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Influence of elevated CO_2 on nutritive value and health-promoting prospective of three genotypes of Alfalfa sprouts *(Medicago Sativa)*

Mohammed S. Almuhayawi[a,*,1], Abdelrahim H.A. Hassan[b,1], Soad K. Al Jaouni[c], Dalal Hussien M. Alkhalifah[d,*], Wael N. Hozzein[e,f], Samy Selim[g,h], Hamada AbdElgawad[f], Galal Khamis[i]

[a] Department of Clinical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[b] Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt
[c] Hematology/Pediatric Oncology, Yousef Abdulatif Jameel Scientific Chair of Prophetic Medicine Application, Faculty of Medicine, King Abdulaziz University, Jeddah 21589, Saudi Arabia
[d] Department of Biology, College of Science, Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia
[e] Bioproducts Research Chair, Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
[f] Botany and Microbiology Department, Faculty of Science, Beni-Suef University, Beni-Suef 62521, Egypt
[g] Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Sakaka, P.O. 2014, Saudi Arabia
[h] Botany Department, Faculty of Science, Suez Canal University, Ismailia, P.O. 41522, Egypt
[i] Department of Laser Applications in Metrology, Photochemistry and Agriculture (LAMPA), National Institute of Laser Enhanced Sciences, Cairo University, Cairo, Egypt

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ABSTRACT

Alfalfa sprouts are well known for their nutritive values. Although there are several studies reported the positive impact of elevated CO_2 (eCO_2) on plants, there are no in-depth, comprehensive studies on how eCO_2 could improve the sprouting of plant seeds. Herein, the production of health-promoting metabolites was determined in eCO_2 (620 ppm)-treated Alfalfa sprout cultivars (Giza 1, Nubaria and Ismailia 1). eCO_2 increased the photosynthetic process and pigment contents, which consequently induced carbohydrates, proteins, fats and fiber accumulation. eCO_2 also boosted the levels of vitamins, phenolics, flavonoids and mineral individuals and enhanced the antioxidant capacity of alfalfa sprouts. Interestingly, eCO_2 reduced the antinutritional factor L-canavanine content in Ismailia 1 cultivar and improved the anti-inflammatory activities through inhibiting cyclooxygenase-2 and lipoxygenase activity. Therefore, eCO_2 is a promising approach to improve the health-promoting prospective of alfalfa sprouts to be a valuable source of nutritious and bioactive compounds in our daily diet.

1. Introduction

Medicago sativa (Alfalfa) is a perennial flowering herbal plant, belongs to the pea family (Fabaceae). Alfalfa leaves contain high levels of vitamins (*e.g.*, vitamin A, B, C, D, E and K) and minerals (*e.g.*, phosphorus, calcium, iron and potassium) (Grela & Pietrzak, 2014), as well as, their anti-inflammatory effect was recorded (Karimi et al., 2013). These major bioactive compounds in Alfalfa and other legumes showed a wide range of biological activities such as antioxidant, anticancer, antiallergenic, antimutagenic, anti-inflammatory, antihepatotoxic and molluscicidal effects (Costa et al., 2015). For instance, the leaves extract of alfalfa was found rich in antioxidants that reduced the inflammation induced by both cyclooxygenase (COX-2) and lipoxygenase (LOX), two enzymes involved in inflammatory pathways (Mukherjee, Nissen, & Topol, 2001).

Human may consume alfalfa as sprouts dehydrated leaves or dietary supplements in the forms of tablets or powder (Karimi et al., 2013). For example, Alfalfa sprouts are usually consumed as a salad and most of their sprouts are approximately fluffy, crunchy and sweet (Hedges, 2006). Sprouts are the young seedling produced through seed germination; they were globally used for thousands of years as vegetables and became more popular in the western countries as a functional food (Mattioli et al., 2019). Sprouts are valuable sources of protein, minerals, vitamins, glucosinolates and phenolic compounds (Marton, Mandoki, Caspo & Caspo-Kiss, 2010). It is well-reputed as healthy food due to the presence of phenolic compounds which revealed high antioxidant

* Corresponding authors.
E-mail addresses: msalmuhayawi@kau.edu.sa (M.S. Almuhayawi), dhalkalifah@pnu.edu.sa (D.H.M. Alkhalifah).
[1] These authors contributed equally to this study.

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activities (Silva et al., 2013). One of the most dominant nutrients in Alfalfa sprouts is B vitamins, mostly thiamine (Hedges, 2006). Thus, sprouting is an inexpensive and efficient mechanism to improve the medical values of legumes. For instance, alfalfa sprouts reduced cholesterol levels, kidney and boil problems and arthritis. They also can reduce the H_2O_2-induced DNA damage (Silva et al., 2013) and protect the human body against several chronic and cardiovascular diseases (Marton, Mandoki, Caspo & Caspo-Kiss, 2010).

During the last few decades, numerous studies intended to further improve the growth and bioactivity of sprouts (Hedges, 2006). Similar to the sprouting process, slightly acidic electrolyzed water, UV-B irradiation (Kim, Feng, Kushad, & Fan, 2006) and high atmospheric CO_2 (eCO_2) are considered innovative approachs to enhance plant growth and its nutritive values (Saleh, Selim, Jaouni, & AbdElgawad, 2018). As a result of climate change and global anthropogenic activities, CO_2 levels will be tremendously increased to double concentration by 2100 (Jing et al., 2016). eCO_2 altered the chemical composition of plant species by changing the carbon and nitrogen metabolism (Li et al., 2017). Thus, the availability and redistribution of the valuable metabolites for growth could be affected (Högy et al., 2009). These studies demonstrated that eCO_2 enhanced the photosynthesis process, which eventually induces the accumulation of carbohydrates in the dark respiration (Azam, Khan, Mahmood, & Hameed, 2013; Li et al., 2017). Consequently, it provides the precursor and metabolic energy required for the production of bioactive phytochemicals (Oh, Trick & Rajashekar, 2009). For example, eCO_2 significantly increased the bioactivity of several herbal plants such as parsley, dill, basil and peppermint (Al Jaouni et al., 2018; Saleh et al., 2018). Several active metabolites such as carbohydrates, amino acids, vitamins (e.g., K1), and antioxidants (e.g, ascorbate) were increased in basil and peppermint through eCO_2 (Saleh et al., 2018). Moreover, it improved the antibacterial and anticancer activities of basil and peppermint (Al Jaouni et al., 2018). Additionally, eCO_2 improved the plant quality and biological activities by increasing the levels of antioxidants and anti-lipid peroxidation in fenugreek seeds (Hozzein, Saleh, Habeeb, Wadaan, & AbdElgawad, 2020). Moreover, eCO_2 showed an increase in the grain quality and gluten content of wheat grains (Högy et al., 2009).

