

SCIENTIFIC REPORTS



OPEN

Hazardous gases (CO, NO_x, CH₄ and C₃H₈) released from CO₂ fertilizer unit lead to oxidative damage and degrades photosynthesis in strawberry plants

Sowbiya Muneer^{1,2,3} & Jeong Hyun Lee¹

CO₂ boilers/direct heating systems used in greenhouses often lead to incomplete combustion, which results in the formation of hazardous gases, such as carbon monoxide (CO), nitroxide (NO_x) and other hydrocarbons. In this study, strawberry plants that were grown on rockwool cubes were transferred to airtight bottles and treated with CO, NO_x, CH₄ and C₃H₈ gases for 1–48 hours. Oxidative damage due to hazardous gases was observed, as indicated by H₂O₂ and O₂⁻¹ determination. Photosynthetic pigments were reduced, and stomatal guard cells were damaged and remained closed compared to the control. The activity of other photosynthetic parameters was negatively related to hazardous gases. Reduction in the expression of multiprotein complexes was highly observed under hazardous gas treatments. This study highlighted that hazardous gases (CO, NO_x, CH₄ and C₃H₈) emitted due to incomplete combustion of CO₂ fertilization units/or direct heating systems resulted in the formation of ROS in shoots and limited photosynthetic metabolism. We predicted that major steps must be incorporated to improve the efficiency of CO₂ boiler/heating systems to decrease the emission of these hazardous gases and other hydrocarbons and to reduce the observed risks that are key to the reduction of crops.

The use of a greenhouse heater for carbon fertilization from combusted gas of a direct heating system or hot water boiler system is increased daily for higher yields of horticultural crops. The increase in the yield of CO₂ supply from the heating system results in a higher concentration of CO₂, thus increasing crop yield¹. However, these systems used for CO₂ supply often recapture low combustion, which results in the formation of potentially harmful gases, such as CO (carbon monoxide), NO_x (nitrogen oxide)² and other hydrocarbons, which could be CH₄ (methane) and/or C₃H₈ (propane)³. It has been reported that up to 12 ppm of these hazardous gases are not potential threats to crops, whereas if the concentration exceeds 20 ppm, they can greatly affect the crop yield^{4,5}. The use of CO₂ fertilization from a direct heating system or combusted gas from a hot water boiler system in greenhouse horticultural crops is still under process, although few reports have reported that increased levels of CO₂ fertilization are harmful to plant productivity⁴. Although several steps have been incorporated in certain countries to develop an efficient carbon fertilization unit or heating system to reduce the emission of these hazardous gases, the use of CO₂ from direct heating systems in Korea is still under process, which has greatly reduced the yield of crops in the past few years. Until now, it has not been documented how these hazardous gases affect photosynthetic metabolism and bio-macromolecules at the protein level and what steps should be taken to reduce the emission of these hazardous gases in several developing/developed countries where efficient CO₂ fertilization units have not yet been incorporated. Here, we present a novel study on the production of reactive oxygen species (ROS), photosynthetic metabolism and thylakoid multiprotein complexes in strawberry plants,

¹Department of Horticulture, College of Agricultural life sciences, Chonnam National University, 300 Young Bong-Dong Buk-Gu, Gwangju, 500-757, Korea. ²Centre for Agricultural Innovations and Advanced Learning [VAIAL], Vellore Institute of Technology, Tamil Nadu, Vellore, 632014, India. ³School of Bioscience and Biotechnology, Vellore Institute of Technology, Tamil-Nadu, Vellore, 632014, India. Correspondence and requests for materials should be addressed to J.H.L. (email: leetag@chonnam.ac.kr)

one of the highest crops grown throughout the year in Korea in greenhouses, as affected by hazardous gases, such as carbon monoxide (CO) and nitroxide (NO_x), and other hydrocarbons, such as CH₄ and C₃H₈.

As described above, CO₂ fertilization from a direct heating system or hot water boiler system due to incomplete combustion results in the emission of hazardous gases, such as carbon monoxide (CO) and NO_x. CO is a fungistatic gas that suppresses fungal growth; its effect is pathogen dependent and is greatly enhanced when CO is combined with O₂ in the atmosphere. Since CO is oxidized to CO₂ and is incorporated into organic acids and other products, it can directly affect the physiology of plants⁶. NO_x is a free radical gas that transfers electrons across biological membranes⁷. The biological function of NO_x is therefore the generation of reactive oxygen species (ROS)⁷. The physiological generation of ROS can occur as a byproduct of other biological reactions. ROS generation as a byproduct occurs with mitochondria, peroxisomes and other cellular elements⁸. In addition to CO and NO_x, the incomplete combustion of the CO₂ supply system used in greenhouses leads to the emission of hydrocarbons; however, these hydrocarbons are still unknown. The assumption is that these hydrocarbon emissions could be CH₄ and C₃H₈ because CH₄ has been observed as a freely available gas in greenhouses and has become a serious threat to crop production and to the outside environment. It is important to observe the effect of CH₄ because of its potential harm to global warming. There is much less CH₄ in the atmosphere than CO₂, with approximately 1800 parts per billion (ppb) compared with an estimated 390 parts per million of CO₂; conversely, it has been estimated that 95% of plant growth reduction is due to CH₄ compared to other greenhouse gases. C₃H₈ is relatively highly contributed by industries for fuel consumption. C₃H₈ is not considered a greenhouse gas, so its effect was considered very low in previous studies. Conversely, in 2008, the United States of Environmental Protection Agency reported that high amounts of C₃H₈ used as fuel could be a factor in CO₂ emissions along with other fossil fuels, which could affect the growth and development of plants. This led to the idea that C₃H₈ might be the other hydrocarbon emitted from the incomplete combustion of the direct heating systems used in greenhouses.

These hazardous gases lead to the development of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) and hydroxyl radical (OH·), as previously observed in our studies⁴. The elevation of these ROS can affect physiological metabolism, causing oxidative damage to nucleic acids, proteins and lipids^{9–11}. These hazardous gases can also influence the content of secondary metabolites in plants, which could therefore affect aquatic and terrestrial ecosystems¹². These hazardous gases affect photosynthesis and can affect multiprotein complex proteins in thylakoids carrying electrons and lipids¹¹. It is well known that thylakoid membranes contain the multiprotein complex photosystems I and II, which include the reaction centers responsible for converting light energy to chemical energy, as well as the cytochrome *b6/f* complex and ATPase¹³.

