Journal of Applied Botany and Food Quality 94, 16 - 25 (2021), DOI:10.5073/JABFQ.2021.094.003

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CO_2 treatment increases glucosinolate hydrolysis products in two *Arabidopsis thaliana* accessions

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(Submitted: October 18, 2020; Accepted: February 24, 2021)

Summary

Brassicales include many vegetables of nutritional interest because the hydrolysis products of their phytochemicals, the glucosinolates, have health-promoting properties. So far, the impact of rising CO_2 concentrations on glucosinolates and their hydrolysis is unclear. Applying a modified atmosphere, we exposed two *Arabidopsis thaliana* accessions that differ in their glucosinolate hydrolysis behavior, namely Hi-0 and Bur-0, to elevated CO_2 concentrations. Glucosinolates and their hydrolysis products were analyzed using UHPLC-DAD-MS and GC-MS.

CO_2 treatment increased indicators of primary production, such as biomass, leaf area and electron transport rate, and increased glucosinolate levels in Bur-0, but not Hi-0. Significantly, released glucosinolate hydrolysis product levels increased by up to 122% in Bur-0 due to increased epithionitrile formation. Likewise, in Hi-0 glucosinolate hydrolysis product levels increased after CO_2 treatment by up to 67%, caused by enhanced nitrile and to some extent isothiocyanate formation. In addition, more alkenyl rather than alkyl glucosinolates were formed in Bur-0 under elevated CO_2, thus changing the glucosinolate profile compositions. As CO_2 treatment enhanced primary production but also overall glucosinolate hydrolysis product formation, it is conceivable to recycle excess CO_2 by using it as supplement greenhouse gas to produce high-quality food.

Keywords: Brassicaceae, glucosinolates, isothiocyanates, nitriles, epithionitriles, CO_2-fertilization

Introduction

In the future, humanity will have to face diverse environmental and socio-economic challenges. In 2019, the CO_2 concentration ($[CO_2]$) in the atmosphere reached 410 ppm (compared to pre-industrial levels of 280 ppm) and a further increase in atmospheric CO_2 content is expected over the coming decades (World Meteorological Organization, 2020). Due to increasing global problems such as growing population combined with the rising trend towards urbanization (United Nations, 2014) and scarcity of resources (Steffen et al., 2015), more efficient use of natural resources, including sustainable food production, will become increasingly important. Thus, the societal challenge is a sustainable plant-based food production of a sustainable human diet ensuring both food and nutritional security. However, current agricultural systems are limited in coping these requirements (Odegard and E., 2013). One concept could be the establishment of urban biospheres as multi-functional and multivariable compartments for plant cultivation in urban areas, such as home gardens and large-scale plant growth chambers on unused urban environments. Thus in the scarce urban areas, food production will not compete with areas for housing and infrastructure, but be integrated into city landscapes (European Commission, 2016). Here, in addition to food production, plants could also be used as an up-regulated CO_2 sink due to a fertilization effect through high CO_2 air concentrations (Lau and Deng, 2012; Teng et al., 2006). Moreover, demand for climate resilient plants with optimized metabolite profile is increasing (Baldermann et al., 2016). Glucosinolates (GLSs) are secondary plants metabolites in Brassicales, including radish, rocket, broccoli or cabbage. Depending on their structure, which is based on their precursor amino acid, GLSs can be classified into aliphatic, benzenic and indole GLSs (Agerbirk and Olsen, 2012). When cells are disrupted, GLSs are hydrolyzed by the enzyme myrosinase, which is located separately in the plant, releasing epithionitriles, nitriles and isothiocyanates (ITCs) (Wittstock and Burow, 2010). The latter are valued for their health-promoting properties such as anti-inflammatory effects (Herz et al., 2016) and especially for their cancer preventive effects (Veeranki et al., 2015). However, due to the presence of epithiospecifier protein (ESP) in many Brassicaceae plants (among them broccoli and cabbage) not ITCs but epithionitriles and nitriles are often released (Hanschen and Schreiner, 2017). Therefore, vegetables with a high content of certain GLSs and a high potential to release ITCs are desirable from a nutritional point of view.

In general, plants benefit from elevated atmospheric CO_2 level by increasing primary production, which leads to an increase in primary metabolites, total biomass and leaf growth (Lau et al., 2007; Teng et al., 2006) as well as to a structure-specific increase in GLSs (Schonhof et al., 2007). As shown with isolated guard cells, elevated CO_2 levels can enhance amino acid metabolic pathways and thus activate GLS biosynthesis (Geng et al., 2016). So far, several studies evaluated the effect of elevated CO_2 on GLS contents of Brassicaceae plants. The effect of elevated CO_2 on GLS can be species specific and some studies reported unaffected or decreased GLS levels (Himanen et al., 2008; Karowe et al., 1997), while in other studies increased GLS formation was observed: For example broccoli grown for 33 days with elevated CO_2 (800 ppm) showed increased indole GLS levels, while 3-butenyl GLS (gluconapin, 3But GLS) levels were not affected (Zaghdoud et al., 2016). Likewise, aliphatic GLS in broccoli were not significantly increased by elevated CO_2 (800 ppm), while some indole GLS increased in the cultivar 'Naxos' but not in cultivar 'Viola' (Rodriguez-Hernandez et al., 2014).

In *A. thaliana* in the accession Can-0 elevated CO_2 induced allyl GLS (sinigrin), while other GLS and GLS of other genotypes were not affected (Bidart-Bouzat et al., 2005). Interestingly, in that study GLS levels, in general, were not significantly affected by herbivory with the diamondback moths (*Plutella xylostella*) at ambient CO_2 conditions but herbivory induced a significant 28-62% increase in GLS concentrations under elevated CO_2 (Bidart-Bouzat et al., 2005) demonstrating that chemical responses to herbivory can be fortified by elevated CO_2 levels.

Nevertheless, there is very limited data on the effect of CO_2 treatment on GLS hydrolysis especially with respect to ITC formation in ESP containing plants. Only recently a study analyzed the effect of elevated CO_2 on the formation of GLS hydrolysis products in broccoli sprouts. The elevated CO_2 (620 ppm) led to an increase in 4-(methylsulfinyl)butyl GLS (glucoraphanin), myrosinase activity

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and increased the formation of the corresponding 4-(methylsulfinyl)-butyl ITC (sulforaphane) while reducing the formation of the corresponding nitrile (ALMUHAYAWI et al., 2020). The reduction in nitrile formation could be linked to a reduction in ESP-activity due to the CO_2 treatment. However, the effect of long-term elevated CO_2 on adult plant performance and GLS hydrolysis is not clear. Moreover, differences in ESP abundance might also affect the response of GLS hydrolysis on CO_2 treatment.

In view of future innovative urban home plant cultivation facilities we hypothesize that in-house vegetable production could be linked with CO_2 sequestration and will lead to vegetables with enhanced or at least maintained nutritious properties.

Here, we investigated the effects of highly elevated CO_2 [20 000 ppm] on GLSs and formation of their hydrolysis products, as well as on plants' primary production at two ontogenetic stages of two accessions of *A. thaliana* selected as a model organism for Brassicaceae vegetables. The selected accessions are both rich in alkenyl GLS, a prerequisite for epithionitrile-formation. While the accession Hi-0 mainly releases ITCs upon GLS hydrolysis, the accession Bur-0 is a producer of epithionitriles (HANSCHEN et al., 2018b). We hypothesize that elevated CO_2 will affect accessions with different ESP activities and therefore different routes of GLS hydrolysis in a differential way.