Although there are several studies addressed the effect of eCO_2 on plant nutritive values, to our knowledge, there are no reports regarding the effect of eCO_2 on the chemical composition and nutritional values of Alfalfa sprouts. Therefore, we carried out this study not only to improve the sprouting conditions through high CO_2 but also to give more in-depth details on how high CO_2 improves the sprouting process. To this end, we assessed the photosynthesis process as the main source of carbohydrates, which consequently provides the precursor of bioactive phytochemicals. Measured health bioactive phytochemicals included pigments, phenolics, flavonoids, vitamins and antioxidants. As well as, mineral profile, the antinutritional factor L-canavanine and the anti-inflammatory properties were evaluated. We presume that eCO_2 can be applied as an innovative approach not only to enhance the nutritional and health-promoting metabolites of Alfalfa sprouts but also to reduce some antinutritional compounds like L-canavanine.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of Alfalfa (Medicago sativa) cultivars were collected from three different locations in Egypt (Giza 1, Nubaria and Ismailia 1). The seeds were washed with distilled water and soaked for 1 h in 5 g L^{-1} sodium hypochlorite, then kept overnight in distilled water. The seeds of each cultivar equally spread on 10 trays filled with vermiculite and watered with Milli-Q water every two days. The trays were moved and kept under controlled growth conditions adjusted to 25 °C air temperature, 16/8 day night photoperiod controlled via cool white fluorescent tubes with photosynthetically active radiation (PAR) of 400 μmol m^{-2} s^{-1} and 60% humidity. According to IPCC-SRES B2-scenario prediction of high CO_2 (Murray & Ebi, 2012), Alfalfa seeds were germinated in growth controlled cabinet under two CO_2 conditions, 1) Ambient CO_2 (aCO_2, 400 ± 27 μmol CO_2 mol^{-1} air); 2) Elevated CO_2 (eCO_2, 620 ± 42 μmol CO_2 mol^{-1}air ppm). Five trays per each cultivar for each CO_2 condition were used. CO_2 concentration was continuously monitored (CO_2 analyser, WMA-4, PP Systems, Hitchin, UK). The sprouts from each cultivar and treatment were taken after 10 days and weighed as fresh mass. Samples were frozen in liquid nitrogen and stored (−80 °C) for biochemical analysis.

2.2. Photosynthesis analysis

Photosynthesis (μmol CO_2 m^{-2} s^{-1}) were determined by EGM-4 infrared gas analyzer (PP Systems, Hitchin, UK). Whole sprouts photosynthetic rate was determined from 180 s measurements of net CO_2 exchange (NE).

2.3. Determination of total carbohydrates, protein, lipids and fibers

Carbohydrates were measured by Nelson's method (Clark & Switzer, 1977) from each Alfalfa sprouts cultivar treated with ambient and eCO_2. Protein concentration was evaluated for each frozen Alfalfa sprouts samples (0.2 g FW) according to the Folin-Lowry method adopted by Hartree (1972). Total lipids were evaluated according to Folch modified by Bligh and Dyer (1959), where Alfalfa sprout samples were homogenized in a solvent mixture of chloroform/ methanol (2:1, v/v). Then, the mixture was centrifuged for 15 min at 3000*g*. A rotary evaporator was used to evaporate the chloroform phase containing lipids. The pellet was re-dissolved in 2 mL of toluene/ethanol mixture (4/1, v/v). The extract was mixed and equilibrated with a saline solution. The extracted lipids were concentrated by a rotary evaporator and extracted lipids were weighed in vials to calculate the total lipid content. Fibers were extracted from Alfalfa sprouts samples and measured following to AOAC (1990) method. Samples were gelatinized with a heat-stable alpha-amylase at pH 6, and 100° for 30 min and then enzymatically digested sequentially with protease at pH 7.5 and 60 °C for 30 min) and amyloglucosidase at pH 6 and 0 °C for 30 min to remove protein and starch. Fiber content was precipitated with ethanol, the residue was weighed after washing.

2.4. Analysis of mineral contents

For evaluation of macro and micro-elements, (200 mg) from each Alfalfa sprouts cultivar treated with eCO_2 and control was digested in a HNO_3/H_2O solution (5:1 v/v) in an oven. The inductively coupled plasma mass spectrometry (ICP-MS, Finnigan Element XR, and Scientific, Bremen, Germany) was used to measure the concentrations of macro-minerals and at 25 °C trace elements, where nitric acid in 1% was used as standards (AbdElgawad et al., 2019).

2.5. Determination of L-canavanine content

The frozen Alfalfa sprout samples were homogenized in aqueous ethanol through a MagNALyser, then centrifuged for 20 min at 14,000*g*/4 °C. The extracts contained canavanine were analyzed by reverse-phase HPLC connected with UV/VIS detector after derivatization using diethyl ethoxymethylenemanolate and 20 μL of the samples wasseparated on a Novapack C18 column (300 × 3.9 mm i.d., 4 μm, Waters) maintained at 18 °C. Two mobile phases were used i.e., A (25 mM glacial acetic acid and 0.02% sodium azide (w/v), pH 6.0) and B (acetonitrile). The two mobile phases were filtered through a 0.45 mm membrane filter and run at the flow-rate of 0.9 mL/min.

2.6. Measurement of the antioxidant capacity, phenols, flavonoids, and antioxidant enzymes

The antioxidant capacity was conducted in vitro through ferric reducing antioxidant power (FRAP) method according to (Al Jaouni et al., 2018, Hamed et al., 2020). About 0.2 g of each Alfalfa sprouts samples was evaluated by 80% ethanol and centrifuged for 20 min at 14,000 rpm. Where 0.1 mL of diluted extract was used to determine the antioxidant capacity after mixing with 0.25 mL of FRAP reagent (mixing FeCl3 (20 mM) in acetate buffer (0.25 M, pH 3.6) at room temperature (Soud, Hegab, AbdElgawad, Zinta & Asard, 2013). The absorbance was taken at 517 nm. The activities of peroxidase (POX) and catalase (CAT) were measured in each frozen Alfalfa sprouts in 1 mL of 50 mM MES/KOH (pH6.0) extraction buffer after 10 min of incubation (Hamed et al., 2017; Sinha et al., 2015; Casasole et al., 2017).

Chromatographic techniques such as HPLC and GC/MS, were used to measure the levels of phenolic acids, flavonoids and vitamins in Alfalfa sprouts treated with eCO_2 and control (Hamad et al., 2015). The metabolites were identified by comparing the standard mixture to the relative retention time of each metabolite from each sample. The concentration of each metabolite was calculated using the peak area of the corresponding standard.

Phenolic acids and flavonoids were measured by HPLC (Hamad et al., 2015). 50 mg of freeze-dried sprouts were mixed in 4:1 v/v acetone–water solution. The Shimadzu HPLC system (SCL-10 AVP, Japan), equipped with a Lichrosorb Si-60, 7 μm, 3 × 150 mm column, diode array detector). 90:10 (v/v) of water-formic acid 85:10:5 (v/v/v) of and acetonitrile/water/formic acid were employed a mobile phase at 0.8 mL/min (flow rate) and the internal standard was 3,5-dichloro-4-hydroxybenzoic. The concentration of each metabolite was calculated using the peak area of the corresponding standard.

