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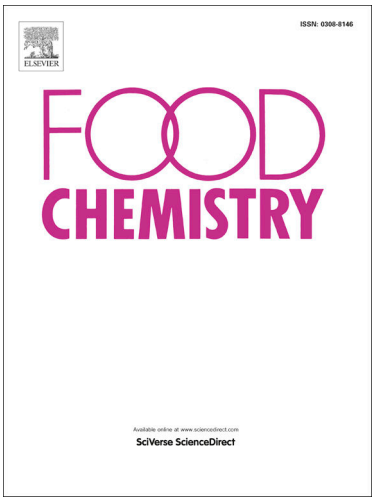

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Elevated CO_2 improves glucosinolate metabolism and stimulates anticancer and anti-inflammatory properties of broccoli sprouts

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Abstract:

Sprouting process enhances plant bioactive compounds. Broccoli (*Brassica oleracea* L) sprouts are well known for their high levels of glucosinolates (GLs), amino acids, and antioxidants, which offer outstanding biological activities with positive impact on plant metabolism. elevated CO_2 (eCO_2, 620ppm) was applied for 9 days to further improve nutritive and health-promoting values of three cultivars of broccoli sprouts *i.e*., Southern star, Prominence and Monotop. eCO_2 improved sprouts growth and induced GLs accumulation e.g., glucoraphanin, possibly through amino acids production *e.g*., high methionine and tryptophan. There was increases in myrosinase activity, which stimulated GLs hydrolysis to yield health promoting sulforaphane. Interestingly low levels of ineffective sulforaphane nitrile was detected and positively correlated with reduced epithiospecifier protein after eCO_2 treatment. High glucoraphanin and sulforaphane levels in eCO_2 treated sprouts improved anticarcinogenic and anti-inflammatory properties of their extracts. In conclusion, eCO_2 treatment enrich broccoli sprouts with health promoting metabolites and bioactivities.

Keywords: Broccoli sprouts, Elevated CO_2, Glucosinolates, anticarcinogenic, anti-inflammatory.

1. Introduction

Sprouts are the young seedling produced through seed germination and sprouting mechanism, it harvested and eaten before turning to true leaves (Benincasa, Falcinelli, Lutts, Stagnari, & Galieni, 2019). Sprouts are healthy foods due to the abundant of several nutrients that have positive impact on human health, such as inducing antioxidant capacity and protecting against cancer and cardiovascular diseases. For example, sprouting increased the levels of oligo- and monosaccharides, free fatty acids, oligopeptides, amino acids, antioxidants and vitamins (Dal Bosco et al., 2015). Interestingly, compared to the seeds and mature plants, they contain low levels of anti-nutritive compounds.

Among high nutritive sprouts, broccoli sprouts (Brassica oleracea) are of great interest due to their antioxidant, anticancer and anti-inflammatory activity, given their high content of antioxidants, vitamins, minerals and glucosinolates (GLs) and their hydroxylated derivatives (Matusheski, Juvik, & Jeffery, 2004). For instance, sprouting induced the parent glucosinolate of sulforaphane to 10 times than its value in seeds and mature plants (Fahey, Zhang, & Talalay, 1997). GLs are released from vacuoles of myrosin cells when the cells of broccoli are crushed. Sulforaphane (SF) is the most dominant and active GLs in broccoli, SF and SF nitrile are products of GLs hydrolysis through endogenous cytosolic myrosinase (Matusheski, Juvik, & Jeffery, 2004). This hydrolysis process and the products that released depends on the activity of myrosinase enzyme, the presence of additional proteins such as epithiospecifier protein (ESP) and hydrolysis conditions such as temperature and pH (Williams, Critchley, Pun, Nottingham, & O'Hare, 2008). The capacity of SF as anti-carcinogen was proved through its effectivity to induce the quinone reductase (QR) in Hepa lclc7 cell culture by saving the mammal DNA against damage through arresting the bioactivated carcinogens (Matusheski, Juvik, & Jeffery, 2004). For example, it showed high activity as antiproliferative by inhibiting the growth and inducing the apoptosis in different cancer cells (Chiao et al., 2002). Isothiocyanates in extracts of

broccoli sprout also enhanced the glutathione S-transferase (GST) and NAD(P)H:quinoneoxidoreductase 1 (NQO1) activity in rat bladder tissues, resulting in reducing cancer risks (Zhang et al., 2006). Moreover, treatment with broccoli extracts decreased the levels of biomarkers of DNA damage and reduced the blood pressure, oxidative stress and kidney inflammations (Fahey, Zhang, & Talalay, 1997). Also, broccoli showed effective anti-inflammatory properties due to the abundant content of sulforaphane (Woo & Kwon, 2007).

Numerous studies have been conducted to improve the levels of the phytochemical compounds in sprouts. In this context, environmental conditions such as abiotic stress and climate change were reported to influence the accumulation of phytochemicals in plants tissues (Björkman et al., 2011). Additionally, application of elicitors was used to improve the secondary bioactive metabolite production in plants (Baenas, García-Viguera, & Moreno, 2014). Under these conditions, plants could allocate their primary less active to structural carbohydrates and nutrients to secondary active metabolites (Hozzein, Saleh, Habeeb, Wadaan, & AbdElgawad, 2020). Consequently, the accumulation of these beneficial metabolites (Saleh, Selim, Jaouni, & AbdElgawad, 2018; Hozzein, Saleh, Habeeb, Wadaan, & AbdElgawad, 2020). could increase the nutritional and health-promoting values of plants. Given its known roles as a growth inducer and metabolism regulator through increasing photosynthesis in plant, high CO_2 is considered as an effective fertilizer to improve the nutritional and health-promoting values of plants (Li et al., 2017). It does not only increase plant growth, but it also induces its metabolism including sugar accumulation and breakdown for energy production (Saleh, Selim, Jaouni, & AbdElgawad, 2018). Thus, this approach could provide both the precursor and metabolic energy needed for bioactive phytochemicals biosynthesis such as antioxidants (Li et al., 2017). For instance, CO_2 fertilization increased health promoting GLs in broccoli (Schonhof, Klaring, Krumbein, & Schreiner, 2007), also improve the total flavonoids, vitamins and biological activity of parsley and dill (Saleh,

Selim, Jaouni, & AbdElgawad, 2018). Although high CO_2 application is an effective approach to stimulate plant nutritive value, to the best of our knowledge, effect of elevated CO_2 (eCO_2) on the chemical composition and nutritive value of sprouts was not previously studied.