Previously, we observed the reduction of photosynthesis in response to hazardous gas stresses in a dose-dependent manner and detected that strawberry plants can act as a hyper-accumulator due to increased levels of antioxidant enzymes to protect against environmental pollution, but the defense ability did not adequately reduce the oxidative damage under long-term exposure to hazardous gas stress (CO, NO_x and SO₂) and effectively reduced plant development¹¹. Since strawberry is a staple fruit for many countries and is grown in greenhouses throughout the year, a CO₂ supply system from a direct heater is used to obtain a higher fruit yield. The present study suggested that the high concentration of these hazardous gases crucially produces reactive oxygen species (ROS) and drastically limits the photosynthetic activity. Therefore, we conclude that major steps should be incorporated to improve the efficiency of CO₂ boilers/heating systems to reduce the emission of hazardous gases and other hydrocarbons, which are key in the reduction of greenhouse-grown crops. In addition, we conclude that efficient and highly advanced CO₂ boilers/heating systems should be installed in greenhouses to elevate photosynthesis in order to improve crop productivity.

Material and Methods

Plant material and treatments. Young strawberry (*Fragaria × anansa*) plants were propagated on rockwool cube (10 × 10 × 6.5 cm) from the runner at 25 °C under fluorescent light (300 μmol m⁻² s⁻¹ of photosynthetic photon flux density) for initiating root and leaves for three weeks (diagrammatic representation in Supplementary Fig. S1) as described in our previous work¹¹. The rooted strawberry plants were grown on the rockwool cubes with hydroponic nutrient solution containing (mM per liter for the macro elements): N 9.5, P 9.5, K 5.5, Ca 5.0, S 1.5, P 1.5 Mg 1.5 and (μM per liter for the micro elements): Fe 33, Mn 22, B 10, Zn 5, Cu 1.0, Mo 0.5. The nutrient solution supplied was regularly with electrical conductivity (EC) 1.0 dS m⁻¹ and pH was maintained to 6.5–7.0. The plants were grown under fluorescent lights with a light intensity of 150 μmol m⁻² s⁻¹ with 16 h photoperiod and 8 h dark conditions. Six-week-old plants grown on rock-wool were transferred to air tight glass bottle (3 litres) and were supplied to 267 ppm of carbon monoxide (CO), 50 ppm of nitroxide (NO_x), 167 ppm of CH₄, and 67 ppm of C₃H₈. The concentration of these hazardous gases are not exactly same seen in the greenhouses what we used, it will vary according the environmental conditions provided or present. The concentration of these gases were selected based on our preliminary experiments¹¹ where we used these hazardous gases in dose dependent manner¹¹. The concentration of treatments were selected based on the records of strawberry growers in Korea. Therefore, different concentration of these hazardous gases: low or high in other countries can be have different effects. The strawberry plants exposed to CO, NO_x, CH₄ and C₃H₈ were harvested from 1–48 hours of treatment, leaves were separated by the order of ontogenic appearance and young leaves were considered for the study. Plant samples were immediately frozen in liquid N₂ and stored in deep-freezer (–80 °C) for further analysis. For determination of physiological measurements such as net-photosynthesis, and Fv/Fm unharvested plants were used.

Determination of H₂O₂ and O₂⁻¹. H₂O₂ and O₂⁻¹ levels were quantified according to Muneer *et al.*¹¹ and Lin and Kao¹⁴. H₂O₂ concentration was measured calorimetrically using titanium sulfate and was expressed as nmol g⁻¹ tissue fresh weight. H₂O₂ concentration was calculated using the extinction coefficient 0.28 mM⁻¹ cm⁻¹

and was expressed as $\mu\text{mole g}^{-1}$ tissue fresh weight. O_2^{-1} determination was measured calorimetrically using alpha aminobenzene sulphonic acid and alpha naphthylamine solution and was expressed as nmol g^{-1} tissue fresh weight. For O_2^{-1} determination leaves of strawberry were extracted in phosphate buffer with pH 7.5, centrifuged for 5 min at 10,000 g. The resultant supernatant was then collected and 1 ml of 10 mM $\text{NH}_2\text{OH-HCl}$ and phosphate buffer (pH 7.8) were added. The solution was incubated at 25 °C for 1 h, incubated samples were added with 0.5 ml of 17 mM alpha aminobenzene sulphonic acid and 0.5 ml of 7 mM alpha naphthylamine solution. The mixture was again incubated for 20 min at 25 °C and absorbance was recorded at 530 nm

Photosynthetic pigments. The content of chlorophyll and carotenoid was estimated by the method of Hiscox and Israelstam¹⁵ as described in our previous studies¹¹.

Measurement of photosynthetic activity, Fv/Fm ratio and Stomatal observation. Photosynthesis rate, were measured using a portable photosynthesis measurement system (LI-6400XT, LI-COR, Inc., Lincoln, NE) and Fv/Fm ratio were measured by modulated fluorimetre (OS1-FL, Optic Science) after 30 min dark adaptation. One of the fully expanded leaves from per plant was measured for 1, 3, 9, 24 and 48 hours after different hazardous gas (CO , NO_x , CH_4 and C_3H_8) treatments.

For stomatal observation, thin layer of leaf tissues was carefully cut and were laid on a glass slide, covered with a cover slip by adding few drops of water and were observed under light microscope under 40X magnification.

RuBisCO activity and sucrose synthase. RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) activity in leaves was estimated by the method given by Parry *et al.*¹⁶. One g of leaf sample was extracted in extraction buffer containing 50 mM Bicine (pH 8.0), 20 mM KCl, 5 mM DTT, 0.1 mM EDTA, 2% PVP and 0.1 mM PMSF and 5 μM luepeptin and was centrifuged at 5 000 g for 5 min. Assays of initial activity were performed at 30 °C, with 100 μL of supernatant added to 400 μL of assay buffer (166 mM Bicine-KOH, pH 8.0, 10 mM MgCl_2 , 5 mM DTT, and 25 mM NaHCO_3). The reaction was initiated with the addition of RuBP to a final concentration of 0.5 mM and terminated after 1 min with 200 μL of 5 N HCl. RuBisCO activity was measured following the oxidation of NADH at 340 nm.