2.7. Determination of the contents of vitamins and pigments

To determine the contents of ascorbate, tocopherols, thiamine, riboflavin and pigments in sprouts, UV and/or fluorescence detectors were used for the separation and detection processes according to AbdElgawad et al. (2015). Ascorbate was extracted in 1 mL of 6% (w/v) meta-phosphoric acid at 4 °C and was separated by reversed-phase HPLC coupled with UV detector (100 mm × 4.6 mm Polaris C18-A, 3 lm particle size; 40 °C, isocratic flow rate: 1 mL min^{-1}, elution buffer: 2 mM KCl, pH 2.5 with *O*-phosphoric acid). Tocopherol was separated on Particil Pac 5 μm column material (length 250 mm, i.d. 4.6 mm) and quantified by HPLC (Shimadzu's Hertogenbosch, normal phase conditions), coupled with fluorometric detector (excitation at 290 nm and emission at 330 nm). Riboflavin and thiamine were separated on a reverse-phase (C18) column (HPLC, methanol:water as mobile phase and fluorescence as a detector). The frozen Alfalfa sprouts plant samples were homogenized in acetone through a MagNALyser (Roche, Vilvoorde, Belgium, 1 min, 7000 rpm), then centrifuged for 20 min at 14 000*g*, 4 °C. The supernatant was taken and filtered (Acrodisc GHP filter, 0.45 μm 13 mm). After that, the solution was analyzed by using HPLC (Shimadzu SIL10-ADvp, reversed-phase, at 4 °C) (AbdElgawad et al., 2015). Carotenoid separation was performed on a silica-based C18 column (Waters Spherisorb, 5 μm ODS1, 4.6 × 250 mm) using two types of solvent; A) consisting of acetonitrile: methanol: water as 81:9:10 and solvent B) consisting of methanol: ethyl acetate as 68:32. The extraction of Chlorophyll *a* and b, beta-carotene and xanthophylls were evaluated through a diode-array detector (Shimadzu SPD-M10Avp) at different four wavelengths (420, 440, 462 and 660 nm, respectively).

2.8. Cyclooxygenase-2 (COX-2) and lipoxygenase (15-LOX) assays

At least three replicates were used from each Alfalfa sprout cultivar treated with ambient CO_2 or eCO_2 to measure COX-2 by the COX assay kit (Cayman chemical company, Ann Arbor, MI, USA). Samples were homogenized in water through a MagNALyser (Roche, Vilvoorde, Belgium, 1 min, 7000 rpm).

According to the manufacturer's instructions, the 96-well microtitre plate was covered with plastic film and maintained at room temperature for 18 h on an orbital shaker, then kept for 90 min at room temperature in dark condition. The plate reading was taken at 420 nm. The inhibition of enzyme activity was calculated according to the following formula: % inhibition = 100-(($^{OD}sample$ - $^{OD}blank$ / $^{OD}control$) × 100).

For the anti-lipoxygenase evaluation, 15-LOX was used as an enzyme and linoleic acid as a substrate. Alfalfa sprout extracts (10 mg/mL) were prepared and 10 μL from each extract were taken and mixed with 90 μL of 15-LOX (400 U/mL) and maintained for 5 min at 25 °C in dark condition. The reaction started when 100 μL (0.4 mM) of linoleic acid solution were added to each well and the reaction kept for 20 min at 25 °C in dark condition. Then, 100 μL of freshly prepared ferrous orange xylenol (FOX) in 90% methanol, 10 μM $FeSO_4$, 100 μM xylenol orange and 30 mM H_2SO_4 were added. After 30 min of reaction at 25 °C, the absorbances were measured at 560 nm and the inhibition percentage was calculated using the following equation: % inhibition = 100-(($^{OD}sample$ - $^{OD}blank$ / $^{OD}control$) × 100)..

2.9. Statistical analysis

To pereform the statistical analyses, we used the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Each experiment was replicated at least two times and for all assays, 3 to 5 replicates were used and each replicate corresponded to a group of sprouts harvested from a certain tray. One-Way Analysis of Variance (ANOVA) was done. Tukey's test was used as the post-hoc test for the separation of means ($P < 0.05$). Hierarchical clustering (Pearson correlation) was generated by MultiExperimental Viewer ((TM4 software package).

3. Results and discussion

3.1. Elevated CO_2 enhanced the growth of alfalfa sprouts by increasing photosynthetic pigments

It is well known that the nutritive value of alfalfa sprouts is strongly related to their contents of bioactive primary and secondary metabolites such as phenols, pigments and vitamins (Karimi et al., 2013). Herein, we investigated the fertilizing impact of eCO_2 on biomass accumulation and the bioactive metabolite contents in alfalfa cultivars. In this regard, eCO_2 was used as a growth promoter for several plants and crops (Azam, Khan, Mahmood, & Hameed, 2013). The exposure of different cultivars of alfalfa sprouts to eCO_2 significantly increased the growth of all cultivars ($p < 0.05$) as compared to control (Table 1). eCO_2 treated Ismailia 1 cultivar showed the highest biomass, followed by Giza 1 and Nubaria. These elevations in biomass were 2.09, 1.83 and 1.6-fold for Ismailia 1, Nubaria and Giza 1, respectively (Table 1). In the line of our findings, eCO_2 improved the growth of three cultivars of broccoli sprouts (Almuhayawi et al., 2020). eCO_2 also increased the yield of carrot (*Daucus carota* L. cv. T-1-111), radish (*Raphanus sativus* L. cv. Mino) and turnip (*Brassica rapa* L. cv. Grabe) by 69, 139 and 72%, respectively (Azam, Khan, Mahmood, & Hameed, 2013).

The improved biomass under eCO_2 can be attributed to improved photosynthesis processes and photosynthetic pigment biosynthesis and accumulation. It is well known that eCO_2 could improve the photosynthesis by suppressing the oxygenation reaction of rubisco, leading to increased C gain (Watanabe et al., 2014). For instance, Gorka, Juan, Pilar, Rafael, & Manuel, (2006) reported that short-term exposure of nodulated alfalfa to eCO_2 increased its net photosynthesis and consequently improved its growth. We found that the exposure of Alfalfa sprouts to eCO_2 increased the levels of all tested pigments (chlorophyll *a*, chlorophyll *b*, β-carotene, lutein, neoxanthin and

Table 1
Effect of elevated CO_2 on the growth (gFW), photosynthesis, total nutrients (mg/gDW), L-canavanine, and contents of minerals of different alfalfa sprouts cultivars. Values are represented by mean ± standard deviation of at least three independent replicates. Means marked by an asterisk (*) are significantly different than control at $p < 0.05$.

Parameters	Control			Elevated CO_2		
	Giza 1	Nubaria	Ismailia 1	Giza 1	Nubaria	Ismailia 1
Growth and Photosynthesis						
Growth (gFW)	0.12 ± 0.01	0.06 ± 0.02	0.13 ± 0.13	0.19 ± 0.04*	0.112 ± 0.01*	0.27 ± 0.08*
Photosynthesis μmol CO_2 m^{-2} s^{-1}	5.1 ± 0.08	6.4 ± 0.6	6.46 ± 1	7.4 ± 0.5*	9.4 ± 1.1*	10.3 ± 1.0*
Total nutrients and L-canavanine						
Total proteins	17.5 ± 0.21	20.2 ± 2.0	24.1 ± 1.7	22.6 ± 1.91*	28.8 ± 2*	32.1 ± 1.9*
Free amino acids	11.1 ± 0.85	13.1 ± 1.1	16.1 ± 0.7	19.5 ± 1.3*	21.8 ± 1.6*	24.9 ± 1.2*
L-canavanine	4.0 ± 0.4	3.89 ± 0. 9	4.4 ± 0.2	4.53 ± 1.0	4.1 ± 1.0	3.2 ± 0.1*
Total lipids	0.87 ± 0.05	0.84 ± 0.03	1.33 ± 0.05	1.24 ± 0.01	1.39 ± 0.1*	2.17 ± 0.03*
Carbohydrates	24.7 ± 1.2	17.7 ± 1.2	25.6 ± 2. 2	34.3 ± 1.2*	28.2 ± 2*	35.5 ± 1.7*
Fibers	13.3 ± 0.9	16.1 ± 1. 2	15.0 ± 0.8	15.1 ± 0.9	21.0 ± 1.7*	26.7 ± 2.3*
Mineral profile						
Ca (mg/gDW)	3.3 ± 0.97	2.67 ± 0.4	2.66 ± 0.4	2.93 ± 0.29*	3.21 ± 0.89*	2.93 ± 0.1*
Cu (μg/gDW)	9.1 ± 2.85	9.68 ± 3.17	13.1 ± 3.7	11.5 ± 1.1	15.8 ± 3.5*	18.9 ± 5.8*
Fe (μg/gDW)	12.9 ± 4.8	16.4 ± 3.84	20.46 ± 3	19.47 ± 2.5	20.4 ± 5.1*	25.3 ± 0.5*
Zn (μg/gDW)	46.9 ± 1.4	53.38 ± 4.8	60.8 ± 5.5	59.6 ± 5.4*	68.21 ± 6.3	78.7 ± 7.3*
Mn (mg/gDW)	2.5 ± 0.03	2.12 ± 0.1	2.39 ± 0.1	2.37 ± 0.1	3.39 ± 0.2*	3.25 ± 0.2
Mg (mg/gDW)	5.69 ± 0.4	15.54 ± 1.3	6.53 ± 0.5	9.8 ± 0.8*	12.84 ± 1.1	14.6 ± 1.2*
K (mg/gDW)	16.3 ± 1.4	11.11 ± 0.9	18.1 ± 1.6	24.6 ± 2.2*	21.23 ± 1.9	28.6 ± 2.5
P (μg/gDW)	47.9 ± 4.4	46.37 ± 4.2	52.7 ± 4.8	73.3 ± 6.6*	81.2 ± 7.3*	65.6 ± 6.2
Na (mg/gDW)	2.56 ± 0.2	1.68 ± 0.1	2.44 ± 0.1	1.97 ± 0.1	3.3 ± 0.2*	2.54 ± 0.1