Materials and methods

Chemicals and enzymes

Benzonitrile (≥ 99.9%), DEAE-Sephadex® A-25 (30,000 Da exclusion limit), allyl ITC (Allyl-ITC; ≥ 99%), 3-butenenitrile (Allyl-CN; ≥ 98%), 4-pentenenitrile (3But-CN; ≥ 97%), and 3-phenylpropanenitrile (≥ 99%) were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany); 3-butenyl ITC (3But-ITC; ≥ 95%) and 4-pentenyl ITC (≥ 95%) were purchased from TCI Deutschland GmbH (Eschborn, Germany); acetic acid (100%) and formic acid (≥ 98%, ROTIPURAN®), methylene chloride (≥ 99.9%), $CaCl_2$ (≥ 98%), ethanol (≥ 96%), imidazole (≥ 99%), HPLC grade 4-hydroxybenzyl-GLS (sinalbin), allyl GLS (sinigrin), and silica gel with indicator were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany); methanol (> 99.95%) and aryl sulfatase (extracted from *Helix pomatia*) from Th. Geyer GmbH + Co. KG (Renningen, Germany); indole-3-acetonitrile (≥ 98%) was acquired from Fischer Scientific GmbH, Schwerte, Germany; Na_2SO_4 (≥ 98.5%) was from VWR GmbH (Darmstadt, Germany); water was of milli-Q quality. 4-(Methylthio)butyl ITC (≥ 98%) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany); 5-(methylsulfinyl)butyl ITC was purchased from Enzo Life Sciences GmbH (Lörrach, Germany). The epithionitrile 1-cyano-2,3-epithiopropane (CETP; ≥ 95%) was synthetized by Taros Chemicals GmbH Co. KG (Dortmund, Germany) and 1-cyano-3,4-epithiobutane (CETB) and 1-cyano-4,5-epithiopentane were synthetized by ASCA GmbH Angewandte Synthesechemie Adlershof (Berlin, Germany). 3-Butenyl GLS (3But GLS), 2-(*R*)-2-hydroxy-3-butenyl GLS (≥ 98%), 2-(*S*)-2-hydroxy-3-butenyl GLS (≥ 98%), and 2-phenylethyl GLS (≥ 98%) were obtained from Phytolab GmbH and Co. KG, Vestenbergsgreuth, Germany.

Plant material and growth conditions

The two *A. thaliana* accessions Hi-0 and Bur-0 were sown and germinated under ambient atmosphere with artificial light (300 $\mu mol \cdot m^{2} \cdot s^{-1}$ with wavelength range of 400-700 nm), 40-60% relative air humidity, an 8 h photoperiod with 22 °C air temperature and a dark period with 18 °C air temperature. After pricking out on potting compost (Fruhstorfer Erde Typ P, HAWITA Gruppe GmbH, Vechta, Germany), the plants were separated and transferred into a control box and a box with modified atmosphere: the CO_2 level was enhanced to 2% while the control plants were kept at ambient atmosphere (0.04% CO_2). The plants were grown as above at 70% relative air humidity. Nematodes (*Steinernema feltiae*; Katz Biotech AG, Baruth, Germany) were applied preventively to avoid *Sciaridae*. The plants were harvested after three and four weeks of CO_2 treatment (plant age four and five weeks). *A. thaliana* rosettes were cropped (shoot) and a minimum of four biological replicas were prepared, each pooled from two plants. The whole experiment was repeated two times.

Measuring plant growth parameters

Biomass

Shoot biomasses were analyzed to assess the effect of CO_2 on primary plant production. For fresh weight two half rosettes were weighed into Polyvials®V (Zinsser Analytic Plastic Vials, 20 mL capacity). The dry weight was measured after flash freezing in liquid nitrogen and lyophilization. Finally, the freeze-dried samples were stored in the dark at room temperature.

Leaf area

Plants were removed from the boxes and photographed from the top view point (Canon EOS 60D + EF 85 mm) in order to assess leaf growth of *A. thaliana* after four weeks of CO_2 treatment. The projected leaf area (cm^2) was determined in three processing steps using the software packages: Digital Photo Professional (version 3.15.0.0, Canon, Tokio, Japan, step 1: raw file conversion), OneCut (Gorelick, L. 2015, step 2: leaf/background segmentation) and Fiji (version 1.51, ImageJ, step3: leaf/pixel area computation). The use of a supervised and global GraphCut segmentation algorithm was essential to discriminate between leaf rosettes and the substrate (background).

Electron transport rate of PSII

The electron transport rate (ETR) of light-adapted leaves is linearly related to CO_2 assimilation and was calculated from chlorophyll a fluorescence. Parameters for calculating ETR were obtained *in situ* after 4 weeks with a slightly adapted fluorometer (LI-6400XT Portable Photosynthesis System with LPL-Software, version 6.3.3, 2014, LICOR, Nebraska, USA, photosynthetically active radiation (PAR): 300 $\mu mol \cdot m^{2} \cdot s^{1}$, leaf to LED source distance: 0.5 cm) one day before the harvest. ETR was calculated by multiplying the quantum-weighted leaf absorption, the effective quantum yield of PSII (ϕPSII), the PSII excitation fraction (0.5) and the incidental PAR on leaf.

Glucosinolate analysis

GLSs were extracted using the method of Wiesner et al. (WIESNER et al., 2013) with small modifications. Briefly, 10 mg of lyophilized and homogenized plant material were extracted in presence of 0.02 µmol of the internal standard 4-hydroxybenzyl-GLS with 70% methanol (LC-MS grade, Th. Geyer GmbH & Co. KG, Renningen, Germany) and samples were prepared as described before (WIESNER et al., 2013). The desulfo-GLSs were analyzed using a 1290 Infinity II UHPLC-DAD coupled with a 6230 ToF-LC/MS (Agilent Technologies, Waldbronn, Germany) with a Poroshell 120 EC-C18 column (Agilent Technologies, Waldbronn, Germany; 100 mm × 2.1 mm, 2.7 µm). UHPLC conditions were as follows: solvent A, MilliQ water; solvent B, 100% v/v acetonitrile. The 19 min run comprised 0.2% (v/v) B (2 min), 0.2% to 19.8% (v/v) B (10 min), a 2 min hold at 19.8% (v/v) B, 19.8% B to 50% (v/v) B (1 min), a 1 min hold at 50% (v/v) B, 50% to 0.2% (v/v) B (1 min), and finally a 2 min hold at 0.2% (v/v) B. The injection volume was 5 µL, and determination was conducted at a flow rate of 0.4 mL min^{-1} and 30 °C and a wavelength of 229 nm. Desulfo-GLSs were identified by comparing retention times, UV absorption spectra, and mass spectral data with

those of individual desulfo-GLSs from authentic standards and with those from standard reference materials of oilseed rape (BCR-190R and BCR-367R). The concentration of desulfo-GLSs was calculated by the peak area relative to the area of the internal standard 4-hydroxybenzyl-GLS.

Analysis of glucosinolate hydrolysis products

GLS hydrolysis products were extracted and quantified according to Hanschen et al. (HANSCHEN et al., 2018a) using the 1 mL He/min flow method with small modifications: 250 mg of plant material was homogenized in the presence of 250 µL of H_2O, the internal standard benzonitrile was added (0.2 µmol), extracted twice after 30 min of incubation with methylene chloride, after which the combined extracts were dried over Na_2SO_4 and concentrated under N_2 gas and analyzed using GC-MS as described previously (HANSCHEN et al., 2018a) [Agilent 7890A Series GC-MS System (MSD: 5975C inert XL) with a SGE BPX5 column (30 m × 0.25 mm × 0.25 µm) (VWR International GmbH, Darmstadt, Germany), He as a carrier gas (1 mL min^{-1}) and an oven program starting from 35 °C (hold 3 min), rising with 9 °C min^{-1} to 90 °C (2 min hold), rising with 3 °C min^{-1} to 110 °C, then with 9 °C min^{-1} to 210 °C, with 3 °C min^{-1} to 223 °C, and with 9 °C min^{-1} to 230 °C. Finally, the column was heated with 35 °C min^{-1} to 310 °C (6 min hold). Mass spectral data (transfer line 270 °C, ion source 230 °C, quadrupole 150 °C) were acquired in the EI mode (70 eV) in full scan (30-240 m/z). Compounds were identified by their mass spectrum and retention time in comparison with those of authenticated standards and with literature data (KJAER et al., 1963; SPENCER and DAXENBICHLER, 1980). In Supplemental Table S2, those compounds that could be identified only based on their EI mass spectra are marked as tentatively identified. Quantification was performed using benzonitrile as internal standard and the response factors calculated from the ratio of the slope of linear calibration curves ($R^2 \geq 0.97$) relative to that of the internal standard. For the commercially unavailable compounds, a response factor equal to that of the chemically most similar compound was assumed.