Sucrose synthase in leaves was estimated spectrophotometrically by the method given by Xu *et al.*¹⁷. Sucrose synthase was assayed in a 1 ml reaction mixture containing 100 mM MES (pH 6.5), 3 mM Mg-acetate, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 0.02 mM glucose 1,6-diP, 0.5 mM NAD, 1 mM UDP, 1 mM PPI, 50 mM sucrose (for crude enzyme), or 200 mM sucrose (for purified enzyme), 1 unit phosphoglucomutase, 2 units glucose-6-P dehydrogenase (from *Leuconostoc*), and 1 unit UDP-glucose pyrophosphorylase was added. Sucrose synthase activity was measured following the oxidation of pyrophosphates (PPI) at 340 nm.

Multiprotein complex proteins (MCPs) (Thylakoid proteins). BN-PAGE of integral thylakoid proteins was performed according to our previous studies Muneer *et al.*⁴. The leaves from strawberry treated plants were collected in a liquid nitrogen and immediately grinded to fine powder in a pestle and mortar. Approximately five to ten gram of powdered samples were suspended in ice cold buffer containing 330 mM sorbitol/50 mM HEPES/5 mM MgCl_2 /2 mM EDTA/2 mM NaF with pH adjusted to 7.8. The resulting suspension was filtered through miracloth (calbiochem, San Diego, CA, USA). The filtered solution were centrifuged at 4,500 rpm for 10 min at 4 degree and resulting pellet were resuspended in same buffer, followed by centrifugation as same in first step. The pellet obtained were resuspended in second buffer containing 20 mM tricine, 70 mM sucrose, and 5 mM MgCl_2 (pH 7.8) and centrifuged at 4,500 rpm for 10 min at 4 degree. The resulting pellet were then washed with washing buffer of 330 mM sorbitol, 50 mM BisTris-HCl, pH 7.0, and 0.1 mg ml^{-1} pefabloc. After washing steps, the purified protein pellet were suspended in 2% w/v n-dodecyl- β -D-maltoside (Sigma, St. Louis, MO, USA) for solubilization. The insoluble material were removed by centrifugation at 13,000 rpm. The resulting protein mixture were mixed with 0.1% loading dye (5% CBB-G250, 100 mM BisTris-HCl, pH 7.0, 30% w/v sucrose and 500 mM ϵ -amino-n-caproic acid) and were loaded on 5–12% w/v acrylamide gradient gel (1 mm). The protein concentration was determined by Bradford and electrophoresis was performed in a ProteanIIxi cell (Bio-Rad, Hercules, CA, USA) at 4 degree by supplying constant voltage of 60 for 60 min and 120 volts until the protein bands were separated. The protein gels were photographed with digital camera and examined for the further analysis.

Statistical analysis. A completely randomized design was used with three replicates for five treatments. An individual Student's *t* test and Tukey's test was employed to compare the means of separate replicates by using software SAS (version 9.1, USA).

Results

Morphology and production of reactive oxygen species. The reactive oxygen species in hazardous gas-treated leaves were determined to be H_2O_2 and O_2^{-1} . The concentration of H_2O_2 in the presence of CO , NO_x , CH_4 , and C_3H_8 increased significantly 24 hours after treatment (−305, −370, −488 and −723%, respectively) (Fig. 1a); however, the induction of reactive oxygen species production was more severe at 48 hours after treatment when compared to the control. Similarly, the O_2^{-1} concentration was increased at 24 hours after treatment under CO , NO_x , CH_4 and C_3H_8 compared to control plants, whereas the concentration was severe at 48 hours after treatment and was increased by −454, −718, −144 and −1442%, respectively (Fig. 1b).

Photosynthetic pigments and stomatal observation. The total chlorophyll and carotenoid contents were significantly decreased by CO , NO_x , CH_4 , and C_3H_8 compared to control plants (Fig. 2a,b). The negative effect of these photosynthetic pigments was more prominent at 48 hours after treatment and was reduced by 50–66% of total chlorophyll content and 40–50% of carotenoid content.

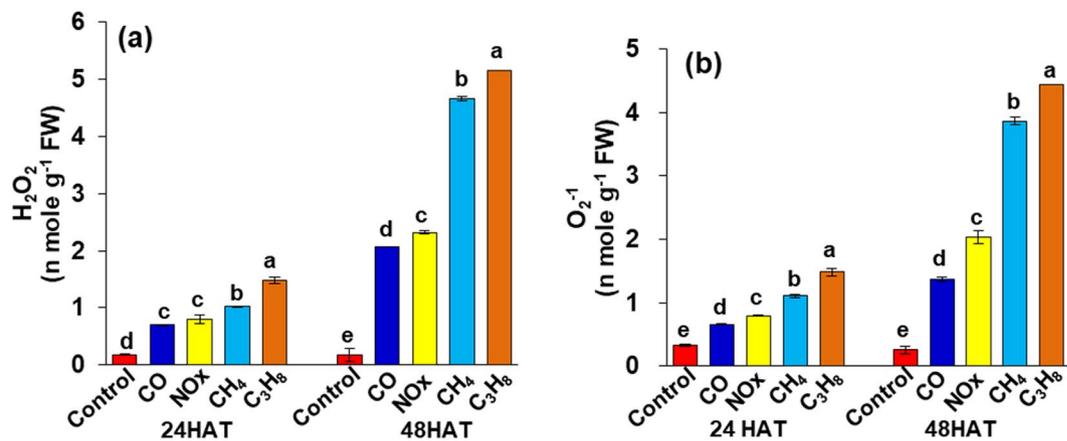


Figure 1. Oxidative damage in leaves indicated by (a) H₂O₂ (b) O₂⁻¹ determination as affected by CO, NO_x, CH₄ and C₃H₈ compared to control at 24 and 48 hours after treatment. Vertical bars indicate ± SE by means with n = 3. Means denoted by the different letter are significantly different at $p < 0.05$ according to the Tukey's studentized range test.