violaxanthin) in all cultivars as compared to their controls, except β-carotene in Giza 1 (Fig. 1). The elevation in pigment contents by eCO_2 was highly pronounced in Nubaria and Ismailia 1 cultivars ($p < 0.05$). For example, Ismailia 1 cultivar showed the highest levels of pigments, except in violaxanthin, which was higher in both Giza 1 and Nubaria. Pigments are not only important for photosynthesis, but pigments such as chlorophyll *a*, chlorophyll *b*, β-carotene and lutein possess a high potential activity as scavengers of the toxic reactive oxygen species (Young, 1991). These plant pigments also previously reported a wide range of biological activities such as the prohibition of cancer risk, reducing the inflammation and enhancing the immune response (Boo et al., 2012). As well as, the tested pigments showed antimicrobial activities against *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, and *Vibrio parahaemolyticus* (Boo et al., 2012). Moreover, plant synthesized β types of carotenoids pigments (β-carotene and β-cryptoxanthin) are a precursor for vitamin A, which is fundamental for the vision, growth and cell differentiation (Fernández-García et al., 2012). Overall, improving the levels of these pigments in alfalfa sprouts by eCO_2 treatment could enhance the benefits of these sprouts as a functional food with health-promoting antioxidants.

3.2. Elevated CO_2 improved the nutritive value of alfalfa sprouts

The development and growth of plants mainly depend on photosynthesis, where plants use CO_2 from the atmosphere through photosynthesis to synthesis energy required for plant metabolism (Al Jaouni et al., 2018). Also, eCO_2 enhances the rate of carbon gain along with improving the efficient use of nitrogen level, thus eCO_2 can boost the rate of photosynthesis resulting in an accumulation of carbohydrates, which may be then broken down by increased dark respiration to produce energy (Noguchi, Watanabe, & Terashima, 2015; Li et al., 2017; Leakey et al., 2009). Thus, it provides the metabolic energy required for the synthesis of various types of nutrients and bioactive compounds.

Accordingly, eCO_2 could be an efficient approach for improving the nutrient contents in sprouts (Li et al., 2017). In this regard, eCO_2 significantly improved the contents of starch and soluble sugars in parsley, dill and alfalfa plants (Saleh, Selim, Jaouni, & AbdElgawad, 2018; Sgherri, Quartacci, Menconi, Raschi, & Navara-Izzo, 1998). Our study indicated a positive effect of short-term exposure of high atmospheric CO_2 on the bioactive primary nutrients such as total protein, total lipids, carbohydrates and fibers in all alfalfa cultivars (Table 1). Total protein content was increased by approximately 73% in all treated alfalfa sprout cultivars after exposure to eCO_2 as compared to control ones. The improvements in the protein content of Ismailia 1, Nubaria and Giza 1 were 1.33, 1.42 and 1.29-fold, respectively. A similar scenario was observed for the total lipid, carbohydrates and fiber contents. The reported elevations of fiber content values in e-CO_2-treated cultivars were 1.78, 1.3 and 1.13-fold in Ismailia 1, Nubaria and Giza 1, respectively. Generally, eCO_2-treated Ismailia 1 alfalfa sprouts had the highest values of each nutrient content as compared to the other cultivars (Table 1). In line with our findings, Al Jaouni et al. (2018) reported an increase in the total values of carbohydrates, proteins and lipids in eCO_2-treated plants (620 ppm). Amongst the factors that affect the nutritional quality of plants are free amino acids, especially in cereals and legumes (Ufaz & Galili, 2008). It was reported that eCO_2 induced the availability of C skeleton and metabolic energy needed for the biosynthesis of amino acids (Noguchi et al., 2015; Nunes-Nesi, Fernie, & Stitt, 2010). On the other side, L-canavanine is a non-protein amino acid found commonly in alfalfa seeds and other legume foods. L-canavanine can prompt inflammation, systemic lupus erythematosus and toxicity as it reduced feed intake and growth rate in chicks (Michelangeli & Vargas, 1994; Constantin et al., 2019). In the current study, we found that eCO_2 did not significantly affect the concentrations of L-canavanine in Nubaria and Giza 1 cultivars but it significantly reduced it by 37% in Ismailia 1 cultivar (Table 1).

In addition to the abovementioned nutrients, alfalfa sprouts are rich in vitamins such as vitamin B (Hedges, 2006). The impact of eCO_2 on the levels of vitamins in plants is poorly studied, whereas very limited studies reported improved vitamin C and E contents in eCO_2-treated medicinal plants (Al Jaouni et al., 2018). Here we also recorded considerable amounts of several vitamins as the most dominant (Fig. 2). eCO_2 treatment significantly ($p < 0.05$) improved almost all investigated vitamin contents in Nubaria and Ismailia 1. For instance, eCO_2-treated Ismailia 1 exhibited the highest values of vitamin C, thiamin and riboflavin. In Giza 1, vitamin C and riboflavin were slightly increased ($p > 0.05$), while vitamin E and thiamin were reduced by eCO_2 treatment. Correspondingly, there are some recent reports on the effect of eCO_2 on vitamin levels in plant tissues such as vitamin K1 (phylloquinone), A, B and E in basil, peppermint, parsley and dill (Al