Statistics

Statistical analyses were conducted with GraphPad PRISM software (version 9.0.0, La Jolla, California, USA). Significant differences between controls and CO_2 treatments were analyzed by unpaired t-test with Welch's corrections ($p \leq 0.05$).

Results

Primary production increased under elevated CO_2 concentrations

In order to evaluate the effect of the highly elevated CO_2 on plant growth and CO_2-fixation, biomass parameters and the effect on electron transport rate as a chlorophyll *a* fluorescence parameter have been analyzed. In both experimental replicas, total biomass increased in both *A. thaliana* accessions (Fig. 1). In Hi-0, the fresh weight increased significantly up to 65% after three weeks in the second experiment, and in both experiments by 46-65% after four weeks (Fig. 1A). In Bur-0, the effects were more pronounced and the fresh weight significantly increased under elevated [CO_2] by up to 69-111% (Fig. 1C).

Consistently, the same effect was observed for the dry weight biomass, which increased in Hi-0 (Fig. 1B) and Bur-0 (Fig. 1D) grown under elevated [CO_2] compared to plants grown under ambient air. Furthermore, in both experiments leaf areas increased significantly

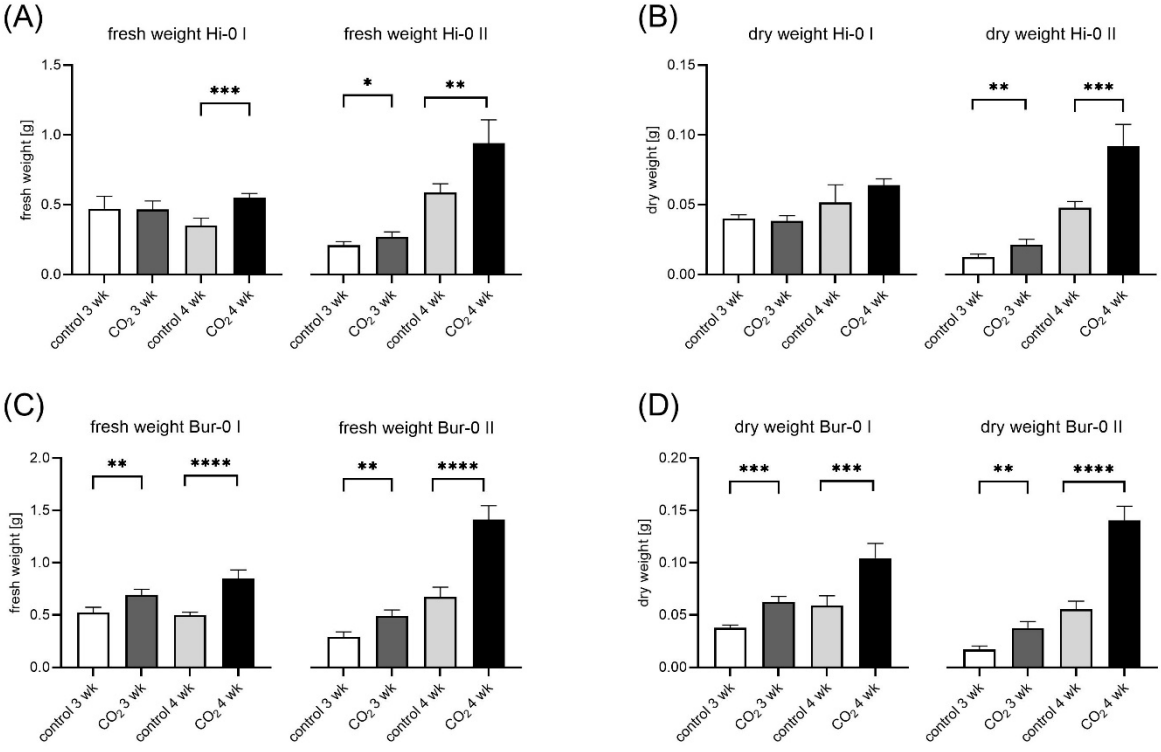

Fig. 1: Primary production of biomass under elevated CO_2 concentrations. (A) Fresh weight and **(B)** dry weight of plants from *A. thaliana* accession Hi-0 and **(C)** fresh weight and **(D)** dry weight of plants from *A. thaliana* accession Bur-0 under elevated (2%) [CO_2] (dark bars) and ambient air (0.04% [CO_2]) (light bars) after three and four weeks (wk). Averages and standard deviations of biological replicates (n = 4-5) are shown for two experimental replications (I and II) separately. Significant differences between CO_2 treated and control group were signed with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (unpaired t-test).

up to 27-38% in Hi-0 (Fig. 2A) and 41-66% in Bur-0 after four weeks under elevated [CO_2] (Fig. 2D).

After four weeks the CO_2-treated plants had significantly higher electronic transport rates (ETR) compared to control plants, e.g. Hi-0 plants showed an increment by 13-16% (Fig. 2B) and Bur-0 plants by 12-23% (Fig. 2E). Also, the effective quantum yield of PSII (ϕPSII) were significantly increased in Hi-0 by 14-18% and in Bur-0 by 12-25% after four weeks compared to control plants (Fig. 2C, 2F). In addition, to some extent, the CO_2-treated plants showed accelerated development, e.g. premature and earlier inflorescence was observed in Hi-0.

Effect of elevated CO_2 on glucosinolates depends on *A. thaliana* accession

In Hi-0 the main GLS was the alkenyl GLS allyl GLS (sinigrin), while Bur-0 contained mainly the alkenyl GLSs allyl- and 3-butenyl GLS (3But GLS) (Supplemental Table S1). The CO_2 treatment affected the GLS levels: the total amount of GLSs in Hi-0 was unaffected, whereas in Bur-0 the total GLS levels increased significantly after the CO_2 treatment (Supplemental Figure S1). In detail, in Hi-0 the levels of alkyl GLSs in the first experiment were significantly reduced (by up to 57%) after three and four weeks compared to the control plants but no significant effect could be found in the second experimental replicate. Likewise, for alkenyl-GLS and indole-GLSs also no clear effect could be found (Fig. 3). These results are in contrast to Bur-0, where the alkenyl and indole GLSs levels were significantly increased by CO_2 treatment by up to 50% and 36% respectively after four weeks of treatment (Fig. 3). These effects are mainly due to the enhanced amounts of the alkenyl GLSs allyl- and 3But-GLSs as well as the indolic GLS indol-3-ylmethyl GLS (Supplemental Table S1).

Elevated CO_2 enhanced the glucosinolate hydrolysis product formation in *A. thaliana*

In both ecotypes elevated CO_2 levels led to a significant increase in the release of GLS hydrolysis products upon homogenization (Supplemental Figure S2). In Hi-0 this increase in GLS hydrolysis product formation due to the CO_2 treatment was mainly due to increased nitrile levels, while in Bur-0 total epithionitrile levels increased (Supplemental Figure S2). Moreover a change in the relative formation of nitriles, epithionitriles and ITC was observed. In Hi-0 the relative formation increased after three weeks of CO_2 (but not after four) which was correlated with reduced relative ITC-formation (Supplemental Figure S3A, S3B). In Bur-0 only in one experimental replicate increased relative epithionitrile and nitrile formation was observed (Supplemental Figure S3C), while in the second experimental replicate the ratio of epithionitriles, nitriles and ITC remained unaffected by CO_2 treatment (Supplemental Figure S3D).

As allyl GLS was the main GLS of Hi-0 and together with 3But GLS the main GLSs of Bur-0, the effect of CO_2 on the levels of these main GLS and their hydrolysis product formation is displayed in Fig. 4 (allyl GLS hydrolysis) and Fig. 5 (3But GLS hydrolysis).