Microscopic observations (40X) showed that the stomata did not show any change in behavior for the first 1 hour after CO, NO_x, CH₄ and C₃H₈ treatment (Fig. 2c). After 3 hours of treatment, the stomata closed, although the guard cells did not show any change. Conversely, 3 to 24 hours after CO, NO_x, CH₄ and C₃H₈ treatment, the stomata were completely closed, and the guard/subsidiary cells were damaged compared to the control. This damage was severely more exaggerated at 48 hours after treatment at a particular magnification chosen under a microscope.

Photosynthetic activity and Fv/Fm ratio. The net photosynthetic rate did not show any changes for the first hour under CO, NO_x, CH₄ and C₃H₈ treatment; however, after 3 to 24 hours of treatment, the net photosynthetic rate decreased continuously (Fig. 3a), and the reduction in photosynthesis was observed to be highest after 48 hours of treatment compared to control plants.

Similarly, the ratio between Fv/Fm under CO, NO_x, CH₄ and C₃H₈ treatment did not change up to 1 hour after treatment (Fig. 3b). After 3 hours of hazardous gas treatment, the ratio of Fv/Fm decreased continuously, and the highest reduction was observed at 48 hours after treatment, at 12.5%, 62%, 50%, 38%, respectively, when compared to the control.

Activity of RuBisCO and sucrose synthase. The response of RuBisCO and sucrose synthase activity to 24 hours of CO, NO_x, CH₄ and C₃H₈ treatment was slightly reduced (Fig. 4a,b), whereas the most severe decrease in activity was found 48 hours after treatment, at 33%, 50%, 66% and 83%, respectively, compared to control plants.

Thylakoid multiprotein complex proteins (MCPs). First-dimensional electrophoresis run under native conditions on BN-PAGE was used to separate multiprotein complexes (MCPs) in intact form from thylakoids that were isolated from leaves affected by CO, NO_x, CH₄ and C₃H₈ (Fig. 5) at 24 and 48 hours after treatment (Supplementary Fig. S2).

Gel portions between 680–340 kDa contained a PSII-core dimer band (band 1) and PSII-core monomer super complex band (band 2). The intensity of these bands was reduced in CO, NO_x, CH₄ and C₃H₈ plants, whereas these bands were abundant in control plants at 48 hours after treatment, although the effect was not observed to be severe at 24 hours after treatment. Analogously strong variation was observed in gel portions between 340–290 kDa (bands 3–4), which contained PSII-monomer/ATP synthase and PSI monomer/cytb6f. These bands were severely reduced at 48 hours after treatment. A LHCII trimer was identified at 140 kDa (band 5). Variation in this band was not observed under hazardous gas treatment. LHC-II monomers identified at 67 kDa (band 6) (bands below 67 kDa) also remained unaffected and were expressed only at 24 hours after hazardous gas stress.

Relationships among descriptive parameters of net photosynthesis and relative parameters. Linear correlations among descriptive parameters of net photosynthetic activity and relative pigments were assessed using the values of plants affected by C, NO, CH₃, and C₃H₈ combined treatments along with the values of control plants (Table 1). Rubisco and total chlorophyll in leaves were closely correlated ($P < 0.001$) after 48 hours of treatment, while significant values were observed 24 hours after treatment. The correlation of carotenoid with Rubisco and total chlorophyll was significant, with $P < 0.01$. The relationships of net photosynthesis with Fv/Fm, Rubisco, total chlorophyll and carotenoid were highly significant after 48 hours of treatment.