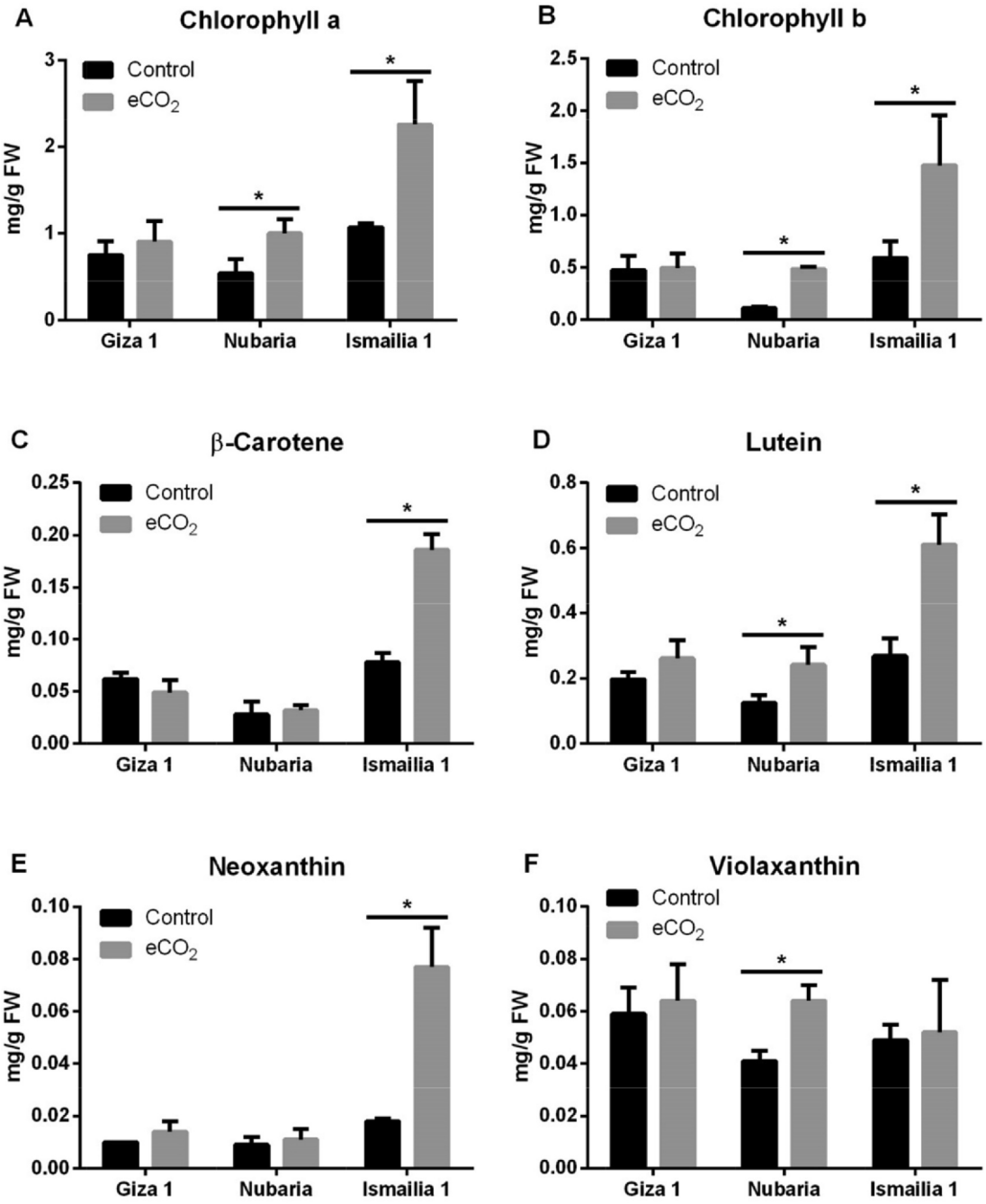

Fig. 1. Effect of elevated CO_2 on the concentrations of pigments (mg/g FW) of different alfalfa sprout cultivars. Values are represented by mean ± standard deviation of at least three independent replicates. Means marked by an asterisk (*) indicate significant differences between control and eCO_2 at $p < 0.05$.

Jaouni et al., 2018; Saleh, Selim, Jaouni, & AbdElgawad, 2018). Also, this positive effect was recorded for the vitamin level in fruits, such as vitamin C in sour orange and strawberry (Idso et al., 2002).

By its effect on primary metabolism and nutrient uptake, eCO_2 has been found to affect the levels of minerals (Watanabe et al., 2014; Noguchi et al., 2015; Asif, Yilmaz, & Ozturk, 2017). In this study, we determined the levels of macronutrients (Ca, K, P, Mg and Na) and micronutrients (Cu, Fe, Zn and Mn) in all cultivars (Table 1). Predominantly, eCO_2 treatment induced an elevation in the macronutrients and micronutrient levels in alfalfa sprouts as compared to control, except for Ca in Giza 1, Mg in Nubaria and Mn in Giza 1. The most dominant macronutrient in alfalfa sprouts was P, whereas Nubaria cultivar showed the highest P accumulation after eCO_2 treatment i.e., 1.43-fold more than control samples. Instead, Ismailia 1 showed the highest content of K after eCO_2 treatment, which represented 1.58-fold of the untreated samples. In accordance with our results, remarkable increases in K, Ca, Mg, Cu, Cd and Zn levels were reported after eCO_2 treatment of herbal plants as compared to the plants grown under ambient CO_2 (Al Jaouni et al., 2018, Saleh, Selim, Jaouni, & AbdElgawad, 2018). As well as, eCO_2 enhanced the uptake of micronutrients and macronutrients (Noguchi, Watanabe, & Terashima, 2015). Moreover, eCO_2 treatment improved the accumulation of Ca, Mn and Cu in carrot, turnip and radish roots (Azam, Khan, Mahmood, & Hameed, 2013). The role of microelements such as Cu, Zn, Se and Mn in free radical scavenging mechanisms was previously reported by Fedor et al. (2017).