Effects on allyl- and 3-butenyl glucosinolate hydrolysis

Regarding hydrolysis of allyl GLS, in Hi-0 mainly Allyl-ITC was released upon its hydrolysis. Allyl-CN formation increased due to CO_2 treatment: up to 95% compared to control after three weeks of CO_2 treatment, while allyl GLS levels remained mainly unaffected (Fig. 4A, 4B). Allyl-ITC formation increased significantly in the first experimental replicate (Fig. 4A) but only by tendency in the other (Fig. 4B). In Bur-0, allyl GLS increased up to 69% compared to control after four weeks of CO_2 treatment (Fig. 4C, 4D). Upon hydrolysis

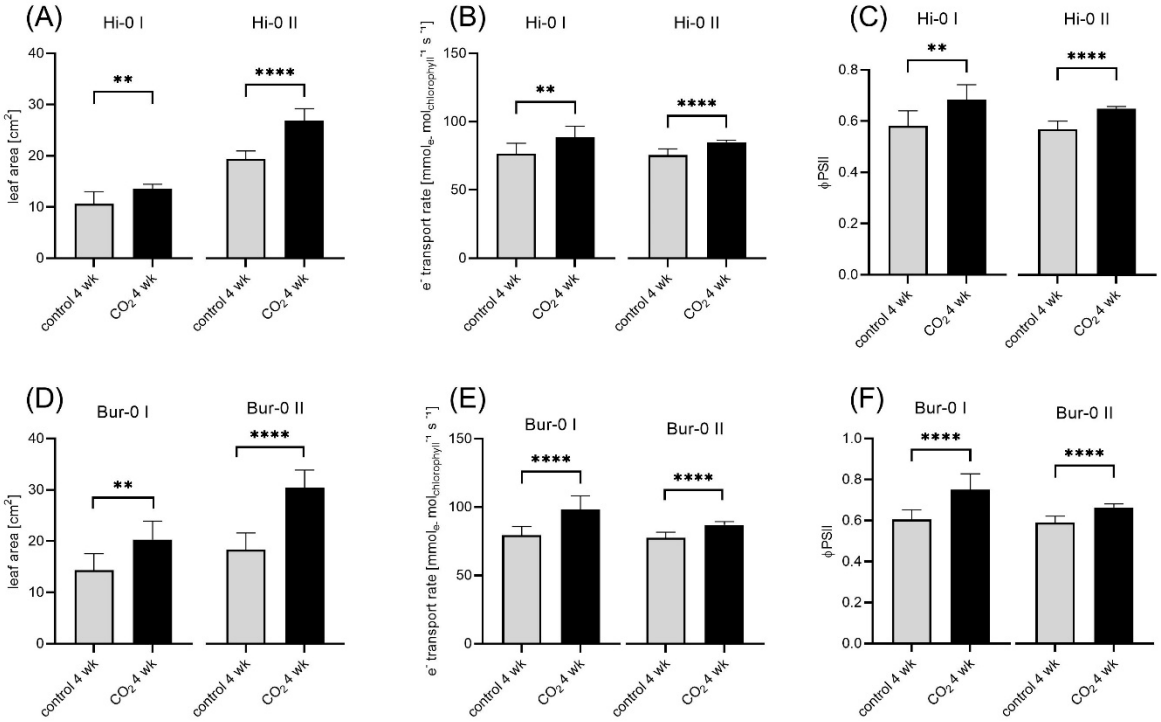

Fig. 2: Leaf area, electron transport rates and effective quantum yield of PSII under elevated CO_2 concentrations. (A) Leaf area, **(B)** electron transport rate (ETR) (ETR = leaf absorbance × ϕPSII × 0.5 × PAR) and **(C)** the effective quantum yield (ϕ) of PSII of *A. thaliana* accession Hi-0 and **(D)** leaf area, **(E)** ETR and **(F)** the effective quantum yield (ϕ) of PSII of *A. thaliana* accession Bur-0 under (2%) [CO_2] (dark bars) and ambient air (0.04% [CO_2]) (light bars) after four weeks (wk). Averages and standard deviations of biological replicates (n = 8-10) are shown for two experimental replications (I and II) separately. Significant differences between CO_2 treated and control groups are indicated with '*' ($p < 0.05$); '**' ($p < 0.01$) and '***' ($p < 0.005$), as tested using an unpaired t-test with Welch's corrections.

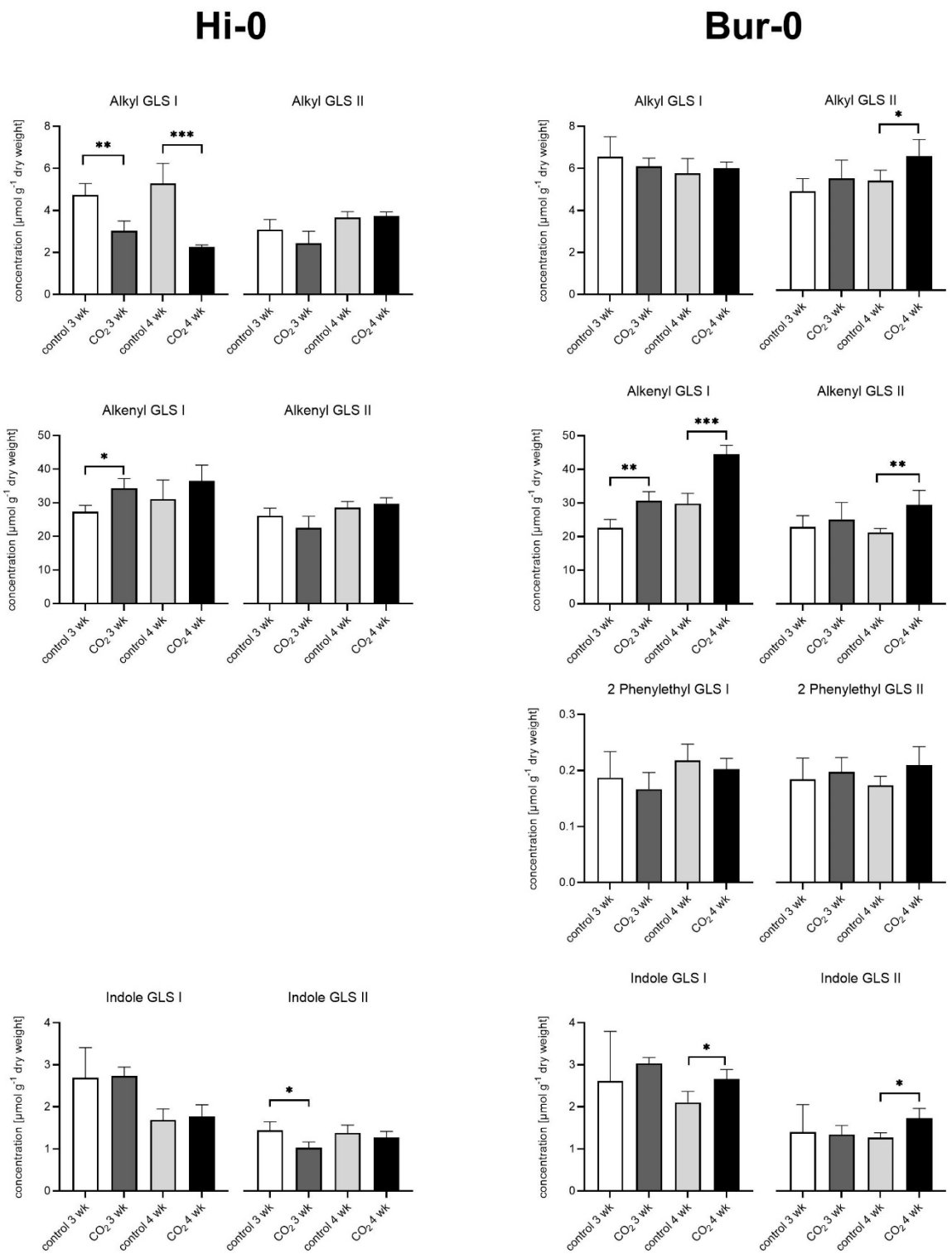

Fig. 3: Effect of elevated CO_2 on glucosinolates in *A. thaliana*. Elevated (2%) [CO_2] effects (dark bars) on glucosinolate (GLS) concentrations in Hi-0 and Bur-0 compared to ambient air (0.04% [CO_2]) (control, light bars) are shown after three and four weeks (wk). Averages and standard deviations of biological replicates (n = 4-5) are shown for two experimental replications (I and II) separately. Significant differences are marked with an asterisk (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; unpaired t-test).

the formation of the main allyl GLS hydrolysis product 1-cyano-2,3-epithiopropane (CETP) significantly increased by up to 133% relative to the control after four weeks of treatment, while the effects on Allyl-CN formations were unreproducible and Allyl-ITC formation was not affected (Fig. 4C, 4D).