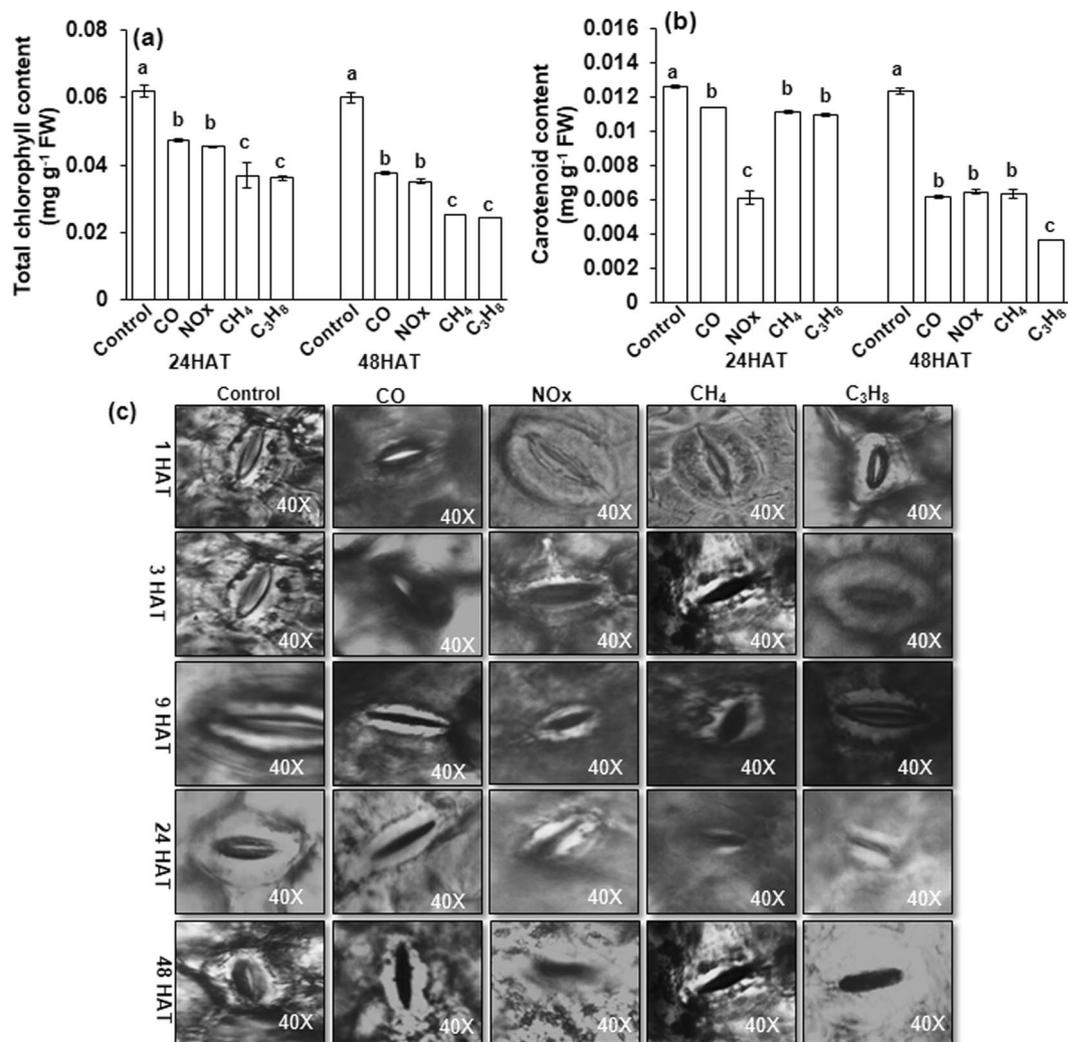


Figure 2. Changes in the content of photosynthetic pigments a total chlorophyll b carotenoid and c representative images of stomata as affected by CO, NO_x, CH₄ and C₃H₈ for 1–48 hours after treatment. Vertical bars indicate \pm SE by means with $n = 3$. Means denoted by the different letter are significantly different at $p < 0.05$ according to the Tukey's studentized range test. For stomatal observation thin layer of leaf outer covering were peeled off carefully and laid on a glass slide, covered with a cover slip and were observed under a light microscope Leica CME, Japan at 40X magnification.

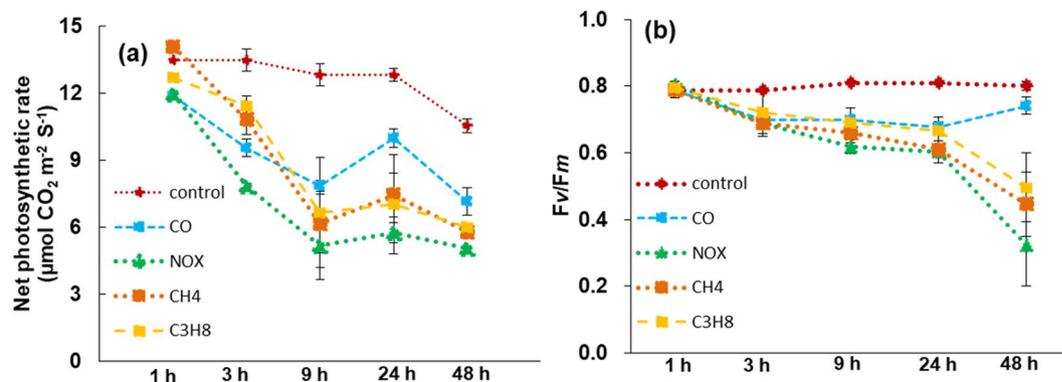


Figure 3. Changes in photosynthetic parameters a photosynthesis rate, b Fv/Fm ratio as affected by CO indicated by blue line, NO_x indicated by green line, CH₄ indicated by orange line and C₃H₈ indicated by yellow line for 1, 3, 9, 24 and 48 hours after treatment. Lines indicate \pm SE of means with $n = 3$.

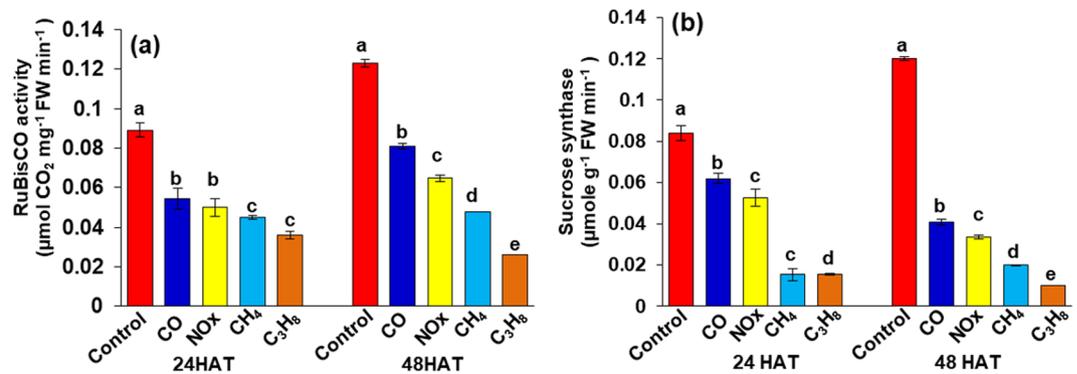


Figure 4. Changes in enzyme activities a RuBisCO activity b Sucrose synthase as affected by CO, NO_x, CH₄ and C₃H₈ for 24 and 48 hours after treatment. Vertical bars indicate \pm SE by means with $n = 3$. Means denoted by the different letter are significantly different at $p < 0.05$ according to the Tukey's studentized range test.

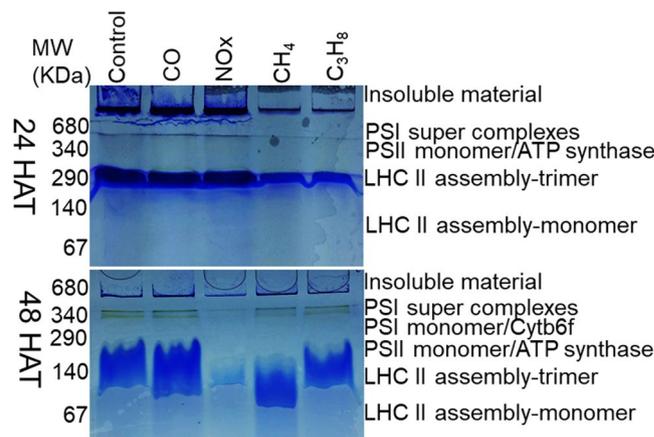


Figure 5. Analysis of thylakoid protein complex by BN-age as affected by CO, NO_x, CH₄ and C₃H₈ for 24 and 48 hours after treatment. Freshly thylakoid membranes from mature leaves were solubilized in 1% BDM at chlorophyll concentration of $1 \mu\text{g} \mu\text{l}^{-1}$, and the protein sample was separated by 7–10% gradient BN-PAGE.