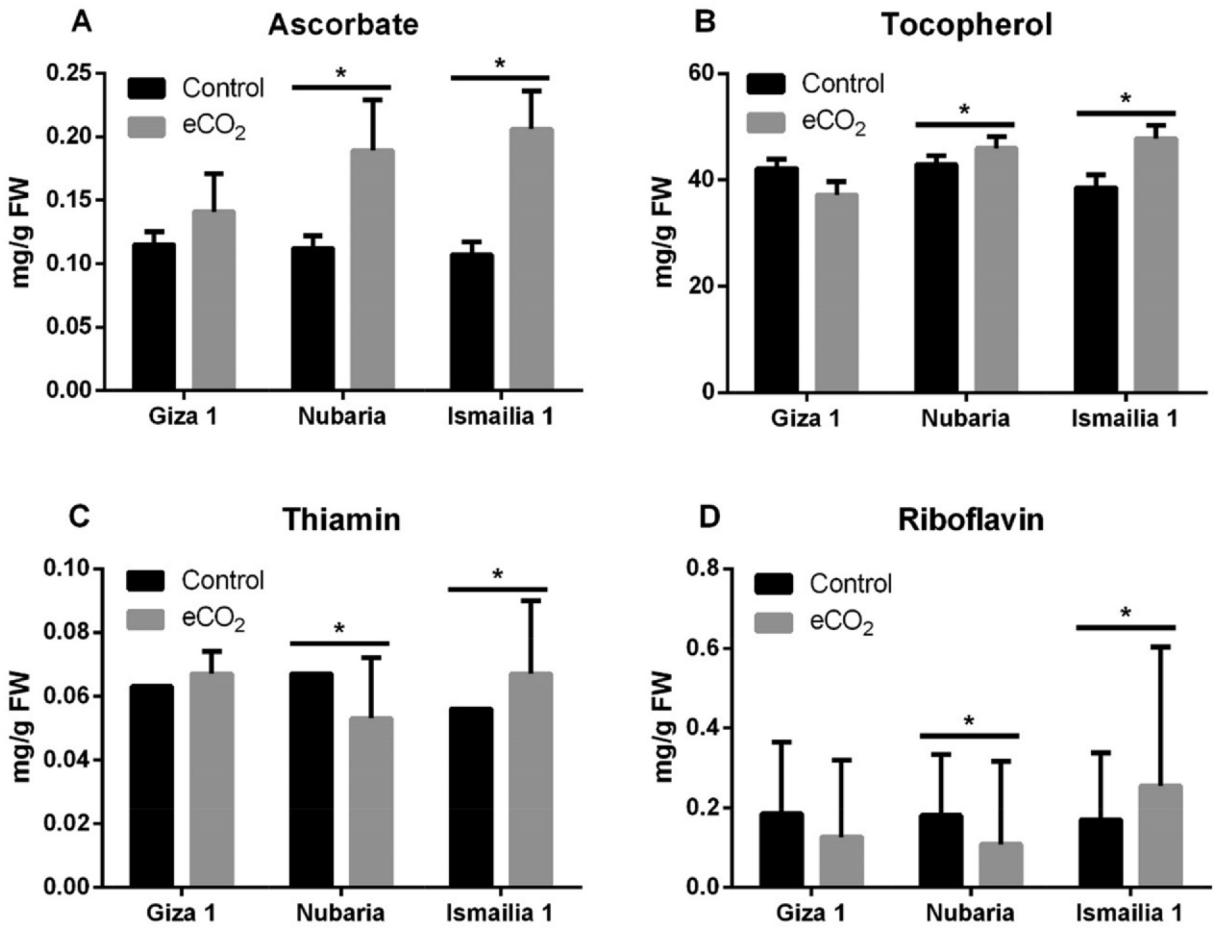

Fig. 2. Effect of elevated CO_2 on the concentration of vitamins (mg/g FW) in different alfalfa sprout cultivars. Values are represented by mean ± standard deviation of at least three independent replicates. Means marked by an asterisk (*) indicate significant differences between control and eCO_2 at $p < 0.05$.

Table 2
Effect of elevated CO_2 on the phenolic and flavonoid profile (mg/gDW) of different alfalfa sprouts cultivars. Values are represented by mean ± standard deviation of at least three independent replicates. Means marked by an asterisk (*) are significantly different than control at $p < 0.05$.

Phenolic and flavonoid profiles	Control			Elevated CO_2		
	Giza 1	Nubaria	Ismailia 1	Giza 1	Nubaria	Ismailia 1
Total phenols (mgGA/gDW)	0.125 ± 0.01	0.084 ± 0.0	0.132 ± 0.01	0.158 ± 0.01	0.107 ± 0.01*	0.209 ± 0.01*
Total flavonoids (μgQA/gDW)	0.021 ± 0.0	0.020 ± 0.01	0.025 ± 0.0	0.028 ± 0.0*	0.038 ± 0.01*	0.04300 ± 0.02
Galic acid	3.59 ± 0.50	5.04 ± 0.40	3.86 ± 0.30	5.2 ± 0.52*	6.38 ± 0.2*	5.61 ± 0.8*
Ferulic acid	0.03 ± 0.0	0.05 ± 0.0	0.05 ± 0.01	0.06 ± 0.0*	0.1 ± 0.01*	0.26 ± 0.02*
p-Coumaric acid	1.87 ± 0.10	1.24 ± 0.22	2.01 ± 0.20	3.11 ± 0.10*	1.54 ± 0.09	2.65 ± 0.30
Caffeic acid	3.63 ± 0.27	1.84 ± 0.10	3.19 ± 0.30	4.32 ± 0.5*	2.2 ± 0.10	3.63 ± 0.30
Catechin	0.68 ± 0.12	1.6 ± 0.10	1.15 ± 0.04	1.2 ± 0.04*	2.2 ± 0.13*	1.5 ± 0.20
kaempferol	1.66 ± 0.08	1.12 ± 0.02	2.47 ± 0.09	1.78 ± 0.13	1.91 ± 0.09*	3.89 ± 0.19*
Quercetin	1.6 ± 0.13	1.8 ± 0.36	1.7 ± 0.10	2.2 ± 0.5*	2.04 ± 0.20	2.9 ± 0.11*
Luteolin	0.05 ± 0.0	0.07 ± 0.01	0.26 ± 0.03	0.12 ± 0.01*	0.19 ± 0.03*	0.35 ± 0.04*
Rutin	1.12 ± 0.10	1.31 ± 0.10	1.3 ± 0.10	1.2 ± 0.10	1.5 ± 0.06	2.5 ± 0.2*
Daidzein	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.05 ± 0.02*	0.03 ± 0.0*	0.01 ± 0.0
Vitexin	0.48 ± 0.04	0.64 ± 0.05	0.58 ± 0.03	0.96 ± 0.04*	1.05 ± 0.01*	1.5 ± 0.19*
Myricetin	0.51 ± 0.05	0.81 ± 0.04	0.6 ± 0.01	1.1 ± 0.03*	1.28 ± 0.2	1.4 ± 0.05*
Apigenin	0.17 ± 0.01	0.22 ± 0.02	0.13 ± 0.03	0.41 ± 0.01*	0.33 ± 0.05	0.25 ± 0.02*
Naringenin	0.6 ± 0.07	0.97 ± 0.05	0.8 ± 0.09	1.3 ± 0.06*	1.4 ± 0.18	1.3 ± 0.2*

3.3. Elevated CO_2 improved the antioxidant capacity of alfalfa sprouts

Legumes, particularly alfalfa, are considered an important healthy food due to their high content of phenolic compounds (Silva et al., 2013). High phenols and flavonoids act as a natural arsenal against oxidative stress through scavenging the free radicals (Masella, Di Benedetto, Vari, Filesi, & Giovannini. 2005). Thus, the nutritive status of plants is correlated with its content of secondary metabolites and antioxidants compounds such as phenols (Idso et al., 2002; Fedor et al., 2017). Interestingly, eCO_2 increased the availability of C skeleton jointly with enough supply of inorganic N that enhances the biosynthesis of antioxidants, such as phenolic compounds (Noguchi, Watanabe, & Terashima, 2015). For instance, Ibrahim & Jaafar (2011) found a positive correlation between the increase in the non-structural