With regard to the response of 3But GLS levels and hydrolysis, in Hi-0, where this GLS is found in low levels, a slight decrease in 3But GLS was observed after four weeks of CO_2-treament (Fig. 5A, 5B), while formation of the hydrolysis products was not significantly altered. In the accession Bur-0, where 3But GLS is the domineering GLS species, this GLS was increased significantly by 54% compared to control after four weeks of treatment (Fig. 5C, 5D). Upon its hydrolysis,

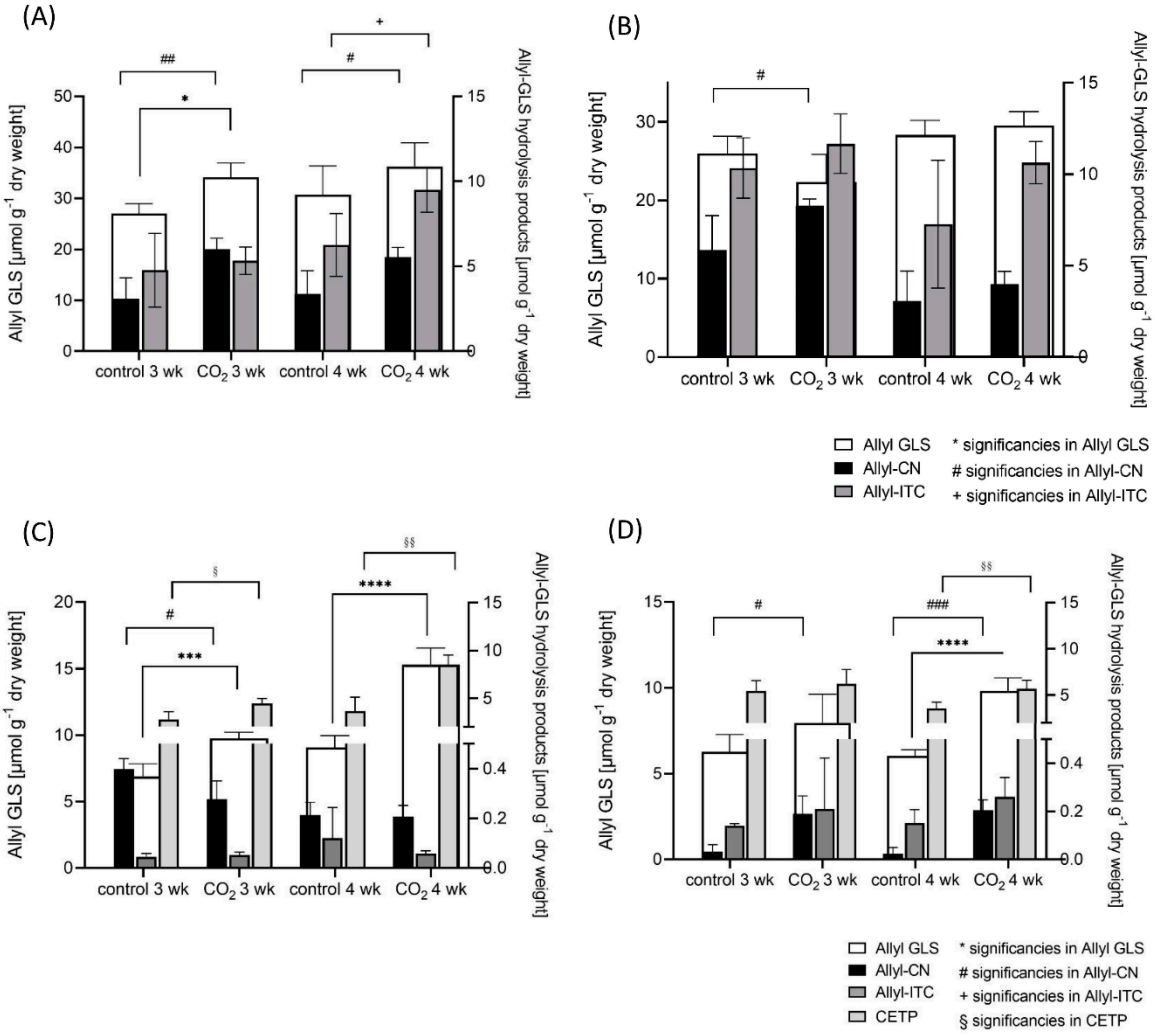

Fig. 4: Effect of elevated CO_2 on allyl glucosinolate (GLS) and the formation of its hydrolysis products from *A. thaliana*. Elevated (2%) [CO_2] effects on allyl GLS and formation of its hydrolysis products in accessions Hi-0 (A, B: experiment I and II) and Bur-0 (C, D: experiment I and II) were compared to ambient air (0.04% [CO_2]) (control) after three and four weeks (wk). Averages and standard deviations of biological replicates (n= 4-5) are shown for two experimental replications (A, B and C, D) separately. Significant differences are marked with symbols (*, #, +, §; for example *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; unpaired t-test). Abbreviations: Allyl-CN: 3-butenenitrile, Allyl-ITC: allyl isothiocyanate, CETP: 1-cyano-2,3-epithiopropane.

3But-ITC was not affected and 3But-CN formation increased only significantly in the first experimental replicate after four weeks (Fig. 5C). The formation of the corresponding epithionitrile 1-cyano-3,4-epithiobutane, which was the main 3But GLS hydrolysis product in Bur-0, increased after four weeks of CO_2 treatment by up to 136% relative to the control (Fig. 5C, 5D).

Discussion

In this study we evaluated the impact of 2% atmospheric [CO_2] on GLS levels, and to our knowledge for the first time, on the formation of their corresponding hydrolysis products in two *A. thaliana* accessions. We also evaluated the effect on the primary production. CO_2 treatment increased biomass production and release of GLSs hydrolysis products in *A. thaliana*, thereby demonstrating the potential in using excess CO_2 for producing high quality vegetables.

Due to the CO_2 treatment, primary production of the two *A. thaliana* accessions Hi-0 and Bur-0 was enhanced, particularly the formation of biomass. This is in agreement with previous reports, which observed an increase in the shoot biomass of *A. thaliana* at elevated CO_2 levels (Lau et al., 2007; Van der Kooij et al., 1999). Furthermore, the electron transport rate and thus primary CO_2 fixation of PSII increased under elevated CO_2 which confirms results of previous investigations (Badger et al., 2009; Wang et al., 2015). In the present study, especially CO_2-treated Hi-0 plants showed accelerated development, e.g. premature and earlier inflorescence was observed. Thus the effect on biomass gain, but not the effects of elevated CO_2 on CO_2 fixation (Fig. 2) and GLS hydrolysis (Fig. 4 and 5) could be explained by a slightly different ontogenetic stage of the CO_2-treated plants. It was demonstrated that increased mitochondrial pyruvate dehydrogenase activity is involved in earlier inflorescence in *Arabidopsis* at elevated CO_2 (700 ppm) (Weraduwage et al., 2016).