| | Net photosynthesis | Fv/Fm | Rubisco | Total chlorophyll | Carotenoid |
|--------------------|--------------------|-------------------|-------------------|-------------------|-------------------|
| (24 HAT) | | | | | |
| Net photosynthesis | — | $r = 0.554^*$ | $r = 0.385^*$ | $r = 0.562^*$ | $r = 0.098$ |
| Fv/Fm | $r = 0.554^*$ | — | $r = 0.62^{**}$ | $r = 0.557^*$ | $r = 0.356$ |
| RuBisCO | $r = 0.385$ | $r = 0.62^{**}$ | — | $r = 0.842^{***}$ | $r = 0.140$ |
| Total chlorophyll | $r = 0.526^*$ | $r = 0.557^*$ | $r = 0.842^{***}$ | — | $r = 0.058$ |
| Carotenoid | $r = 0.098$ | $r = 0.399$ | $r = 0.1688$ | $r = 0.058$ | — |
| 48 HAT | | | | | |
| Net photosynthesis | — | $r = 0.962^{***}$ | $r = 0.934^{***}$ | $r = 0.953^{***}$ | $r = 0.931^{***}$ |
| Fv/Fm | $r = 0.411$ | — | $r = 0.395$ | $r = 0.387$ | $r = 0.258$ |
| RuBisCO | $r = 0.934^{***}$ | $r = 0.395$ | — | $r = 0.931^{**}$ | $r = 0.848^{**}$ |
| Total chlorophyll | $r = 0.953^{***}$ | $r = 0.387$ | $r = 0.931^{**}$ | — | $r = 0.869^{**}$ |
| Carotenoid | $r = 0.931^{**}$ | $r = 0.258$ | $r = 0.848^{**}$ | $r = 0.869^{**}$ | — |

Table 1. Linear correlations among the descriptive parameters of Net-photosynthesis, and photosynthetic parameters. The values measured at 24 and 48 HAT after CO, NO_x, CH₄ and C₃H₈ treatment were used for correlation analysis. The correlation coefficient (r) and significant differences are given; $n = 16$. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Discussion

The positive effect of CO₂ fertilization and/or a direct heating system on several horticultural crops has been reported previously^{18,19–21}. However, the emission of several hazardous gases by inefficient CO₂ fertilization/or heating systems has caused a great threat of low crop production in certain countries. In our study, we described

that these hazardous gases emitted by CO₂ supply from a direct heating system cause unavoidable by-products of aerobic metabolism, as also described in our previous studies under the influence of CO, NO_x and SO₂¹¹. Under normal growth conditions, ROS levels are modest, and cells experience only mild oxidative stress, whereas many stresses enhance ROS production^{10,22}. The results of our study clearly showed that hazardous gases in the form of CO, NO_x, CH₄ and C₃H₈ generated a rapid escalation in H₂O₂ and O₂⁻¹ content in strawberry shoots (Fig. 1). The enhanced production of ROS can pose a threat to cells, but it is also believed that ROS serve as signaling molecules to activate the stress response and defense pathways^{22,23}. Thus, the CO₂ supply from a direct heating system or combusted gas from a hot water boiler system used in greenhouses induced ROS, which can be regarded as a cellular display of stress and as ancillary messengers involved in the stress response signal transduction pathways, causing reduced crop yield. This also led to the hypothesis that hazardous gases result in proteolytic damage and, in turn, oxidative stress¹¹.

In this study, the first and foremost effect of hazardous gases (CO, NO_x, CH₄ and C₃H₈) was a reduction in photosynthetic pigment content, such as total chlorophyll and carotenoid content (Fig. 2a,b). The loss of chlorophyll and carotenoid content indicates that photodamage might have occurred in chloroplasts²⁴. The loss of chlorophyll under other abiotic stresses, such as drought, salinity, and other heavy metal toxicities, has been rigorously documented in several plant species^{25–29}. From previous studies, such a decrease in chlorophyll content may be attributed to a reduction in hazardous gases for the formation of precursor molecules, such as δ-aminolevulinic acid and protochlorophyllide^{11,30}. An additional loss of photosynthetic pigments leads to damage in stomatal guard cells (Fig. 2c) and a reduction in stomatal density. Previous studies on stomata under abiotic stresses have also indicated an effect on the opening and closing of stomata^{30–34}. In our studies, we observed that hazardous gases not only affected the opening and closing of stomata but also severely damaged guard cells/subsidiary cells. This negative effect on guard cells and density in our results indicates that leaves might have been defoliated during the growth conditions due to high temperatures under hazardous gas treatments^{35–37}. The closure of stomata due to hazardous gases might be due to the synthesis and mobilization of abscisic acid (ABA) due to key physiological actions in plants, which result in the closure of stomata^{38–40}. Based on the current knowledge of stomatal behavior, it is quite obvious that gaseous exchange was likely to be affected by the reduction in stomatal conductivity and the transpiration rate⁴¹, which leads to the reduction in net photosynthesis and the ratio of Fv/Fm (Fig. 3). The reduction in net photosynthesis and the ratio between Fv/Fm has been observed under various other abiotic stresses, such as in green algae under salinity⁴², tomato⁴³, oilseed rape under Fe deficiency⁴⁴ and strawberry under CO, NO_x and SO₂ stresses¹¹. The reduction in net photosynthesis and Fv/Fm might be due to inactivation of photosynthesis reaction centers, which obtain a preliminary quantity of light energy that cannot exploit efficiently due to the presence of oxidative stress. This ineffective energy exploitation⁴⁵ leads to an extreme increase in the dissipated energy and therefore decreases photosynthesis. The decrease in photosynthetic pigments and stomatal behavior under hazardous gases resulted in the reduction in the activity of important enzymes such as RuBisCO and sucrose synthase. The degradation of RuBisCO and sucrose may imply that other housekeeping enzymes may lead to a decline in multiprotein complexes, which are important for light and dark reactions during photosynthesis, as also shown by our results (Fig. 5).

