrosoaniline with and without the addition of fruit extract. The scavenging capacity of β-carotene at various concentrations (1 to 10 μg) on singlet oxygen ($^1O_2$) was measured and used for determining the $^1O_2$ scavenging capacity of fruit extract. The antioxidant capacity of fruit extract against $^1O_2$ was expressed as μmole of β-carotene equivalent per gram dry weight.

HPLC Analysis of Strawberry Anthocyanins and Phenolic Compounds. HPLC was used to separate and quantity individual anthocyanins and phenolic compounds in strawberry tissue samples. Fruit samples of 0.5 g (powders) were extracted twice with 15 mL of acetone. The mixtures were shaken at 400 rpm at room temperature on an orbital shaker for 30 min. Extracts (30 mL) were combined and concentrated to 1 mL, using a Buchler Evapomix (Fort Lee, NJ) in a water bath at 35 °C. The concentrated sample was dissolved in 10 mL of acidified water (3% formic acid) and then passed through a C_{18} Sep-Pak cartridge (Waters), which was previously activated with methanol followed by water and then 3% aqueous formic acid. Anthocyanins and other phenolics were adsorbed onto the column, while sugars, acids, and other water-soluble compounds were eluted with 10 mL of 3% aqueous formic acid. The anthocyanins and other phenolics were then recovered with 2.0 mL of acidified methanol containing 3% formic acid. The methanolic extract was passed through a 0.45-μm membrane filter (Millipore, MSI, Westboro, MA), and 20 μL was analyzed by HPLC. The samples were analyzed using a Waters (Waters Associates, Milford, MA) HPLC system equipped with two pumps (600 E system Controller) and coupled with a photodiode array detector (Waters 990 Series). Samples were injected at ambient temperature (20 °C) onto a reverse phase NOVA-PAK C_{18} column (150 × 3.9 mm, particle size 4 μm) with a guard column (NOVA-PAK C_{18}, 20 × 3.9 mm, particle size 4 μm) (Sentry guard holder universal) (Waters Associates, Milford, MA). The mobile phase was acidified water containing 2.5% formic acid (A) and acetonitrile (B) in a linear gradient from 5% to 20% B in the first 15 min, followed by a linear gradient from 20 to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30 to 90% B for 5 min, an isocratic mixture for 2 min before returning to the initial conditions. The flow rate was 1.0 mL/min, and the wavelengths of detection were set at 320, 350, and 510 nm. Scanning between 240 and 550 nm was performed, and data were collected by the Waters 990 3-D chromatography data system. Retention times and spectra were compared to pure standards.

Statistical Analysis. Sixteen plants were transplanted into each chamber. Two replicate chambers were used per CO_2 treatment (ambient, ambient + 300, and ambient + 600 μmol mol^{-1} CO_2). Three samplings (4 fruit/sampling) were taken from each chamber for chemical analyses. The experiments were replicated for two years. Data from two years were combined and were subjected to analysis of variance, and means were compared by least significant difference (LSD). The effect of CO_2 concentrations on the values of AsA, DHAsA, GSH, GSSG, and flavonoid concentrations in strawberry fruit samples and their antioxidant capacity to $ROO^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, and $^1O_2$ radicals were evaluated by the Tukey-Kramer multiple-comparison test used in NCSS (*23*). Differences at $P < 0.05$ were considered significant.

RESULTS

Strawberries had higher concentrations of AsA and GSH when plants were grown under enriched CO_2 environments (**Table 1**). An increase of CO_2 level (ambient + 300, and ambient + 600 μmol mol^{-1} CO_2) resulted in an increase in AsA and GSH and a decrease in DHAsA in strawberries. AsA concentration increased from 32.3 μmol/g dry weight in ambient atmosphere grown strawberries to 36.6 μmol/g dry weight in the 600 μmol mol^{-1} CO_2-enriched environment. The highest DHAsA level was at 6.0 μmol/g dry weight for ambient fruit, whereas the lowest DHAsA content occurred in the 600 μmol mol^{-1} CO_2-enriched condition. GSH content was 745.2 nmol/g dry weight in ambient fruit and increased to 2019.9 nmol/g dry weight in the 600 μmol mol^{-1} CO_2-enriched fruit. Strawberry growth under CO_2 enrichment conditions enhanced the ratio of AsA to DHAsA. The ratio of the GSH to GSSG was also found increased in the 600 μmol mol^{-1} CO_2-enriched environment. The effect of CO_2 enrichment conditions on GSSG content showed no significant difference in strawberries.