In our studies, the impact of elevated CO_2 on the absolute GLS concentrations differed depending on the ecotype. In general, total GLS levels increased significantly by up to 41% in Bur-0, but seemed unchanged in Hi-0 compared to the control group. Karowe and coworkers found a species-dependent shift in total leaf GLS content when treating three *Brassica* species with a rather gradual enrichment of 700 ppm CO_2 (Karowe et al., 1997). In young mustard and turnip leaves the total GLS content significantly decreased after treatment, while in treated young radish leaves the total GLS level increased. However, their results also showed an ontogenetic effect: in treated

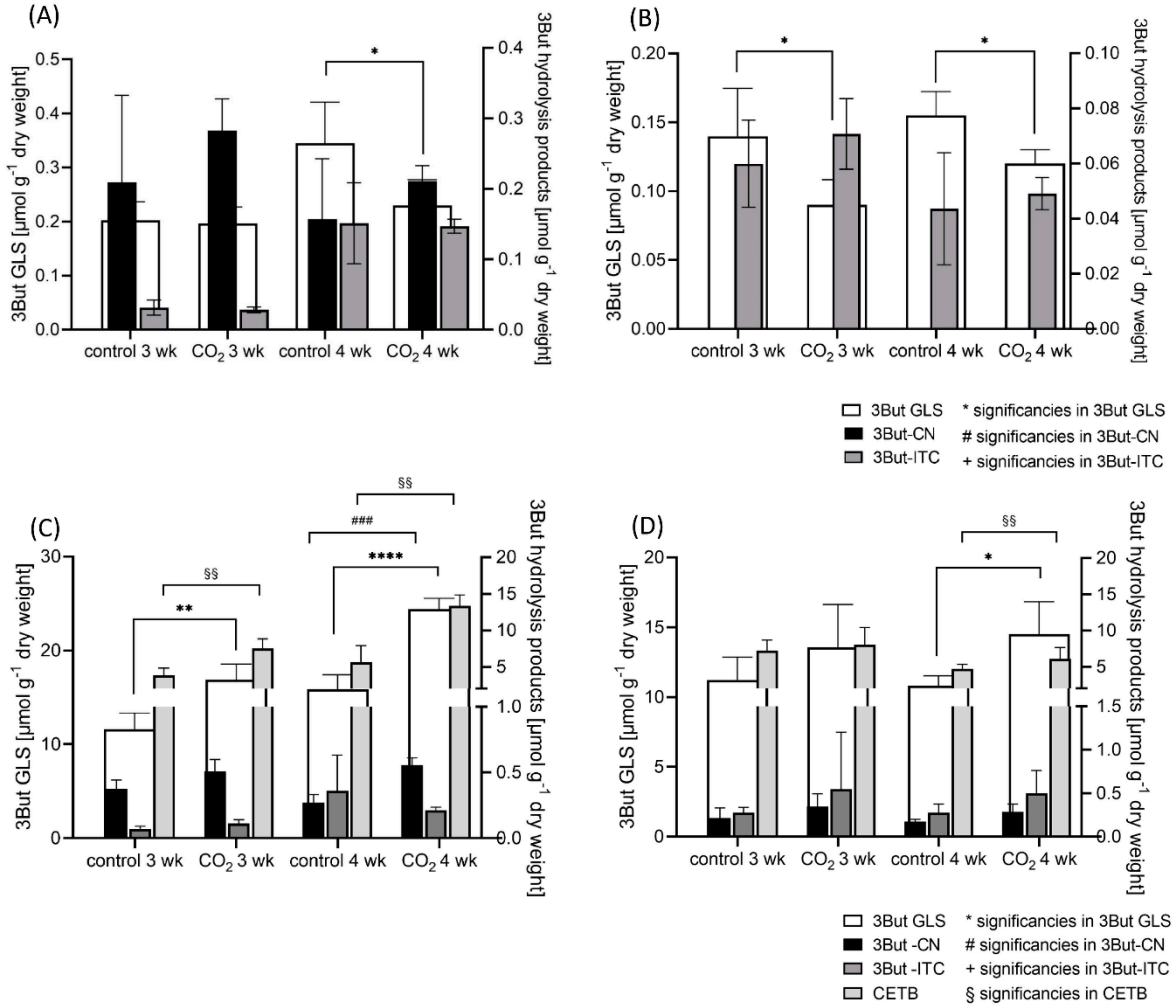

Fig. 5: Effect of elevated CO_2 on 3-butenyl glucosinolate (3But GLS) and the formation of its hydrolysis products from *A. thaliana*. Elevated (2%) [CO_2] effects on 3But GLS and formation of its hydrolysis products in accessions Hi-0 (A, B: experiment I and II) and Bur-0 (C, D: experiment I and II) were compared to ambient air (0.04% [CO_2]) (control). Averages and standard deviations of biological replicates (n = 4-5) are shown for two experimental replications (A, B and C, D) separately. Significant differences are marked with symbols (*, #, +, §; for example *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; unpaired t Test). Abbreviations: 3But-CNs: 4-pentenenitrile, 3But-ITC: 3-butenyl isothiocyanate, CETB: 1-cyano-3,4-epithiobutane.

older leaves total GLS levels decreased in all three *Brassica* species (Karowe et al., 1997). Moreover, enhanced GLS biosynthesis was found in guard cells under short-term treatment of elevated CO_2 (800 ppm) due to increasing levels of primary metabolites such as sugars and amino acids which are needed to form GLSs (Geng et al., 2016). The different response of both ecotypes to the elevated CO_2 is also seen within the different groups of GLSs. In Hi-0 no effects on GLS-groups that were consistent over the two experimental replicates were observed. In contrast, in Bur-0, while 2-phenylethyl GLS and alkyl GLS remained mainly unaffected, alkenyl GLS as well as indole GLS increased significantly after four weeks of CO_2 treatment, which was linked to increment in the alkenyl GLSs, allyl and 3But GLS, as well the indole GLS indol-3-ylmethyl GLS. This elevation may be related to increased expression or activity of GLS biosynthesis enzymes, such as methylthioalkylmalate synthases (MAM) (Redovnikovića et al., 2012), 2-oxoglutarate depending dioxygenases (Neal et al., 2010) and sulfotransferases (Klein and Papenbrock, 2009; Piotrowski et al., 2004). In accession Col-0, for example, the expression of *MAM1* increased under elevated CO_2 (Li et al., 2008). In a further study it was found that *A. thaliana* plants grown under higher atmospheric CO_2 levels showed increased transcription levels of *MYB76*, a transcription factor inducing the biosynthesis of aliphatic GLSs (Paudel et al., 2016). Allyl GLS seems to be more toxic than alkyl GLSs and thus may have a greater defense effect (Witzel et al., 2013). In that study it was demonstrated that extracts of freeze-dried leaves of six *A. thaliana* accessions, including Hi-0 and Bur-0, inhibited the growth of the fungus *Verticillium longisporum* by more than 50%, saying the allyl GLS-derived degradation product Allyl-ITC was responsible for the antifungal effect (Witzel et al., 2013). Elevated CO_2 could thus have improved the defensive ability of *A. thaliana* plants by increasing the percentage of alkenyl GLSs.

Due to increased CO_2 in the present study, the amount of released total GLS hydrolysis products increased in both replicates and *A. thaliana* accessions. This is probably linked to the higher GLS concentrations, as in case of Bur-0, but could also depend on other factors, like increased myrosinase activity or expression, which could apply for Hi-0. For example, the myrosinase thioglucoside glucohydrolase2 (TGG2) was incrementally expressed in accession Col-0 under elevated CO_2 (Li et al., 2008). Moreover, in broccoli sprouts grown under elevated CO_2 myrosinase activity also increased up to two-fold (Almuhayawi et al., 2020). Nevertheless, the recovery of the GLS hydrolysis products of a specific GLS relative to the corresponding GLS in general was similar between treatments and controls (Supplemental Figure S4) thereby making the increased myrosinase hypothesis less likely as explanation. Moreover, while in general in the *Arabidopsis* accessions nitriles, epithionitriles and

ITCs increased, in Hi-0 particularly nitriles were increased after three weeks. Since Hi-0 was very low in epithionitriles (although it is rich in alkenyl GLS), which is caused by very low expression of the ESP protein (Hanschen et al., 2018b), the increment in nitriles cannot be attributed to ESP. However, the breakdown of GLS is also affected by other factors. Since *A. thaliana* can also have nitrile specifier proteins (NSPs), which especially in the roots alter GLS hydrolysis in favor of nitriles (Wittstock et al., 2016), it is conceivable here that CO_2 increased the expression of NSPs in *A. thaliana*. However, shifts in the pH value or Fe^{2+} concentrations could also affect the ratio of nitriles, ITCs and epithionitriles (Burow et al., 2009; Hanschen et al., 2017). Li et al. found an increase in intercellular CO_2 by up to 32% even under 0.055% CO_2 (Li et al., 2008), which can acidify the cytoplasm (Assmann, 1999). Lower pH may not only affect the conformation and activity of enzymes (Martinière et al., 2013), but also increase nitrile formation due to the chemical degradation of GLS-aglucon, since the Lossen-like rearrangement to ITC is blocked by protons (Uda et al., 1986). Higher Fe^{2+} concentrations will also increase nitrile formation from myrosinase-only hydrolysis, and it also increases the activities of NSPs and ESP (Burow et al., 2009; Burow et al., 2006; Uda et al., 1986).