The abovementioned properties of broccoli sprouts, besides the unavailability of data on the effect of eCO_2 on growth of broccoli sprouts inspired us to conduct this study. Herein, we aimed at investigating the effect of eCO_2 exposure on improving the nutritive and health promoting values of broccoli sprouts through determining its effect on the contents of bioactive compounds, mainly amino acids-derived GLs. The effect on enzymes activity involved in GLs hydrolysis and the potentiality of using broccoli extract rich with GLs and its hydrolyzed products as anticarcinogenic and anti-inflammatory. We hypothesized that eCO_2 will not only improve broccoli sprouts growth, but also it will improve its nutritive and health-promoting values.

2. Materials and methods:

2.1. Plant material and experimental conditions

Seeds of three broccoli cultivars (Southern star, Prominence and Monotop) were collected and rinsed in distilled water for a few minutes. The seeds were soaked in 5 g L^{-1} sodium hypochlorite for 1 h and drained. Then, the seeds were kept and rinsed in distilled water overnight. After pouring off the rinsing water the seeds equally were spread on trays lined with vermiculite and watered (every two days) with Milli-Q water. The trays were transferred and maintained at 25˚C air temperature on a controlled growth chamber under 16 h light/8 h dark cycle managed through cool white fluorescent tubes with photosynthetically active radiation (PAR) of 400 µmol m^{-2} s^{-1} and relative humidity (RH) of 60% per day. Where, the seeds were subjected to two climate conditions; 1) ambient CO_2 (aCO_2, 400 ± 27 µmol CO_2 mol^{-1} air); 2) eCO_2, 620 ± 42 µmol CO_2 mol^{-1} air). Seeds were subjected to the selected eCO_2 that

shown to improve the plant growth and nutritive values (e.g., (Hozzein, Saleh, Habeeb, Wadaan, & AbdElgawad, 2020; Saleh, Selim, Jaouni, & AbdElgawad, 2018) and it also follows the expected IPCC-SRES B2-scenario prediction of eCO_2 of the year 2100 (Murray & Ebi, 2012). CO_2 concentration was continuously monitored (CO_2 analyser, WMA-4, PP Systems, Hitchin, UK). 24 trays, each tray contains 10 seeds, were subjected to aCO_2 or eCO_2 (12 trays/treatment). After 9 days the sprouts from each tray (a biological replicate) were taken and weighed as fresh mass then frozen in liquid nitrogen and stored at -80 °C for further biochemical analysis. Three to five biological replicates (each biological replicate was a pool of 10 plants of the same tray) were used for each measurement.

2.2. Glucosinolates analyses

2.2.1 Extraction and determination of total glucosinolates

We extracted and determined total glucosinolates using modified methods of Rossetto et al., (2013). According to Rossetto et al, (2013), the thioglucosidase coupled assay was validated by measuring the glucosinolates levels in different Brassicaceae species and comparing the values with those values reported in the literatures. Before glucosinolates extraction, fresh samples were steamed for 2 min on a perforated tray over boiling water. According to Pongmalai et al. (2016), this steaming duration was adequate to inactivate myrosinase activity. About 1g of sample was homogenized 3 mL of MeOH: water (70:30; v:v) containing trifluoroacetic acid (TFA, 1.5 g/L) using a porcelain mortar (Sigma). The obtained extracts were moved into stoppered Erlenmeyer flasks to be conditioned under constant agitation in a thermostatic bath. This extraction was performed for 30 min at 70 °C. After cooling and centrifugation (8000 × g for 20 min), the collected supernatants were filtered (qualitative filter papers, Whatman) and evaporated at 40 °C until solvent was completely evaporated (approximately 72 h). The dry precipitate was reconstituted in HEPES–KOH (0.2 mM, pH 7.0) in the same container.

To enzymatically determine total glucosinolate concentrations, 10 µL of the extract, was mixed with thioglucosidase (0.12 U) contained myrosinase enzyme in HEPES–KOH (0.2 mM pH 7.0) at 37 °C for 24 h (Li and Kushad 2005). The reaction was terminated by adding of perchloric acid solution (18 mM). Control samples were performed by replacing sample extracts with buffer or immediately adding the stopping solution. Glucose produced from glucosinolates hydrolysis by thioglucosidase was measured by applying the stoichiometry (Palmieri et al., 1987), where 1 mol of produced glucose is equivalent to 1 mol of total glucosinolate. Total glucose was assayed enzymatically by using a glucose oxidase/peroxidase kit and sinigrin and allyl-glucosinolate were used as a calibrant and as a positive control, respectively.

2.2.2 Extraction and determination of glucoraphanin

Similar to total glucosinolates, fresh samples were steamed for 2 min on a perforated tray over boiling water which proved to yield the highest maximum content of glucoraphanin by rapidly inactivate myrosinase activity. Glucoraphanin content was extracted from 0.5g of steamed samples in 5 mL methanol (70%) and the mixture was then stirred in a stirring water bath for 15 min at 70 °C to assure maximum recovery. The mixture was cooled, filtered (Whatman No. 1 filter paper) and washed with 5 mL of methanol (70%). The methanol fraction was dehydrated using the rotary evaporator for 30 min at 50 °C. The residue was re-dissolved in 5mL methanol.

For quantification of glucoraphanin content (Celik et al., 2014), the obtained extract was first introduced to Sep-Pak! Vac 6 cc cartridge and the eluate was filtered through nylon filter (0.2-um). Twenty uL of the filtrate was injected into Zorbax Eclipse SB- aq column (150 X 4.6 mm i.d., 5 mm), using a mobile phase consisted of mixture of acetonitrile: water: formic acid (1:99:0.1v/v/v) and the flow rate was 1 mL/min. We detect glucoraphanin using DAD detector (235 nm), and the concentrations was calculated from a standard curve of glucoraphanin concentrations.