The effect of enrichment CO_2 on oxygen radical absorbance activity against $ROO^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, and $^1O_2$ radicals in strawberries were significant (**Table 2**). Strawberry growth under CO_2 enrichment conditions significantly enhanced strawberry fruit $ROO^{\bullet}$ absorbance capacity, as well as $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, and $^1O_2$. Plants grown in the 600 μmol mol^{-1} CO_2 enrichment conditions had the highest oxygen radical absorbance capacity. In strawberry, the antioxidant capacity values against $ROO^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, and $^1O_2$ in the fruit grown in ambient conditions were 131.9 μmol Trolox/g dry weight, 42.1 μmol α-tocopherol/g dry weight, 19.6 μmol ascorbate/g dry weight, 43.3 μmol chlorogenic acid/g dry weight, and 5.1 μmol β-carotene/g dry weight, respectively (**Table 3**). Elevation of CO_2 over the concentration range from 300 to 600 μmol mol^{-1} all resulted in an increase in values of oxygen radical absorbance capacity. The highest CO_2 enrichment (600 μmol mol^{-1}) yielded fruit with the most $ROO^{\bullet}$ absorbance capacity, as well as $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, and $^1O_2$ absorbance capacity (**Table 2**).

Table 2. Effect of Elevated Carbon Dioxide Treatments on Antioxidant Activity Against Peroxyl Radicals (ROO•), Superoxide Radicals ($O_2^{\bullet -}$), Hydrogen Peroxide (H_2O_2), Hydroxyl Radicals (OH•), and Singlet Oxygen ($^1O_2$) in Fruit of Strawberry[a]

CO_2 treatment (μmol mol^{-1})	ROO• ORAC (μmol TE/g dry wt)[b]	$O_2^{\bullet -}$ (μmol α-tocopherol/g dry wt)[c]	H_2O_2 (μmol ascorbate/g dry wt)[d]	OH• (μmol chlorogenic acid/g dry wt)[e]	$^1O_2$ (μmol β-carotene/g dry wt)[f]
ambient (350)	131.9 ± 6.4	42.1 ± 0.7	19.6 ± 0.9	43.3 ± 2.3	5.1 ± 0.3
ambient +300	145.8 ± 7.6	44.6 ± 0.6	21.3 ± 0.2	51.2 ± 0.2	6.2 ± 0.2
ambient +600	181.9 ± 2.5	47.2 ± 0.8	22.6 ± 0.6	52.2 ± 0.3	7.1 ± 0.4
$LSD_{0.05}$	3.48	0.75	0.43	0.37	0.54
significance[g]	sig	sig	sig	sig	sig

[a] Data expressed as mean ± SEM (n=12). [b] Data expressed as micromoles of Trolox equivalent per gram of dry weight. [c] Data expressed as micromoles of α-tocopherol equivalent per gram of dry weight. [d] Data expressed as micromoles of ascorbate equivalent per gram of dry weight. [e] Data expressed as micromoles of chlorogenic acid equivalent per gram of dry weight. [f] Data expressed as micromoles of β-carotene equivalent per gram of dry weight. [g] sig = significant at $p \leq 0.05$.

Table 3. Effect of Elevated Carbon Dioxide Treatments on Flavonoid Content in Fruit of Strawberry[a]

	CO_2 treatment (μmol mol^{-1})				
compound	ambient (350)	ambient (+300)	ambient (+600)	$LSD_{0.05}$	signif[e]
p-coumaroylglucose[b]	417 ± 25.1	591 ± 32.4	735 ± 23.8	58.0	sig
dihydroflavonol[b]	152 ± 17.3	177 ± 12.1	561 ± 15.3	7.3	sig
quercetin 3-glucoside and quercetin 3-glucuronide[c]	27 ± 11	89 ± 9	108 ± 18	6.3	sig
kaempferol 3-glucoside[c]	52 ± 2	67 ± 3	84 ± 5	5.4	sig
kaempferol 3-glucuronide[c]	56 ± 1	57 ± 4	54 ± 2	3.2	ns
cyanidin 3-glucoside[d]	561 ± 24	817 ± 35	1153 ± 57	102.4	sig
cyanidin 3-glucoside-succinate[d]	67 ± 9	73 ± 4	75 ± 8	5.1	ns
pelargonidin 3-glucoside[d]	2124 ± 115	3224 ± 122	3669 ± 131	178.1	sig
pelargonidin 3-glucoside-succinate[d]	323 ± 14	537 ± 21	681 ± 29	57.6	sig

[a] Data expressed as mean ± SEM (n = 12). [b] Data expressed as micrograms of *p*-coumaric acid equivalent per gram of dry weight. [c] Data expressed as micrograms of quercetin 3-glucoside equivalent per gram of dry weight. [d] Data expressed as micrograms of cyanidin 3-glucoside equivalent per gram of dry weight. [e] sig = significant, ns = nonsignificant at $p \leq 0.05$.