In Bur-0 grown under elevated CO_2 the percentage of epithionitriles only increased in the first experimental replicate (forcing down nitriles) but not in the second. Such a shift indicates increased activity or biosynthesis of ESP. ESP possesses a catalytic function and can alter the decomposition of alkenyl GLS-aglucons to produce epithionitriles (Burow et al., 2006). Moreover, in *A. thaliana* Cvi-0 Li et al. (2008) found higher expression of ESP under elevated CO_2 (Li et al., 2008). In contrast, in broccoli sprouts CO_2-treatment reduced ESP activity and increased the formation of the health promoting 4-(methylsulfinyl)butyl ITC (Almuhayawi et al., 2020). However, taken together all the results of the present study, in Bur-0 the increase in epithionitrile formation was mainly linked to overall increment of the corresponding alkenyl GLSs.

The question is whether CO_2 can be used for the cultivation of nutritionally valuable plants for humans in a strongly demanded sustainable food production system. We conclude that elevated CO_2 content increases biomass production as well as GLS levels and the formation of their hydrolysis products, when enhanced CO_2 treatment is already started at an early stage of plant development and continued to the end of the vegetative plant phase. However, with species that are epithionitrile forming, high CO_2-levels can even further promote epithionitrile formation, while maintaining ITC formation on a low basis. In contrast to ITC, the effects of epithionitriles so far are still not fully explored. Epithionitriles are linked to nephrotoxicity in rodents (VanSteenhouse et al., 1999) and cytotoxic effects *in vitro* (Hanschen et al., 2015), but also chemopreventive effects have been reported (Kelleher et al., 2009). Similarly to ITCs, epithionitriles are bioavailable and rapidly metabolized via the mercapturic acid pathway (Hanschen et al., 2019). As a conclusion, CO_2-rich atmosphere is beneficial for both plant biomass production and enrichment with GLS but with regard to health-promoting GLS hydrolysis products the ratio of ITC was not increased but epithionitriles and nitriles were favored. Nevertheless, preparation conditions and changes in pH during hydrolysis (for example due to saliva in the mouth or due to acids added to a salad) have a strong effect on GLS hydrolysis and thus using favorable conditions, ITC-formation from epithionitrile producing plants can be easily promoted (Hanschen, 2020; Hanschen et al., 2017).

It should be investigated whether consumed vegetables of the Brassicaceae family behave similarly to *A. thaliana* under increased CO_2 level. In particular, the CO_2 recycling capacity of *Brassica* species such as cabbage and broccoli should be studied. As rising CO_2 levels also activate defense strategies in the plants and enhance resistance against pathogens such as bacteria and fungi or herbivores by increasing GLS levels after infestation or leaf injury (Bidart-Bouzat et al., 2005; Himanen et al., 2008; Paudel et al., 2016), growth under elevated CO_2 is a promising strategy for future food production systems.

Acknowledgements

The authors thank Elke Büsch and Andrea Jankowsky for excellent technical help, and Thomas Runge and Ingo Hauschild for help with the experimental facility.

Funding

The experimental facility (carbon dioxide chamber) was funded by the strategy fund of the Leibniz Institute for Vegetables and Ornamental Crops (IGZ) e.V.; Franziska S. Hanschen is funded by the Leibniz-Association (Leibniz-Junior Research Group OPTIGLUP; J16/2017).

Conflict of interest

There author declares no conflict of interest.

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Supplementary material

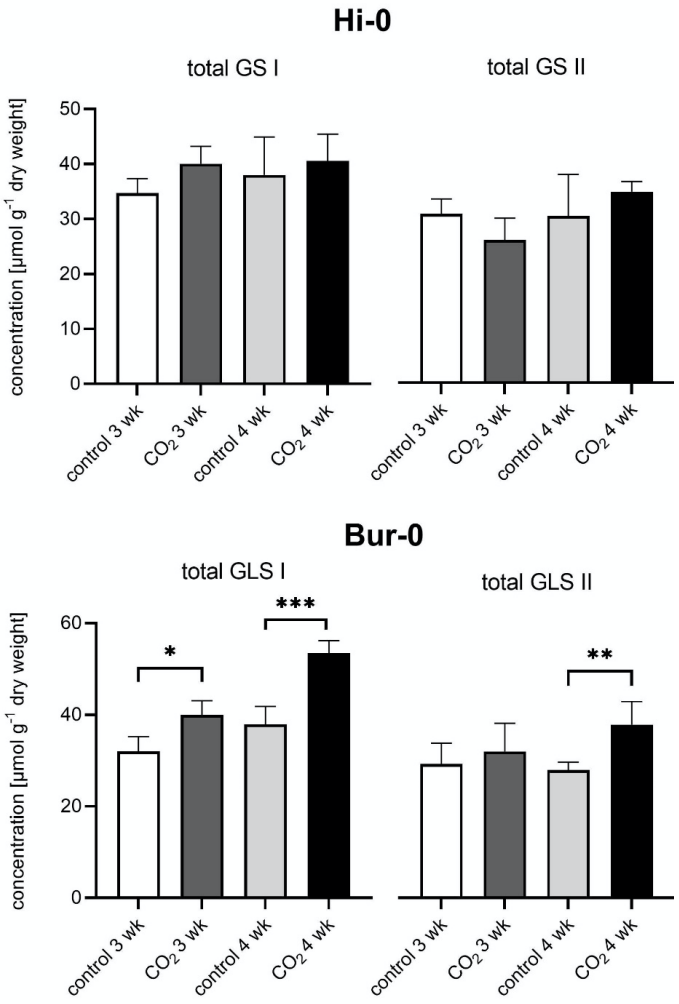

Fig. S1: Changes in total glucosinolate (GLS) levels in *Arabidopsis thaliana* Hi-0 and Bur-0 after CO_2 treatment for three or four weeks (wk). Averages and standard deviations of biological replicates (n = 4-5) are shown for two experimental replications (I and II) separately. Significant differences are marked with an asterisk (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ; unpaired *t*-test).