2.2.3 Extraction and determination of sulforaphane and sulforaphane nitrile content

Sulforaphane (SF) and sulforaphane nitrile (SFN) were extracted from broccoli sprouts according to (Matusheski et al., 2001). Broccoli sprouts were ground in liquid N_2 to a fine powder and mixed with deionized water. The mixture was allowed to autolyse at room temperature for 8 hours to achieve complete myrosinase catalyzed conversion of glucosinolates (glucosinolates conversion to SF and SFN using Na-phosphate buffer (Ph7.4, 0.01M), for 3 hours at 37C were also tested and give very similar results). NaCl and Na_2SO_4 were then added (1:0.75:1, w/w/w). The resultant paste was extracted with methylene chloride for three times and the methylene chloride layers were combined. To remove traces of water, $MgSO_4$ was added to the methylene chloride extraction, this was then filtered and dried using a rotatory evaporator. The obtained residue was re-dissolved in 5 % acetonitrile in water (v/v) and filtered (0.22-µm nylon membrane). To Quantify SF content (Celik et al., 2014), the extract was introduced to Oasis HLB, 3 cc cartridge. The eluate was purged with N_2 and dissolved in 0.5 mL of 1% (v/v) acetic acid. The whole content was filtered through a 0.2-l m nylon filter. The filtrate was injected into Zorbax Extend-C18 column (250 X 4.6 mm i.d., 5 mm). The elution was performed using a mixture of acetonitrile: water (30:70, v/v) and the flow rate was set as 0.8 mL/min. A DAD detection at 202 nm was performed and analyses were carried out at 20 C. SF content was calculated from a standard curve of sulforaphane. Extraction contained SF were concentrated and analyzed by LC-ESI- MS/MS for SFN using LC-MS analysis (Q-Tof Premier mass spectrometer, attached to Alliance 2695 HPLC system. Separation was conducted using Atlantis T3 C18 column (100 mm x 2.1 mm; 3 µm) and column temperature was maintained at 40 °C. The elution was carried by applying 10 mM ammonium acetate buffer (pH = 4.5) and 0.1% formic acid in acetonitrile at flow rate was 0.2 mL/min. Mass spectral data (positive mode with a mass range of m/z 100 to m/z 1000), capillary voltage and cone voltage (3 kV and 30 V respectively) and collision induced fragmentation (MSe mode, 12 eV to 20 eV energy with helium as the collision gas). Leucine-Enkephalin was used as internal reference compound.

2.2.4 Extraction and determination of amino acids

Amino acids were extracted by homogenizing 100mg of broccoli sprouts in 1 mL of aqueous ethanol (80%, v/v) using norvaline as internal standard to increase the accuracy of quantitation as well as to correct for different mass spectrometry responses. After centrifugation at 14000 rpm for 30 min, the supernatant was transferred to new tubes and dried, and the pellet was resuspended in chloroform (1 mL). After centrifugation at 14000 rpm for 30 min, the plant residue re-extracted in water was mixed with the pellet suspended in chloroform. Then the extracts were centrifuged for 10 min at 20000 g and the aqueous phase was filtered by Millipore microfilters (0.2 μm pore size) before assaying amino acid concentrations. Amino acids were measured by using a Waters Acquity UPLC-tqd system (Milford, MA, USA) equipped with a BEH amide 2.1 × 50 column (Abdelgawad et al., 2015).). 10 μL of each sample was injected and eluted with a gradient of solvent A (0.1% formic acid in H_2O) and solvent B (0.1% FA in acetonitrile) over 7.5 min at 0.5 mL/min. Flow rate was set at 0.3 mL min^{-1}, the column temperature was maintained at 30 °C, the sample temperature at 20 °C.

2.3. Protein extraction and myrosinase assay

Samples of broccoli sprouts subjected to eCO_2 or ambient CO_2 (control) from each cultivar were homogenized in 0.1 M phosphate buffer pH 6.0 including 5 mM benzamidine. The mixture was kept in a gentle and continues stirring at 4 °C for 30 min. After the centrifugation at 13,000 rpm for 15 min, the supernatant was taken and filtered through a 0.45 μm filter then precipitated in 70 % saturated ammonium sulfate. Then, the precipitate was dissolved in 10 mM phosphate buffer and purified against the same buffer, whereas the pellet was removed through the centrifugation at 4,000 g for 10 min at 4 °C. The activity of myrosinase was evaluated by measuring the hydrolysis of sinigrin (sigma) through monitoring the reduction in absorbance at 227 nm (Schwimmer, 1961). The evaluation was conducted at 27 °C in 0.75 mL of 33 mM sodium phosphate pH 6.0 including 20±50 μg of protein extract and 0.24

mM sinigrin. Molar extinction coefficient was e=7800 and e=564 at 277 nm for subtract and product respectively.

2.4. Lipoxygenase (LOX) assay

The linoleic acid was used as a substrate and LOX as an enzyme to evaluate the anti-lipoxygenase activity. The extracts of broccoli sprouts (10 mg/mL) from each cultivar subjected to eCO_2 or control one were performed, whereas (10 µL) from each extracts were mixed with 90 µL of LOX (400 U/mL) and kept in a dark condition for 5 min at 25 °C. To start the reaction, 100 µL of linoleic acid solution (0.4 mM) were added to each well, then the reaction maintained in a dark condition for 20 min at 25 °C. Afterwards, a 100 µL of freshly prepared ferrous orange xylenol (FOX) reagent containing (90% methanol, 10 µM FeSO4, 100 µM xylenol orange, 30 mM H2SO4) was added. The reaction incubated for 30 min at 25 °C and absorbance was measured at 560 nm and percentage inhibition was calculated.

2.5. Evaluation of Cyclooxygenase-1 and cyclooxygenase-2

At least three replicates from each cultivar of broccoli sprouts subjected to eCO_2 or control were used for this experiment. The experiment was conducted according to the manufacturer's instructions of COX assay kit (Cayman chemical company, Ann Arbor, MI, USA). The microtitre plate was covered with a plastic film and maintained at room temperature on an orbital shaker for 18 hr. According to the manufacturer's instructions, the microtitre plate was incubated in a dark condition for 90 min at 25^0C. Then, the reading of the plate was performed at 420 nm and percentage inhibition was calculated.

2.6. Induction of QR in Hepa lclc7 cells

The cells of Hepa lclc7 were placed into 96 well plates as 10,000 cells/well (Costar # 3595, Corning, Inc., Corning, NY). The α-minimum essential medium without ribonucleosides or deoxyribonucleosides was used and supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (final concentrations). Cells were grown for 24 h, afterwards the medium was changed

with a medium containing sterile filtered aqueous extracts of broccoli sprouts (0.57 mg fresh weight/mL) from each cultivar treated with eCO_2 or control. For each experimental replicate, one plate was assayed and β-Naphtho-flavone (l μM) was used as a positive control. The cells were lysed with digitonin after 24 h and QR was determined by a common 96 well plate assay procedure (Prochaska and Santam- aria, 1988).