High CO_2 growing conditions significantly enhanced the strawberry fruit content of *p*-coumaroylglucose, dihydroflavonol, quercetin 3-glucoside, quercetin 3-glucuronide, and kaempferol 3-glucoside, as well as those of cyanidin 3-glucoside, pelargonidin 3-glucoside, and pelargonidin 3-glucoside-succinate. Fruit from plants grown under ambient conditions had the lowest phenolic acids, flavonols, and anthocyanins. An enriched CO_2 environment resulted in an increase in phenolic acid, flavonol, and anthocyanin contents of fruit. The highest CO_2 enrichment (600 μmol mol^{-1}) condition yielded fruit with the highest levels of these compounds (**Table 3**).

DISCUSSION

The results indicated that rising atmospheric concentrations of carbon dioxide could have a major impact on antioxidant capacities of strawberry fruit. Elevated CO_2 concentrations enhanced the content of AsA and DHAsA and the ratio of AsA/DHAsA in strawberries. AsA is an outstanding antioxidant and reducing agent, and may play a major role in protecting cells and cell components against free radicals and oxidant damage (*24*). AsA provides electrons for enzymes, for chemical compounds that are oxidants, or for other electron acceptors (*25*). The AsA oxidation product DHAsA is efficiently reduced by cells back to AsA, which then becomes available for reuse (*26*). AsA reduces superoxide radical [with a rate constant of 2.7×10^5 M^{-1} s^{-1} ($2\ O_2^{\bullet -}$ + ascorbate + $2H^+ \rightarrow$ dehydroascorbate + $2\ H_2O_2$)], hydrogen peroxide (H_2O_2 + 2 ascorbate $\rightarrow$ 2 monodehydroascorbate + $2\ H_2O$], hydroxyl radical, hypochlorous acid, and other reactive oxidant species (*24*). Increased AsA, DHAsA, and the ratio of AsA/DHAsA in elevated CO_2-treated strawberries may allow for the removal of free radicals, which is associated with increased antioxidant capacity.

GSH is a tripeptide, composed of cysteine, glutamic acid, and glycine, and is the most abundant nonprotein thiol in cells. Its active group is the thiol (−SH) of cysteine. The majority of glutathione in the cell is maintained in the reduced state. The GSH plays an important role in the stabilization of many enzymes. In addition, as an oxidant scavenger, it serves as a substrate for DHAsA reductase and is also able to react directly with free radicals, including the hydroxyl radical, to prevent the inactivation of enzymes by oxidation of an essential thiol group (H_2O_2 + 2GSH $\rightarrow$ 2 H_2O + GSSG) (*27*). GSH also directly reduces OH• radicals (*28*) and scavenges $^1O_2$ with a rate constant of 10^6 M^{-1} s^{-1} (*29*). GSSG consists of two GSH molecules joined by their −SH groups into a disulfide bridge and was found to be present in low quantities compared to GSH. We found that CO_2 enhancement increased GSH concentration and the GSH/GSSG ratio. A high GSH and GSH/GSSG ratio is necessary for several physiological functions. Those include activation and inactivation of redox-dependent enzyme systems (*30*) and regeneration of cellular antioxidant AsA under oxidative conditions (*24*). Increased GSH and the ratio of GSH/GSSG in CO_2-enhanced conditions was associated with increased antioxidant activity.

Strawberries possess antioxidant activities against active oxygen species (*9*, *12*). Strawberry growth under CO_2 enrichment conditions significantly enhanced strawberry fruit on antioxidant capacity (inhibition of the free radicals ROO•, $O_2^{\bullet -}$, H_2O_2, OH•, and $^1O_2$). This indicated that strawberry fruit grown

with CO_2 enrichment had high scavenging activity for chemically generated active oxygen species.