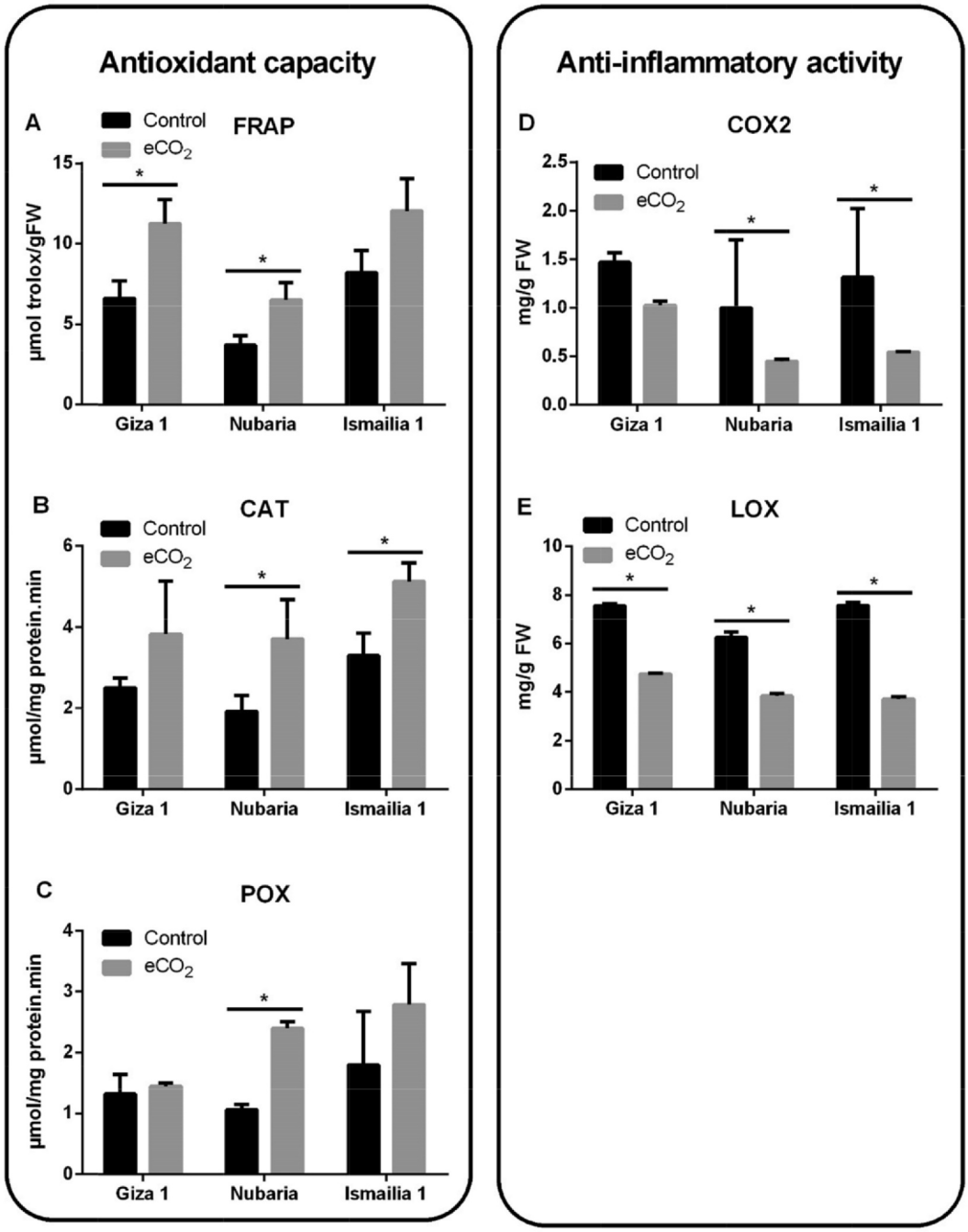

Fig. 3. Effect of elevated CO_2 on the antioxidant capacity (left) and anti-inflammatory activity (right) of different alfalfa sprout cultivars. A) Total antioxidant capacity (FRAP), B) Catalase activity (CAT), C) Peroxidase activity (POX), D) COX-2 and E) LOX. Values are represented by mean ± standard deviation of at least three independent replicates. Means marked by an asterisk (*) indicate significant differences between control and eCO_2 at $p < 0.05$.

carbohydrates and the enhancement of secondary metabolites production under eCO_2. Herein, we found that alfalfa sprouts contain considerable levels of antioxidant metabolites and there was a noticeable variation among cultivars in their antioxidant levels. Total phenols value was significantly raised ($p < 0.05$) in Nubaria and Ismailia 1 cultivars as a result of exposure to eCO_2. Additionally, total flavonoid content was significantly boosted ($p < 0.05$) in Giza 1 and Nubaria cultivars because of eCO_2 treatment. Ismailia 1 had the highest values of both total phenols and flavonoids after eCO_2 treatment (Table 2). Besides, individual phenolic compounds were determined for sprouts of Giza 1, Nubaria and Ismailia 1 cultivars. For all alfalfa cultivars, gallic acid was the most abundant phenolic acid, followed by caffeic acid, whereas quercetin and rutin were the predominant flavonoids. Also, in response to eCO_2, the levels of the individual phenolic acids were enhanced, particularly in Ismailia 1 cultivar. Similarly, most of the detected flavonoids were significantly elevated in all treated sprouts in response to eCO_2 (Table 2). Similarly, eCO_2 improved the contents of phytochemicals such as total phenolics in parsley and dill compared to the control (Saleh, Selim, Jaouni, & AbdElgawad, 2018), and consequently improved the antioxidant, anti-lipid peroxidation and antibacterial activities in fenugreek seeds (Hozzein, Saleh, Habeeb, Wadaan, & AbdElgawad, 2020). This elevation in these bioactive compounds in response to eCO_2 could be attributed to the abundant of C and N intermediates that are used for the biosynthesis of these bioactive compounds, given that high CO_2 was reported to affect the metabolism of C and N (Noguchi, Watanabe, & Terashima, 2015).