2.7. Measuring of the Glutathione-S-transferase

For evaluating the Glutathione-S-transferase; 100 mg (FW) homogenate of broccoli sprouts from each cultivar treated with eCO_2 or control was prepared in one mL of 50 mM potassium phosphate buffer (pH 7.0), containing 10% (w/v) polyvinylpyrrolidone (PVP), 0.25% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride(PMSF) and 1 mM ASC, by using a MagNA Lyser (Roche, Vilvoorde, Belgium). The activity of GST was determined by measuring the conjugation of GSH with excess 1-chloro-2, 4-dinitrobenzene (CDNB) at 340 nm ($\varepsilon 340 = 0.0096\ \mu M^{-1}\ cm^{-1}$) (Habig et al., 1974).

2.8. Statistical analyses

Statistical Analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). One-way Analysis of Variance (ANOVA) was applied to all data. Tukey's Test ($P \leq 0.05$) was carried out as the post-hoc test for mean separations. Each experiment was replicated three times (n = 3-5).

3. Results and discussion:

3.1. eCO_2 improved the broccoli sprouts growth and photosynthesis

Sprouts growth was expressed as fresh biomass of the 9 days old sprouts after eCO_2 treatment. High CO_2 treatment improved the growth of all cultivars of broccoli sprouts as compared to the control (Table 1). In contrast to Southern star cultivar, high CO_2-treated Prominence showed the highest increase (1.78

fold, $p<0.05$) in biomass, followed by cultivar Monotop (1.46 fold, $p<0.05$) compared to corresponding ambient CO_2 treated cultivars. In this regard, CO_2 is considered as an essential substrate for plant photosynthesis mechanism, so that increasing in the CO_2 level directly promotes the plant photosynthesis, even after short exposure to 700 mmol mol^{-1} CO_2 (Gorka, Juan, Pilar, Rafael, & Manuel, 2006). Consequently, increasing the level of photosynthesis will induce an accumulation of soluble sugars, starch and organic acids that increase the plant growth and metabolites (Li et al., 2017). For example, high CO_2 in 400-700 ppm range, significantly increased the biomass of *Hymenocallis littorali* by 48% and the bulb growth by 56% (Idso et al., 2000). Atmospheric CO_2 enrichment also increased the photochemical efficiency in broccoli leaves (Krumbein, Kläring, Schonhof, & Schreiner, 2010) and soluble carbohydrates in parsley and dill shoots ((Saleh, Selim, Jaouni, & AbdElgawad, 2018).

3.2. eCO_2 increased amino acid derived GLs in broccoli sprouts

It is well known that improving the nutritional and health-promoting values of plants is highly related to their content of the essential amino acids. Several amino acids are greatest ample and have multi-purpose in the body (Cruzat, Macedo Rogero, Noel Keane, Curi, & Newsholme, 2018). Free amino acids are also precursors for health promoting metabolites such as GLs. Therefore, further improving of the amino acids levels in broccoli sprouts by applying promising approach such as eCO_2 will increase the nutritional values of treated sprouts, accordingly, will enhance the potentiality of using these sprouts as valuable food additives to many food products and meals.

In the current study, we mesured the profile of several amino acids in the three different cultivars of broccoli sprouts grown under control and eCO_2 conditions (Figure 1 and Table 2). Apparently, after eCO_2 treatment the levels of most amino acids increased in each cultivar of broccoli sprouts compared to the control. Monotop genotype significantly possessed the higher values ($p<0.05$) of tryptophan, methionine, tyrosine, asparagine and glutamic acid, as they were 4.14, 2.32, 2.1 and 1.8-fold more than corresponding

control values, respectively. In all cultivars, glutamic acid was the major amino acid followed by asparagine. These results are in consistent with that reported by López-Cervantes et al. (2013) who reported that the most dominant amino acids in broccoli sprouts were glutamic acid and asparagine, where the sprouts significantly showed higher contents of the essential amino acids (ranging between 3% and 45%) compared to the broccoli seeds. (Cruzat, Macedo Rogero, Noel Keane, Curi, & Newsholme, 2018) reported that the High levels of glutamic acid and asparagine suggest the potential role of broccoli, particularly after CO_2 treatment, in protecting nervous system function. As glutamine is an important nutrient needed for immune cells, lymphocyte proliferation, the production of cytokine. Also, controlling the neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Kanunnikova, 2012). As well as, asparagine is also a crucial amino acid for keeping the equilibrium of human nervous system and increasing the fatigue resistance to stress (Lomelino, Andring, McKenna, Michael, & Kilberg, 2017).

Additionally, eCO_2 significantly increased levels of methionine, tryptophan and phenylalanine in the three cultivars (Figure 1, $p<0.05$), as compared to corresponding controls. GLs originate from methionine and tryptophan, as they are precursors for indolic and alphatic glucosinolates (Grubb, & Abel, 2006). These increases are correlated with the high GLs levels, where two precursors are highly induced by high CO_2 in all genotypes, particularly Monotop and Prominence. Also, high levels of valine, serine and glutamine (2.2, 1.91 and 1.34-fold, respectively) were detected in CO_2-treated prominence cultivar. The Southern star cultivar displayed significantly higher induction in leucine after eCO_2 treatment, as it represented a 1.6-fold higher than the value at the control. Overall, the variation in the amino acid composition in sprouts could be related to the high metabolic activities in the sprouting mechanism, which involved in the seedling development (Taiz & Zeiger, 2002).

3.3. GLs production and hydrolysis were increased by high CO_2 treatment

Biological activities of plants depend on their contents of bioactive compounds such as the secondary bioactive GLs, which can serve as antioxidants and anticarcinogenic agents (Idso et al., 2002). According to our data and previous studies, broccoli plants are rich in glucosinolate contents (Figure 2). High CO_2 treatment further increased the levels of total GLs, as well as the dominant GLs (glucoraphanin). Moreover, eCO_2 increased myrosinase enzymes activity involved in glucosinolate hydrolysis (Figure 2). The activity of myrosinase enzyme controlled the hydrolysis of glucosinolates to isothiocyanates (ITCs) products i.e., effective GLs (sulforaphane) and ineffective GLs (sulforaphane nitrile) (Williams, Critchley, Pun, Nottingham, & O'Hare, 2008). Broccoli sprouts is well known as a rich source of various ITCs, especially sulforaphane (Zhang et al., 2006). For instance, Lopez-Cervantes et al. (2013) reported that the levels of sulforaphane in the seeds and sprouts of broccoli were ranged from 273 to 3632 μg g^{-1}, respectively, whereas the highest levels of sulforaphane were found in the 8- and 11-day-old sprouts. eCO_2 treatment insignificantly increased the myrosinase enzymes in Southern star and significantly increased it in both Prominence and Monotop ($p<0.05$). Consequently, eCO_2 increased sulforaphane in all sprout cultivars, particularly in Prominence and Monotop cultivars ($p<0.05$).