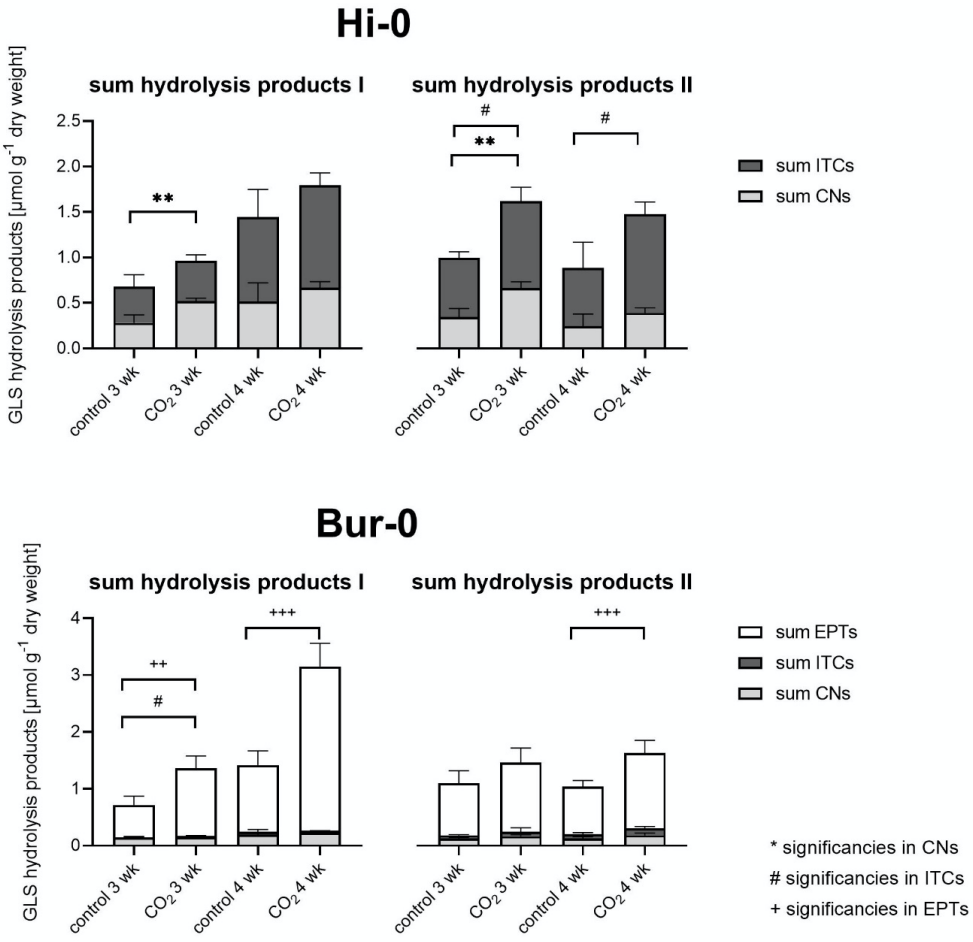

Fig. S2: Effect of elevated CO_2 on the sum of glucosinolate (GLS) hydrolysis products. Hi-0 and Bur-0 after CO_2 treatment (2%) for three or four weeks (wk) compared to ambient air (control). Averages and standard deviations of biological replicates (n = 4-5) are shown for two experimental replications (I and II) separately. Significant differences are marked with symbols (*, #, +, §; for example *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; unpaired *t*-test). CNs: nitriles, ITCs: isothiocyanates, EPTs: epithionitriles

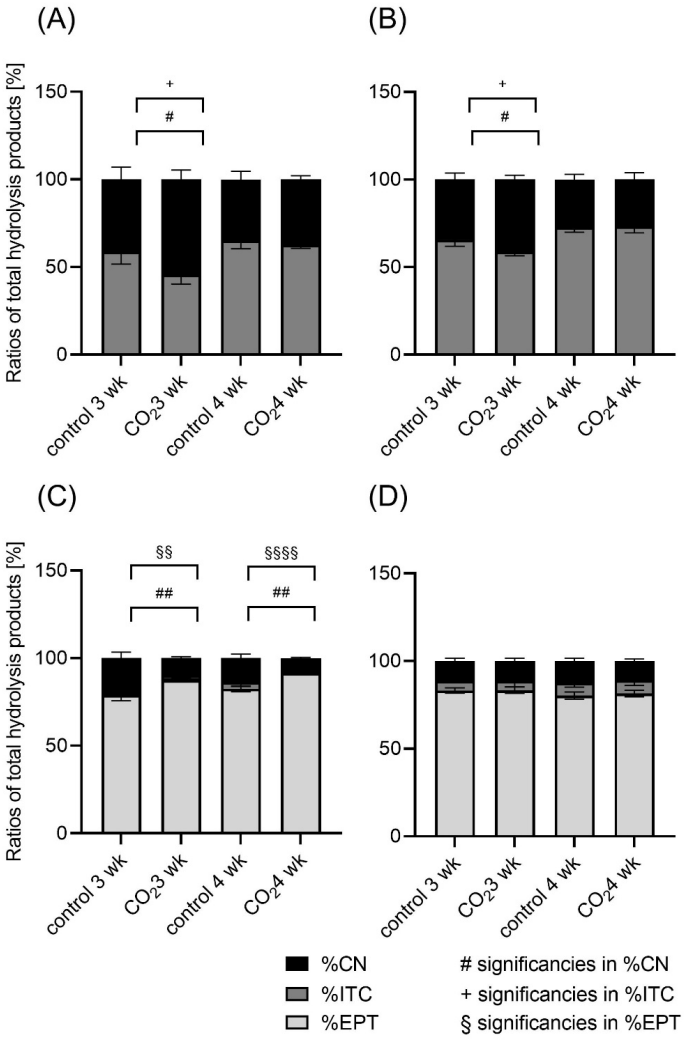

Fig. S3: Effect of elevated CO_2 on the percentage of nitriles (%CN), epithionitriles (%EPT) and isothiocyanates (%ITC) relative to the total glucosinolate hydrolysis products in Hi-0 and Bur-0 after CO_2 treatment for three or four weeks (wk) compared to ambient air (control) shown as relative to the control. Averages and standard deviations of biological replicates (n = 4-5) are shown for two experimental replications (I and II) separately. Significant differences are marked with symbols (*, #, +, §; for example *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; unpaired *t*-test).

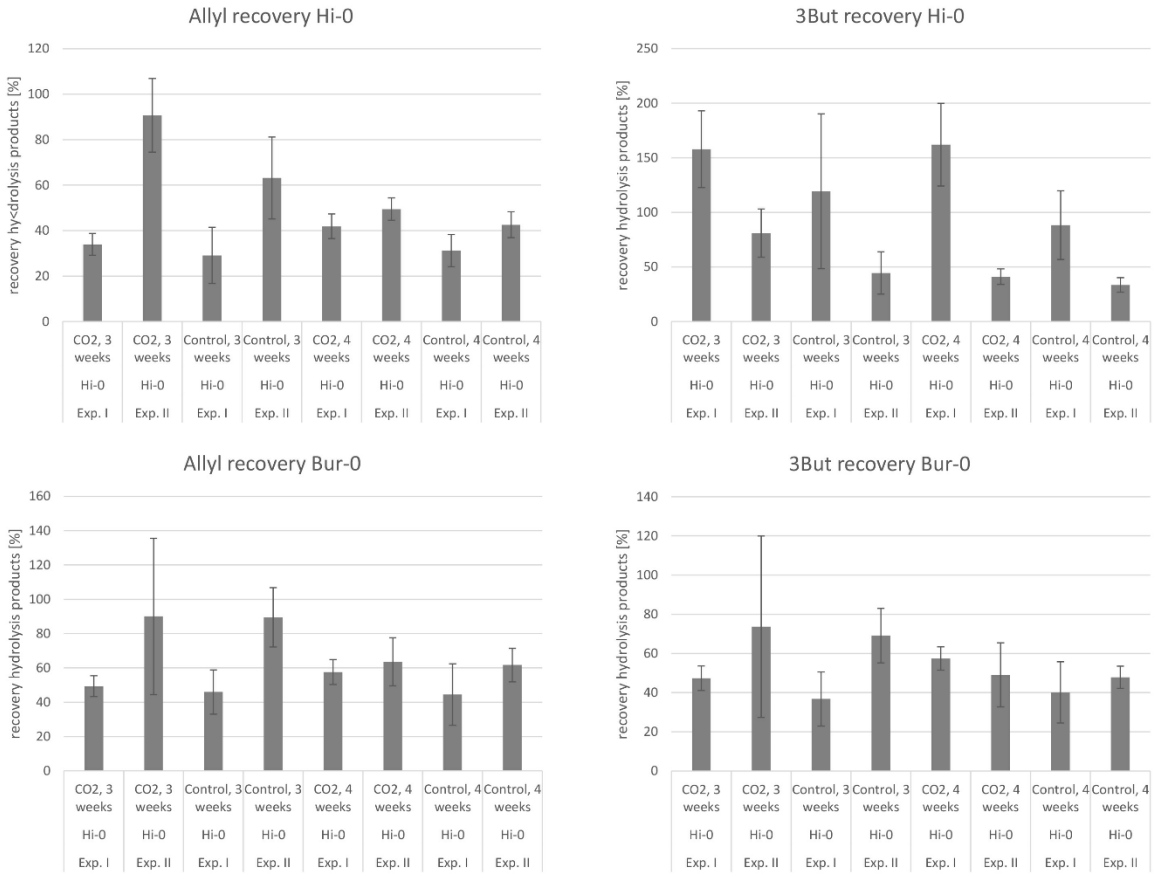

Fig. S4: Recovery of total allyl (Allyl) and 3-butenyl (3But) glucosinolate (GLS) hydrolysis products relative to the amount of the respective GLS values. Averages and standard deviations of biological replicates (n = 4-5) are shown for two experimental replications (I and II) separately.