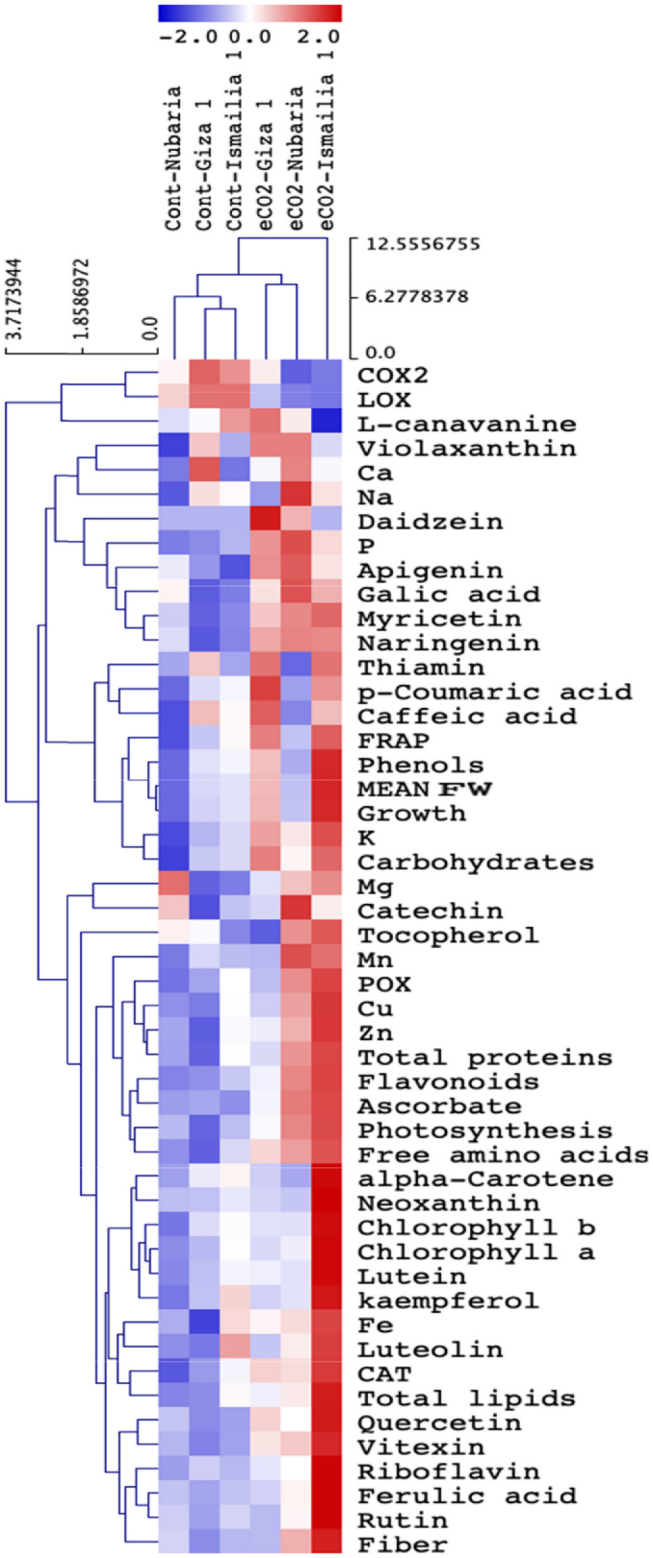

Fig. 4. Hierarchical analysis of macronutrients, micronutrients, antioxidants, pigments and enzymes of the three Alfalfa sprout cultivars (Giza 1, Nubaria and Ismailia 1) under control and elevated CO_2 treatments. The number of replicates of each parameter was at least 3. Red and blue colors revealed low and high concentrations, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These increases in the antioxidant metabolites, due to eCO_2 treatment, improved the total antioxidant capacity as indicated by the significantly higher FRAP level, an assay used to measure the efficiency of antioxidants in fruits and vegetables (Karimi et al., 2013), in treated samples than control ones (Fig. 3). In this context, Hollman (2001) demonstrated the role of increased antioxidant potential as antibacterial, antifungal and anticancer agents. The total antioxidant capacity represented by FRAP was found the highest in Ismailia 1, followed by Giza 1 and then Nubaria under both ambient and eCO_2 conditions. Remarkably, eCO_2 treatment significantly improved FRAP levels ($p < 0.05$) in Giza 1 and Nubaria cultivars, however in Ismailia 1 genotype, it was slightly enhanced. eCO_2-induced antioxidant capacity was ascribed to the enhanced levels of flavonoids and phenolic acids such as gallic and caffeic acids (Idso et al., 2002; Wang, Bunce, & Maas, 2003). Supporting this explanation, phenolic compounds have been recognized for their antioxidant properties (Almuhayawi et al., 2020). Farfan-Vignolo and Asard (2012) and Karimi et al. (2013) reported that eCO_2 increased the antioxidant capacity in Medicago lupulina and Alfalfa plant crude and high reactive oxygen species (ROS) detoxification. Moreover, the treatment with eCO_2 improved the antioxidant capacity, as well as the contents of phenols, flavonoids and vitamin C in ginger (Idso et al., 2002). As well as improved antioxidant status under eCO_2 treatment could also be attributed to the increased macroelements and microelements such as Zn, Cu and Mn and pigment levels (Fedor et al., 2017). Macromolecules could act as scavengers of reactive oxygen species, thus prohibiting the oxidative damage of necessary biomolecules such as proteins, lipids and DNA (Hollman, 2011). Similarly, the induction in antioxidants by accumulating various pigments was reported in strawberry after eCO_2 treatment (Wang et al., 2003).

Additionally, it is well known that ROS accumulation induces aging and several human diseases such as cancer, ischemia and immunity failure, thus ROS level is controlled through the activity of antioxidant enzymes such as catalase (CAT) and peroxidase (POX) (Matés, Pérez-Gómez, & De Castro, 1999). eCO_2 treated alfalfa sprouts could be a good source of these enzymes, particularly Ismailia 1 which showed the highest values of both CAT and POX after eCO_2 treatment compared to other cultivars (Fig. 3B and 3C). Regarding CAT, the genotypes Ismailia 1 and Nubaria were significantly higher than their controls, while in POX, only Ismailia 1 showed significantly higher results than control one ($p < 0.05$). Therefore, this increase may protect against oxidative stress-induced cardiovascular, cancer and aging diseases (Dai & Mumper, 2010).

3.4. *The anti-inflammatory activity of eCO_2-treated alfalfa sprouts*

Several reports investigated the anti-inflammatory potential of Alfalfa leave extracts due to their high antioxidant capacity (Karimi et al., 2013). Cyclooxygenase (COX-2) and lipoxygenase (LOX) are known as inflammatory markers, where COX-2 is linked to the inflammatory tissue as an inducible isoform (Mukherjee et al., 2001). COX-2 induces gastrointestinal irritation symptoms that are considered as a side effect of these analgesic products (Ondua, Adebayo, Shai, & Lebelo, 2016). Numerous inflammatory diseases and skin inflammations were linked to LOX products (Wedi & Kapp, 2001; Ondua, Adebayo, Shai, & Lebelo, 2016). Our results revealed that eCO_2 treatment significantly inhibited ($p < 0.05$) the COX-2 and LOX activities in all Alfalfa sprout extracts except COX-2 activity in Giza 1, which was slightly reduced. Ismailia 1 showed the lowest COX-2 and LOX activities then Nubaria cultivars following the eCO_2 treatment (Fig. 3D and 3E). In this regard, eCO_2 treatment of three cultivars of broccoli sprouts improved the levels of glucosinolates, amino acids and antioxidants, which consequently improved the anticarcinogenic and anti-inflammatory properties of their extracts (Almuhayawi et al., 2020). This high anti-inflammatory potential of Alfalfa sprouts could be attributed to high antioxidant capacity induced by the enhanced accumulation of phenols, flavonoids, micro minerals and pigments (chlorophyll *b*, β-carotene and lutein) under eCO_2 treatment. More depth, these metabolites have high potential as scavenging of reactive oxygen species

(Young, 1991; Matés, Pérez-Gómez, & De Castro, 1999; Fedor et al., 2017), which prohibit the synthesis of lipid hydroperoxide via scavenging of the synthesized lipid proxy radical. This mechanism reduces the presence of lipid hydroperoxide substrate which is needed for the catalytic cycle of LOX (Wedi & Kapp, 2001).

3.5. Cultivar-specific responses

Hierarchical clustering analysis (Fig. 4) revealed cultivar-specific variations in the effect of eCO_2 treatment on the levels of macronutrients, micronutrients, antioxidants, pigments and vitamins in alfalfa sprouts. The clustering analysis resulted into four main clusters. The first cluster represents COX-2, LOX and L-canavanine which were significantly reduced in eCO_2-treated samples. The second cluster consisted of violaxanthin, Ca, Na, P, daidzein, apigenin, galic acid, myricetin, and naringenin which were significantly enhanced in eCO2-treated samples, particularly Giza 1 and Nubaria cultivars. The third cluster contained FRAP, total phenols, thiamin, p-coumaric acid, caffeic acid, growth, carbohydrates and K, which were significantly improved due to high CO_2 treatment, especially in Giza 1 and Ismailia 1 cultivars. While the fourth cluster represented the rest of the measured parameters which were apparently higher in treated samples than control ones, particularly in Ismailia 1 cultivar.

4. Conclusion

To conclude, the exposure of alfalfa sprouts to high CO_2 is a promising innovative approach to enhance the levels of nutrients, essential minerals, vitamins, pigments and antioxidant metaboties/enzymes levels. It consequently, improved the overall antioxidant capacity, represented by FRAP and anti-inflammatory through inhibiting the inflammatory indicators; COX-2 and LOX. This study, therefore, recommends eCO_2 as a simple and costless way with minimal challenges of application to improve the nutritive value, functionality and health-promoting prospective of alfalfa sprouts to be a cheap but valuable source of bioactive compounds in the daily diet or used as a functional food additive to improve the nutritive values and health-promoting effects of food products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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