On the other hand, sulforaphane nitrile production is stimulated by epithiospecifier protein (ESP), where it boosts the synthesis of simple nitriles, for instance, that is formed from the endogenous glucoraphanin in place of sulforaphane (Matusheski et al., 2006; Williams, Critchley, Pun, Nottingham, & O'Hare, 2008). In this regard, Matusheski et al. (2006) showed that the formation of health-promoting phytochemical sulforaphane is negatively correlated to ESP activity in broccoli. Moreover, in broccoli, ESP enzyme is considered as a key factor guiding the formation of sulforaphane nitrile, so that a cultivar with low expression level of ESP accumulates more sulforaphane and showed increased potential as an anti-carcinogenic food (Matusheski et al., 2006). We found that the activity of epithiospecifier protein in broccoli sprouts was dropped significantly in each of Southern star and Monotop cultivars by eCO_2

treatment, nonetheless it significantly increased in Prominence as compared to the control ($p<0.05$), as displayed in Figure 2. This was consistent with the decreased level of sulforaphane nitrile in Southern star and Monotop cultivars.

Overall, eCO_2-treated broccoli sprouts showed an increase in the myrisinase activity induced hydrolysis to effective sulforaphane and a decreased in ESP activity reducing sulforaphane conversion to an active form (sulforaphane nitrile).

3.4. eCO_2 improved the anticancer and anti-inflammatory potential of broccoli sprouts

Due to high antioxidants, vitamins, minerals and the GLs, broccoli sprouts showed high antioxidant, anticancer and anti-inflammatory activity (Matusheski, Juvik, & Jeffery, 2004). In our study, we reported high ITC level in the extract of eCO_2 treated broccoli. ITC from broccoli sprouts significantly promoted the detoxification enzymes glutathione S-transferase (GST) and quinone reductase (QR) thus reduced the risk of urothelial bladder cancer in human (Zhang et al., 2006). Herein, it is obvious from our data that growing the target broccoli sprouts cultivars at high levels of CO_2 significantly increased ($p<0.05$) their levels of QR and GST anticancer indicators (Figure 3A and 3B). High CO_2-treated Monotop cultivar exhibited the highest level of QR induction among broccoli sprouts cultivars. Whereas, Prominence cultivar showed the highest level of GST induction after eCO_2 treatment. We also found reduced levels of sulforaphane nitrile, which has been found to be ineffective as anti-carcinogens, as well as less effective for inducing the detoxification enzymes (GST and QR) in the rat (Matusheski et al., 2001). High anticancer activity of the atmospheric CO_2 treated plants (Saleh, Selim, Jaouni, & AbdElgawad, 2018). High CO_2 promoted the levels of numerous therapeutic compounds in the bulb of *Hymenocallis littorali*, which improved the anticancer and antiviral activities of bulb extract (Idso et al., 2000). Also, broccoli sprouts extract had a strong bactericidal effect on reducing the infection by *Helicobacter pylori*, which is correlated with gastric cancer (Yanaka et al., 2015).

Several studies revealed that the cruciferous vegetables such as broccoli have significant anti-inflammatory properties (Tilg, 2015). The inflammation is intended to minimize the infections and damage after tissue injuring. There are numerous inflammatory pathways involving cyclooxygenase (COX) and lipoxygenase (LOX) pathways. Cyclooxygenase-2 (COX-2) is considered as an inducible isoform related to inflammatory tissue (Mukherjee, Nissen, & Topol, 2001). Analgesics containing anti-inflammatory products such as COX-2 selective drugs are needed for pain relief; however, these analgesic products showed several side effects such as gastrointestinal irritation (Ondua, Adebayo, Shai, & Lebelo, 2016). So that, there is a demand for an alternative anti-inflammatory product from natural sources such as the herbal medicine. Our results showed that, eCO_2 treatment inhabited COX-2 and LOX activities in broccoli sprouts extracts by increasing sulforaphane, indicating high anti-inflammatory potential. Similarly, sulforaphane supressed the expression of COX-2 through the modulation of several core promoter elements (NF-κB, C/EBP, CREB and AP-1) in the COX-2 transcriptional regulation (Woo & Kwon, 2007). The highest inhibition in COX-2 activity was reported in Monotop then Prominence cultivars, where the highest inhibition in LOX activity was reported in Prominence followed by Monotop cultivars after the eCO_2 treatment (Fig 3C and 3D).

Lipoxygenase is a key factor in several inflammatory diseases (Wedi & Kapp, 2001). Moreover, several skin inflammations were linked to products of LOX (Ondua et al., 2016). In this regard, growing the three broccoli sprouts cultivars at high levels of CO_2 significantly increased ($p<0.05$) the anti-inflammatory properties of broccoli sprouts extracts; under eCO_2 treatment the activity of lipoxygenase was decreased through the three broccoli cultivars compared to their corresponding controls (Fig 3D). Broccoli sprouts extracts showed a promising anti-LOX effect, which could be attributed to polyphenolic content and antioxidants properties of the broccoli sprouts extract. Whereas, antioxidants inhibit the lipid hydroperoxide synthesis through the scavenging the formed lipid peroxy-radial, this process decreases the

availability of lipid hydroperoxide substrate which is required for the catalytic cycle of LOX (Wedi & Kapp, 2001). Thus, eCO_2 induces antioxidant bioactive compounds and increase the effectively of the broccoli sprouts as a functional food with high anti-inflammatory properties.

To conclude, since, eCO_2 increased QR and GST enzymes activities and glucosinolates and sulforaphane levels, but reduced COX-2 and LOX enzymes activity. These changes increased the anticancer and anti-inflammatory properties of broccoli sprouts. Therefore, broccoli sprouts grown under eCO_2, besides their high nutritive values, are recommended as a potential functional food possess significant anti-inflammatory properties, beside the prohibition and treatment of bladder tumors.