Flavonoids consist of at least two phenyl rings separated by a pyran ring. The antioxidant activity of flavonoids critically depends on the part of the polyphenol molecule with better electron-donating properties. The ability of flavonoids to scavenge superoxide, $O_2^{\bullet-}$, and alkyl peroxyl radicals is particularly important. The reduction rate constant ($k \sim 10^2 - 10^6$ M^{-1} s^{-1}) of superoxide radicals by flavonoids is the highest found among biological compounds. The quenching of singlet oxygen, $^1O_2$, by flavonoids is also very fast and efficient ($k \sim 10^4 - 2.2 \times 10^8$ M^{-1} s^{-1}) and comparable to that of vitamin E (*31*). These flavonoids have been shown to have anticancer properties and also to have applications for use as antibiotics, antidiarrheals, antiulcer, and antiinflammatory agents, as well as in the treatment of diseases such as hypertension, vascular fragility, allergies, and hypercholesterolemia (*10*, *11*, *32*, *33*). *p*-Coumaroylglucose, dihydroflavonol, quercetin 3-glucoside, quercetin 3-glucuronide, kaempferol 3-glucoside, and kaempferol 3-glucuronide have been detected in strawberries (*34*−*36*). These flavonols are effective antioxidants (*37*). Kaempferol and quercetin are potent quenchers of $ROO^{\bullet}$, $O_2^{\bullet-}$, and $^1O_2$ (*33*). Quercetin and other polyphenols have been shown to play a protective role in carcinogenesis by reducing bioavailability of carcinogens (*38*). Quercetin, with 3′,4′-dihydroxy substitution in the B-ring and conjugation between the A- and B-rings, has high antioxidant potential (*39*). The antioxidant capacities measured by the ORAC assay for quercetin and kaempferol are 3.29 and 2.67, respectively (*40*). CO_2 enrichment conditions significantly enhanced flavonol content in strawberry fruit, and high flavonol content was associated with high antioxidant activity.

Anthocyanin and phenolic compounds are largely responsible for the antioxidant capacity in plant tissues (*40*). Anthocyanidins are hydroxylated and methylated derivatives of phenyl-2-benzopyrrylium. In the typical U. S. diet, daily intake of anthocyanins is approximately 180−215 mg/day and represents the largest group of phenolic compounds in the human diet (*32*). Natural anthocyanins are glycosides, which release aglycone forms (anthocyanidins) by hydrolysis. Some common anthocyanidins have varying hydroxyl or methyl substitutions on their basic structure, flavylium ion. Over 250 naturally occurring anthocyanins exist, and they are differentiated further by their *O*-glycosylation with different sugar substitutes (*41*). The common anthocyanins are either 3- or 3,5-glycosylated. Free radical scavenging properties of the phenolic hydroxyl groups attached to ring structures are responsible for the strong antioxidant properties of the anthocyanins (*11*, *42*). Differences in antioxidant capacity of anthocyanins could be ascribed to individual molecular structures with the possible following explanations: (i) Increasing the number of hydroxyl groups may increase antioxidant activity (*40*). (ii) The *o*-dihydroxy structure in the B ring confers higher stability to the radical form and participates in electron delocalization (*43*). Thus, the dihydroxylation in the 3′, 4′ positions of the B ring play an important role in antioxidant activity. (iii) The glycosylation of flavonoids may reduce their activity when compared to corresponding aglycones (*44*). (iv) The unsaturation in the C ring allows electron delocalization across the molecule for stabilization of aryloxy radicals due to existing conjugation. Anthocyanins have been reported to help reduce damage caused by free-radical activity, such as low-density lipoprotein oxidation, platelet aggregation, and endothelium-dependent vasodilation of arteries (*10*, *11*). Anthocyanin and phenolic contents in strawberry fruit grown under 600 μmol $mol^{-1}CO_2$ enrichment conditions were significantly higher than those for ambient fruit and 300 μmol $mol^{-1}CO_2$-enriched fruit. The increase in anthocyanins and phenolics in elevated CO_2-treated strawberries associated with increased antioxidant capacities may allow for quenching of the excited state of active oxygen species (*33*, *45*).

In summary, strawberry fruit contain flavonoids with potent antioxidant properties, and under CO_2 enrichment conditions, increased the AsA, GSH, phenolic acid, flavonol, and anthocyanin concentrations, as well as the ratios of AsA/DHAsA and GSH/GSSG. Plants grown under CO_2 enrichment conditions also had higher oxygen radical absorbance activity against $ROO^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, and $^1O_2$ radicals in the fruit.

ABBREVIATIONS USED

AAPH, 2′, 2′ Azobis (2-amidinopropane) dihydrochloride; ORAC, oxygen radical absorbance capacity; R-PE, (*R*)-phycoerythrin; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TE, Trolox equivalent.

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