The study of biomacromolecules (multiprotein thylakoid membrane proteins), which are essential proteins for photosystems I and II, under hazardous gases has not been conducted until now. To determine whether the multiprotein complexes of chloroplasts were affected, 1st-dimensional BN-PAGE of chloroplast/thylakoid complexes in strawberry leaves under hazardous gas treatments was performed (Fig. 5). We studied thylakoid proteins after 48 hours of hazardous gas treatment, and strong reductions in PSII-core dimer, PSII-core monomer/ATP synthase, PSI monomer/cytb6f and LHCII trimer complex were observed. The consequent high availability of hazardous gases (CO, NO_x, CH₄ and C₃H₈) may limit the activity of (4Fe-4S) and (2Fe-2S) clusters, which are associated with chloroplast membranes, especially in the leaves of strawberry, one of the highest demanding plants for fruits throughout the year. Thus, it can be concluded that chlorosis is possibly due to the proteolytic loss of photosystems, the cytb6/f complex⁴⁶ and the light-harvesting chlorophylls and carotenoids. It has been generally established that leaf chlorosis is accompanied by a reduction in the number of granal and stromal lamellae per chloroplast and is due to a lower transpiration rate and stomatal conductivity⁴⁷.

The present data clearly showed that hazardous gases crucially produce ROS and drastically limit photosynthesis by disruption of the chloroplast membrane and by the accompanied proteolytic losses of photosystems I and II in the presence of CO, NO_x, CH₄ and C₃H₈. In addition, the decrease in the activity of RuBisCO and sucrose synthase, net photosynthesis, the Fv/Fm ratio and stomatal behavior in response to hazardous gases was paralleled with the responses of thylakoid protein complexes, and the structure of the stem was observed to determine the influence of hazardous gases on cortical cells. CO₂ fertilization from a direct heating system greatly emits hazardous gases, which lead to a reduction in crop yield. From the present study, we conclude that the older CO₂ boilers/heating systems installed in greenhouses need improved efficiency, and this can be done by using sensors to analyze the emission of hazardous and hydrocarbons. Therefore, the present results can be used to prepare manuals for protecting horticulture to improve agricultural products, particularly for commercial purposes. Moreover, the results of chloroplast protein analysis revealed an initial step towards the molecular pathways of the photosynthetic mechanisms of strawberry grown inside a closed environmental system. Overall, the current results revealed that major steps must be taken to introduce resourceful CO₂ fertilization from a direct heating system to reduce the risks to crop yield globally, particularly in those countries where the risk of these hazardous gases is still causing considerable crop productivity loss. More studies on molecular mechanisms, such as chloroplast proteomics, will be useful to identify stress-responsive proteins affected by hazardous gases, and indeed, those results can also be used to identify resistant and sensitive cultivars. In addition, plant breeders can select specific cultivars for particular environmental systems.