3.5. Genotype specific responses to high CO_2

Our hierarchical clustering confirmed that the effect of eCO_2 condition was cultivar specific (Figure. 4). The variations among the three cultivars could be attributed to the ontogeny and species diversity, as the highest contents of amino acids and total glucosinolate were found in Monotop, followed by Prominence under eCO_2 condition. The content of bioactive compounds in broccoli (*Brassica oleracea var. italica*) differs with growth conditions, environmental conditions, stress, food processing and storage, besides the genotype (Perez-Balibrea, Moreno, & Garcıa-Viguera, 2008). Several reports have been investigating the ontogeny and species diversity, generally in vegetables and particularly in Brassicas, based on their contents of bioactive compounds. For instance, Perez-Balibrea, Moreno, & Garcıa-Viguera, (2011) found that the bioactive compounds in commercial seeds and sprouts cultivars of broccoli (*Brassica oleracea* var. italica; cv. Nubia, cv.Marathon and cv. Viola) are varied depending on the genotype, moreover, the cultivar Marathon showed the higher level of total glucosinolate. Moreover, the genetic effect was more pronounced in the aliphatic glucosinolates than the indolic ones.

4. Conclusions

To the best of our knowledge, this is the first report which investigated the effect of eCO_2 interaction with sprouting of broccoli on enhancing the growth, nutritive value, anti-cancer and anti-inflammatory properties. The activity of myrosinase was significantly increased through broccoli sprouts and that consequently enhanced the amino acid derived glucosinolates induction by eCO_2 with low levels of ineffective sulforaphane nitrile. Moreover, the broccoli cultivars showed reduction in GST and QR activity and reduced COX-2 and LOX in most target cultivars of broccoli sprouts. The variation among broccoli cultivars in growth and the contents of bioactive compound was cultivar specific. These results increase the potentiality of using broccoli sprouts grown under eCO_2 as a promising nutritional and health-promoting functional food or food additive.

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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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Table. 1. Effect of elevated CO_2 (eCO_2) on fresh total biomass (g FW) of different cultivars of broccoli sprouts.

Genotypes	Control	eCO_2
Southern star	0.200 ± 0.04	0.255 ± 0.012
Prominence	0.189 ± 0.02	0.338* ± 0.022
Monotop	0.284 ±0.02	0.415* ± 0.026

Values represent means ± standard deviations of three independent replicates. Means that marked by asterisk (*) are significantly different than control at the 0.05 probability level.

Table 2. Effect of elevated CO_2 (eCO_2) on the amino acid concentrations through different cultivars of broccoli sprouts.

Amino acids	Southern star		Prominence		Monotop	
	Control	eCO_2	Control	eCO_2	Control	eCO_2
Leucine	10.57 ± 0.7	17.28* ± 0.2	8.74 ± 0.3	16.02* ± 0.6	8.08 ± 0.3	18.51* ± 0.6
Tyrosine	7.61 ± 0.27	10.68 ± 0.47	7.89 ± 0.28	17.42* ± 1.7	6.71 ± 0.25	15.71* ± 0.82
Glutamic acid	122.8 ± 6.3	106.01 ± 7.6	68.1 ± 3.3	101.97 ± 26.2	99.6 ± 4.8	181.62* ± 5.5
Glutamine	43.24 ± 5.0	95.38 ± 10.4	99.27 ± 2.7	58.81 ± 20.3	98.1 ± 4.1	116.65 ± 2.2
Asparagine	59.15 ± 15.7	82.26 ± 3.7	89.62 ± 4.6	154.44* ± 11	65.19 ± 16.9	175.61* ± 4.9
Valine	12.86 ± 0.4	16.09* ± 0.1	10.6 ± 0.5	21.76* ± 0.9	13.8 ± 0.5	10.43 ± 3.1
Proline	29.34 ± 2.2	28.39 ± 1.0	35.56 ± 0.5	35.56* ± 0.5	23.61 ± 0.2	40.75* ± 1.8
Serine	16.27 ± 0.4	10.58 ± 1.5	20.38 ± 1.2	42.33* ± 6.1	20.14 ± 1.1	31.91* ± 4.6
Alanine	2.01 ± 0.3	1.83* ± 0.3	0.76 ± 0.1	0.68 ± 0.6	1.03 ± 0.1	0.92 ± 0.2
Phenylalanine	5.59 ± 1.2	9.85* ± 1.4	10.20 ± 2	15.63* ± 2.6	7.56 ± 1.1	18.67* ± 5.3
Glycine	0.56± 0.00	0.30 ± 0.50	0.36 ± 0.0	0.15 ± 0.03	0.65 ± 0.1	0.45 ± 0.08

Values are represented by means ± standard deviations of three independent replicates. Means that marked by asterisk (*) are significantly different than control at the 0.05 probability level.

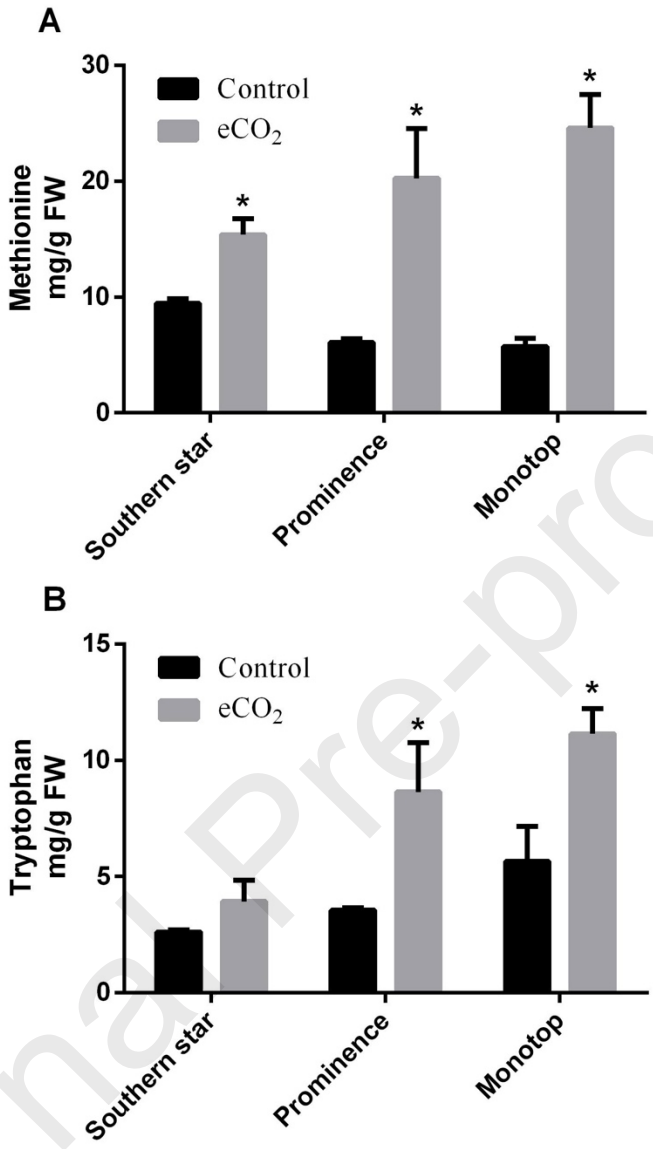

Fig 1. Effect of elevated CO_2 (eCO_2) on A) methionine and B) tryptophan concentrations through different cultivars of broccoli sprouts. Values are represented by means ± standard deviations of three independent replicates. Means that marked by asterisk (*) are significantly different than control at the 0.05 probability level.