References

- Jiang, J. Z., Huang, X. P. & Zhang, J. P. Effects of CO₂ enrichment on photosynthesis, growth and biochemical composition of seagrass *Thalassia hemprichii* Ehrenb. *Aschers. J of Int. Biol.* **5210**, 904–913 (2010).
- Guo, H., Small wood, G. J. & Gulder, O. L. The effect of reformate gas enrichment on extinction limits and NO_x formation in counterflow CH₄/air premixed flames. *Proceeding of the Comb. Inst.* **31**, 1197–1204 (2007).
- Feilberg, K. L., Sellevag, S. R., Nielsen, C. J., Griffith, D. W. T. & Johnson, M. S. CO₂ + OH!CO₂ + H: The relative reaction rate of five CO. *Phy. Chem. Chem. Phys.* **4**, 4687–4693 (2002).
- Muneer, S., Kim, E. J., Park, J. S. & Lee, J. H. Influence of green, red and blue light emitting diodes on multiprotein complex proteins and photosynthetic activity under different light intensities in Lettuce Leaves *Lactuca sativa* L. *Int. J of Mol. Sci.* **15**, 4657–4670 (2014).
- Wang, H., Lustig, W. P. & Li, J. Sensing and capture of toxic and hazardous gases and vapors by metal-organic frameworks. *Chemical Soc. review*, <https://doi.org/10.1039/C7CS00885F> (2018).
- Gladon, R. J. & Staby, G. L. Carbon monoxide formation in tomatoes. *J of American Soci. of Horticult. Sci.* **104**, 74–76 (1979).
- Xu, J., Yin, H. & Liu, X. Nitric oxide is associated with long term zinc tolerance in. *Solnum nigrum*. *Plant Physiol.* **1543**, 1319–1334 (2010).
- Bedard, K. & Krause, K. H. The NO_x family of ROS generating NADPH oxidase: physiology and pathophysiology. *Physical Rev.* **87**, 245–313 (2010).
- Mittler, R., Vanderauwera, S., Gollery, M. & Van-Breusegem, F. Reactive oxygen gene network of plants. *Trends in plant sci.* **9**, 490–498 (2004).
- Foyer, C. H. & Noctor, G. Oxidant and antioxidant signaling in plants: a reevaluation of the concept of oxidative stress in a physiological context. *Plant Cell and Environ.* **28**, 1056–1071 (2005).
- Muneer, S., Kim, T. H., Choi, B. C., Lee, B. S. & Lee, J. H. Effect of CO, NO_x and SO₂ on ROS production, photosynthesis and ascorbate–glutathione pathway to induce *Fragaria × annasaas* a hyperaccumulator. *Redox Biol.* **2**, 91–98 (2014).
- Halitschke, R. & Baldwin, I. T. Jasmonates and related compounds in plant insect interactions. *Journal of Plant Grow. Regul.* **23**, 238–245 (2004).
- Timperio, A. M., D'Amici, G. M., Barta, C., Loreto, F. & Zolla, L. Proteomics, Pigment composition, and Organization of thylakoid membranes in iron deficient Spinach leaves. *J of Exp. Bot.* **5813**, 3695–3710 (2007).
- Lin, C. C. & Kao, C. H. Abscisic acid induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings. *Plant Sci.* **160**, 323–9 (2001).
- Hiscox, J. D. & Israclstam, G. F. A method for interaction of chlorophyll from leaf tissue without maceration. *Canadian J of Bot.* **57**, 1332–1334 (1979).
- Parry, M. A. J. *et al.* Regulation of RuBisCO by inhibitors in the light. *Plant Cell and Environ.* **20**, 528–534 (1997).
- Xu, P., Sung, S. J. S., Loboda, T., Kormanik, P. P. & Black, C. C. Characterization of sucrolysis via the Uridine diphosphate and pyrophosphate dependent sucrose synthase pathway. *Plant Physiol.* **90**, 635–642 (1989).
- Cramo-Silva, A. E. *et al.* Decreased CO₂ availability and inactivation of RuBisCO limit photosynthesis in cotton plants under heat and drought stress in the field. *Environ. Exp. Bot.* **83**, 1–11 (2012).
- Pandey, R. *et al.* Elevated CO₂ improves growth and phosphorus utilization efficiency in cereal species under suboptimal phosphorus supply. *Journal of Plant Nutr.* **38**, 1196–1217 (2015a).
- Pandey, R. *et al.* Physiological and molecular alterations in plants exposed to high [CO₂] under phosphorus stress. *Biotechnology Advances* **33**, 303–316 (2015b).
- Parmesan, C. & Hanley, M. E. Plants and climate change: complexities and surprises. *Annals of Botany* **116**, 849–864 (2015).
- Mittler, R. Oxidative stress, antioxidant and stress tolerance. *Trends in plant Sci.* **7**, 153–158 (2002).
- Apel, K. & Hirt, H. Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annual Reviews of Plant Biol.* **55**, 373–399 (2004).
- Armstrong, G. A. & Hearst, J. E. Carotenoids 2: Genetics and molecular biology of carotenoid pigment biosynthesis. *The J of Federation of American Soc. for Exp. Biol.* **10**, 228–237 (1996).
- Herrera, A., Fernández, M. D. & Taisma, M. A. Effects of drought on CAM and water relations in plants of *Peperomia carnevalii*. *Annals of Bot.* **86**, 511–517 (2000).
- Higbie, S. M. *et al.* Physiological response to salt NaCl stress in selected cultivated tetraploid Cottons. *Int. J of Agro.* Article ID 643475, 12 pages (2010).
- Saleh, B. Salt stress alters physiological indicators in cotton *Gossypium hirsutum* L. *Soil Environ.* **312**, 113–118 (2012).
- Sakuraba, Y. *et al.* Delayed degradation of chlorophylls and photosynthetic proteins in *Arabidopsis* autophagy mutants during stress induced leaf yellowing. *J of Exp. Bot.* **65**(14), 3915–25 (2014).
- Muneer, S., Ahmad, J. & Qureshi, M. I. Involvement of Fe nutrition in modulating oxidative stress and the expression of stress responsive proteins in leaves of *Vigna radiata* L. *Australian J of Crop Sci.* **79**, 1333–1342 (2013).
- Marschner, H. Mineral Nutrition in Higher Plants. Academic Press, London (1986).
- Mori, I. C. *et al.* CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S⁻-type anion- and Ca²⁺-permeable channels and stomatal closure. *Plos Biol.* **4**, e327 (2006).
- Khokon, A. R. *et al.* Involvement of extracellular oxidative burst in salicylic acid–induced stomatal closure in *Arabidopsis*. *Plant Cell and Environ.* **34**, 434–443 (2011).
- Laanemets, K. *et al.* Calcium-dependent and -independent stomatal signaling network and compensatory feedback control of stomatal opening via Ca²⁺ sensitivity priming. *Plant Physiol.* **163**, 504–513 (2013).
- Li, Y., Xu, S. S., Gao, J., Pan, S. & Wang, G. X. Chlorella induces stomatal closure via NADPH oxidase dependent ROS production and its effect on instantaneous water use efficiency in *Vicia faba*. *Plos One.* **9**, e93290 (2014).
- Desikan, R. *et al.* Hydrogen peroxide is a common signal for darkness- and ABA-induced stomatal closure in *Pisum sativum*. *Funct. Plant Biol.* **31**, 913–920 (2004).
- Neill, S. *et al.* Nitric oxide, stomatal closure, and abiotic stress. *J of Exp. Bot.* **59**, 165–176 (2008).
- Fernandez, V. *et al.* Leaf structural changes associated with iron deficiency chlorosis in field-grown pear and peach: Physiological implications. *Plant and Soil* **311**, 161–172 (2008).
- Seo, M. & Koshiba, T. Complex regulation of ABA biosynthesis in plants. *Trends in Plant Sci.* **7**, 41–48 (2002).
- Christmann, A. *et al.* Integration of abscisic acid signaling in to plant responses. *Plant Biol.* **8**, 314–325 (2006).
- Pauli, M. R. & Raghavendra, A. S. Pyrabactin, an ABA agonist, induced stomatal closure and changes in signalling components of guard cells in abaxial epidermis of *Pisum sativum*. *J of Exp. Bot.* **633**, 1349–1356 (2012).
- Anderson, C. A. Development of leaf water deficits in detached green and lime–chlorotic leaves of seedlings from populations of *Eucalyptus oblique* L'Herit. *Plant and Soil* **77**, 171–181 (1984).
- Demetriou, G., Christina, N., Eleni, N. & Kiriakos, K. Salt stress impact on the molecular structure and function of the photosynthetic apparatus—The protective role of polyamines. *Bioch. et Biophys. Acta* **1767**, 272–280 (2007).
- Wargent, J. J., Pickup, D. A., Paul, N. D. & Roberts, M. R. Reduction of photosynthetic sensitivity in response to abiotic stress in tomato is mediated by a new generation plant activator. *BMC Plant Biol.* **13**, 108 (2013).
- Muneer, S. *et al.* Involvement of sulphur nutrition in modulating iron deficiency responses in photosynthetic organelles of oilseed rape *Brassica napus* L. *Photosynthesis Res.* **119**, 319–329 (2014).

45. Lu, C. & Vonshak, A. Effects of salinity stress on photosystem II function in cyanobacterial *Spirulina platensis* cells. *Physiol. Plant.* **114**, 405–413 (2002).
46. Briat, J. F., Curie, C. & Gaymard, F. Iron utilization and metabolism in plants. *Current Opin. in Plant Biol.* **10**, 276–282 (2007).
47. Spiller, S. & Terry, N. Limiting factors in photosynthesis. II. Iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. *Plant Physiol.* **65**, 121–125 (1980).

Acknowledgements

This study was supported by iPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Author Contributions

S.M. and J.H.L. designed the experiments, S.M. performed the experiments, S.M. and J.H.L. analyzed the data and wrote the manuscript.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-30838-3>.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2018