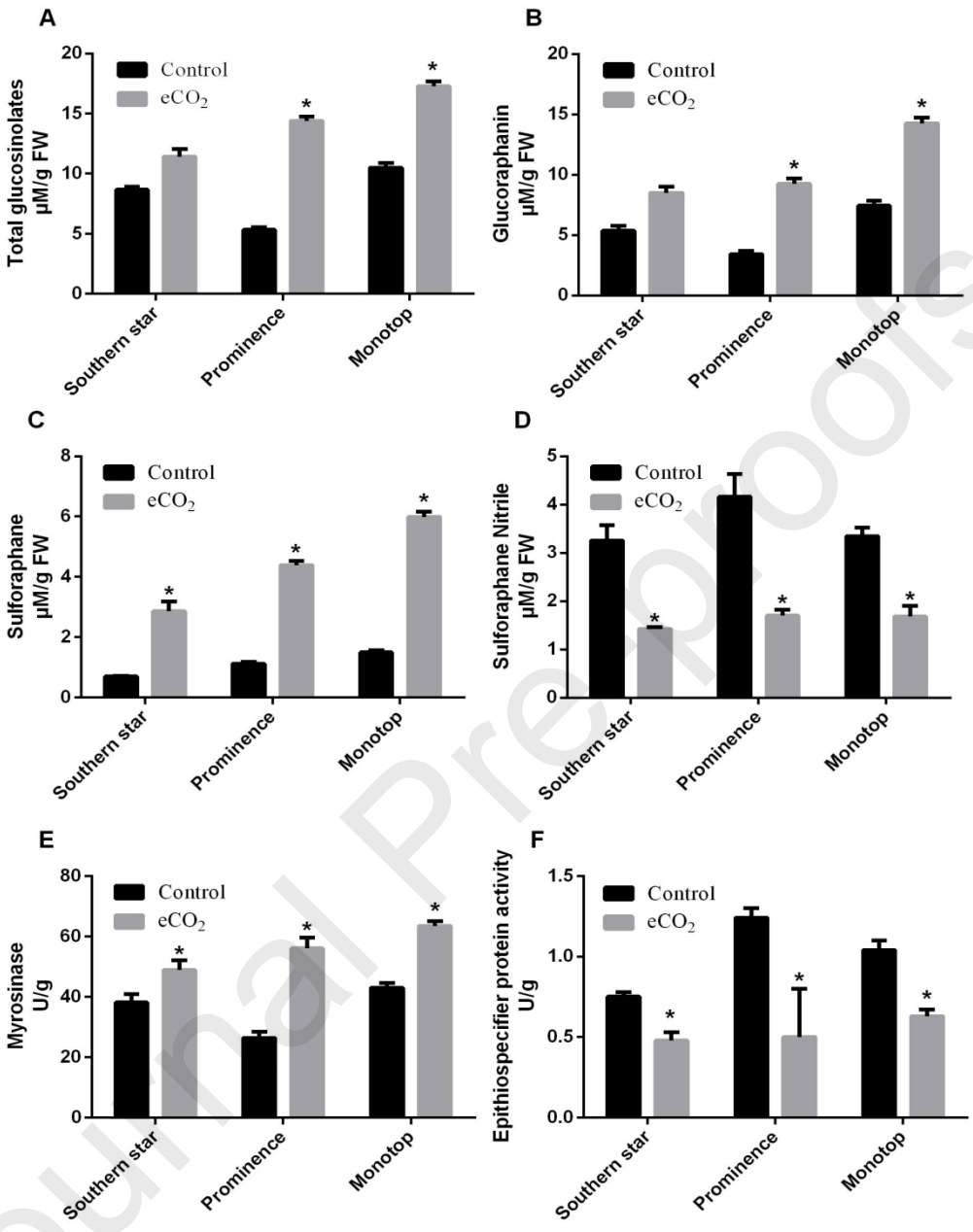

Fig 2. Effect of elevated CO_2 (eCO_2) on the levels of A) total glucosinolates, B) glucoraphanin, C) sulphoraphane, and D) sulforaphane Nitrile, and E) myrosinase, F) epithiospecifier protein activity in different cultivars of broccoli sprouts. Values are represented by means ± standard deviations of three independent replicates. Means that marked by asterisk (*) are significantly different than control at the 0.05 probability level.

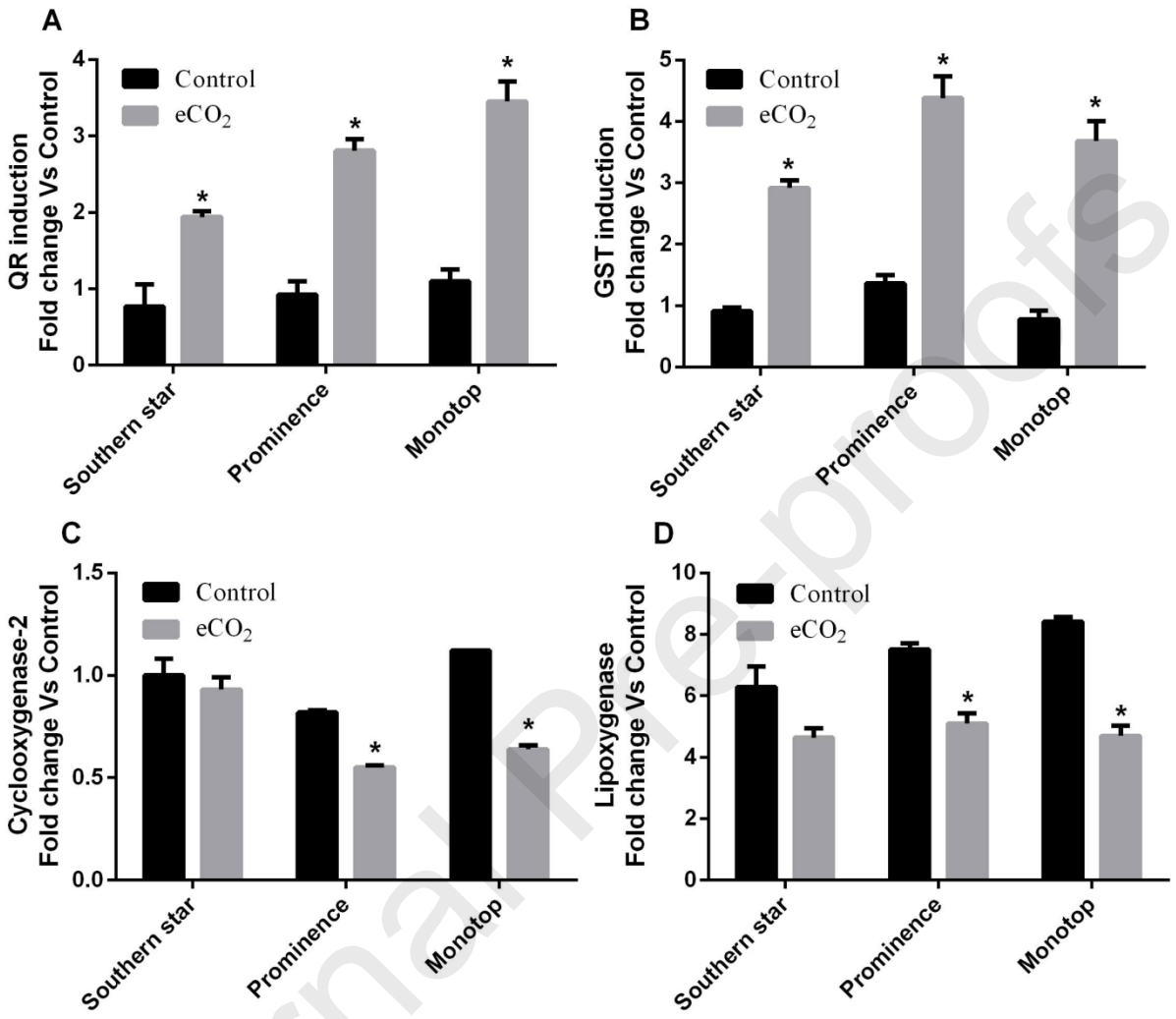

Fig 3. Effect of elevated CO_2 (eCO_2) on A) quinone reductase (QR) induction, B) Glutathione-S-transferase (GST) induction, C) cyclooxygenase (COX-2) and D) lipoxygenase (LOX) activity in different cultivars of broccoli sprouts. Values are represented by means ± standard deviations of three independent replicates. Means that marked by asterisk (*) are significantly different than control at the 0.05 probability level.

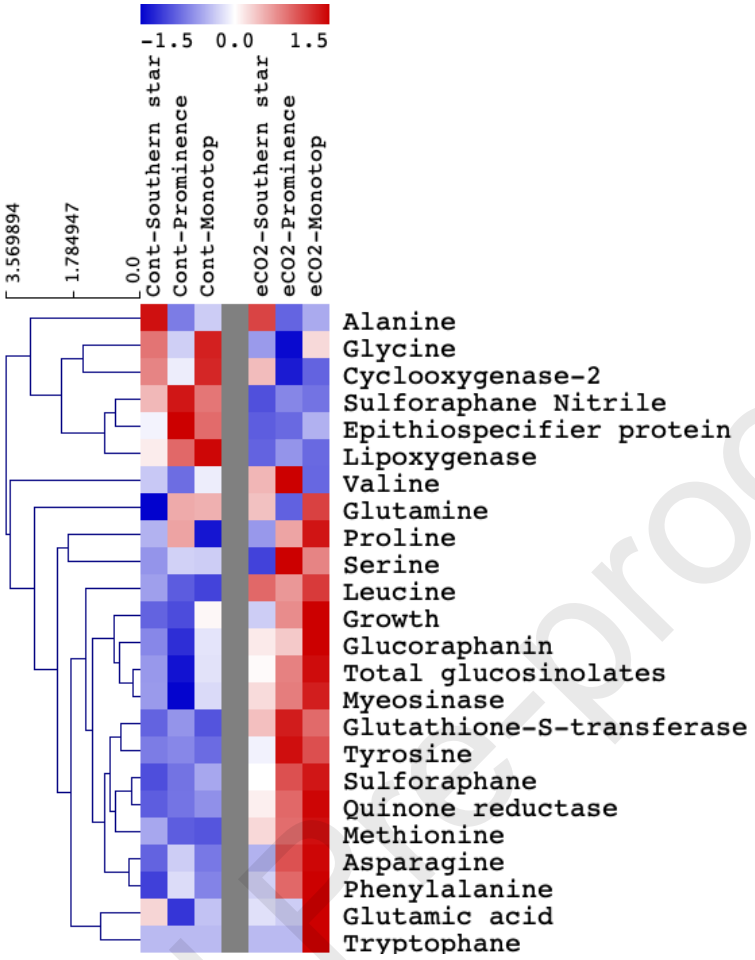

Fig 4. The variations in growth rate, amino acid contents, total glucosinolate and biological activity among the three broccoli sprouts cultivars grown under control and elevated CO_2 (eCO_2) conditions. Each parameter is representing at least 3 replicates. Red and blue color ranges revealed the low and high values, respectively.

High lights

- eCO_2 treatment increased glucosinolate accumulation in broccoli sprouts.
- eCO_2 increased glucosinolate hydrolysis to the health promoting sulforaphane.

- eCO_2 reduced epithiospecifier protein and ineffective sulforaphane nitrile levels.
- Anticarcinogenic and anti-inflammatory properties of broccoli are promoted by eCO_2.

Credit Author Statement

MA, SS and HA planned and designed the research; **MA, HA, AH and GK** performed the experiments; **SS, HA, SA, AH and GK** analysed the data; **MA, SS and SA** contributed to the reagents/chemicals. **GK and MA** provided a draft version of the manuscript, and **AH and HA** revised and finalized the manuscript.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: