Research Paper

Effect of CO, NO\textsubscript{x} and SO\textsubscript{2} on ROS production, photosynthesis and ascorbate–glutathione pathway to induce Fragaria × annassa as a hyperaccumulator

Sowbyia Muneer\textsuperscript{a}, Tae Hwan Kim\textsuperscript{b}, Byung Chul Choi\textsuperscript{c}, Beom Seon Lee\textsuperscript{d}, Jeong Hyun Lee\textsuperscript{a,\times}

\textsuperscript{a} Department of Horticulture, College of Agricultural Life Sciences, Chonnam National University, 300 Young Bong-Dong Buk-Gu, Gwangju 500-757, Republic of Korea
\textsuperscript{b} Department of Animal Science, Institute of Agricultural Science and Technology, College of Agriculture & Life Science, Chonnam National University, 300 YoungBong-Dong Buk-Gu, Gwangju 500-757, Republic of Korea
\textsuperscript{c} School of Mechanical Systems Engineering, College of Engineering, Chonnam National, University, 300 Young Bong-Dong Buk-Gu, Gwangju 500-757, Republic of Korea
\textsuperscript{d} Dayung GS Co., Ltd., Damyang, Jeonnam 517-922, Republic of Korea

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\textbf{A B S T R A C T}

A study was conducted to determine the effect of carbon monoxide (CO), nitroxide (NO\textsubscript{x}) and sulfur dioxide (SO\textsubscript{2}) on ROS production, photosynthesis and ascorbate–glutathione pathway in strawberry plants. The results showed that both singlet oxygen (O2\textsuperscript{−1}) and hydrogen peroxide (H2O2) content increased in CO, NO\textsubscript{x}, and SO\textsubscript{2} treated strawberry leaves. A drastic reduction of primary metabolism of plants (photosynthesis), with the closure of stomata, resulted in a reduction of protein, carbohydrate and sucrose content due to production of reactive oxygen species (ROS) under prolonged exposure of gas stress. The resulting antioxidant enzymes were increased under a low dose of gas stress, whereas they were decreased due to a high dose of gas stress. Our results indicate that increased ROS may act as a signal to induce defense responses to CO, NO\textsubscript{x} and SO\textsubscript{2} gas stress. The increased level of antioxidant enzymes plays a significant role in plant protection due to which strawberry plants can be used as a hyperaccumulator to maintain environmental pollution, however, the defense capacity cannot sufficiently alleviate oxidative damage under prolonged exposure of CO, NO\textsubscript{x} and SO\textsubscript{2} stress.

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\textbf{Introduction}

Environmental pollution from industrial effluents and other organic pollutants has become a major environmental and human concern worldwide [1,2]. The major hazardous gases released from industries are abundant in number, among which carbon monoxide (CO) is a piousness gas to mammals, and has many similar qualities and effects to those of carbon dioxide (CO\textsubscript{2}), except for its flammability at high concentration [3]. As an important signaling molecule in physiological activity, CO has a similar toxic effect to that of cyanide, the inhibitory effect of a higher ratio of CO to O\textsubscript{2} (above 4:1) on cytochrome c oxidase in the mitochondrial electron transport chain at the cellular level or the effect on the detoxification enzyme P-450 in the monooxygenase system and multiprotein complex proteins in chloroplasts. CO can have important signaling roles as well as toxic effects, but this depends upon the amount produced and context, as plants also contain a variety of heme moiety-containing proteins (catalase, peroxidase, cytochromes, etc.), and produce a number of important, biliverdin-related tetrapyrrole pigments, such as phycocyanobilin [4] and phytochromes [5].

On the other hand nitrogen oxides (NO\textsubscript{x}) are produced by combustion processes and NO\textsubscript{x} is usually >90% nitric oxide (NO). With the balance being composed of nitrogen dioxide (NO\textsubscript{2}). In the atmosphere, chemical reactions convert NO to NO\textsubscript{2}, which can further react to produce O\textsubscript{3}. While NO\textsubscript{x} emission rates vary from plant to plant, largely according to the design of the plant and the characteristics of the fuel, an uncontrolled emission rate of 1.0 lb mmBtu\textsuperscript{−1} is typical for coal-fired generators. NO\textsubscript{x} is a free radical gas which transfers electrons across biological membranes [6].
In general the electron acceptor is oxygen and the product of the electron transfer reaction is superoxide. The biological function of NO₃ is therefore the generation of reactive oxygen species (ROS) [6]. The physiological generation of ROS can occur as a byproduct of other biological reactions. ROS generated as a byproduct occurs within mitochondria, peroxisosomes and other cellular elements [7].

Sulfur dioxide (SO₂) is one of the most common harmful air pollutants. Thephytotoxicity of SO₂ depends on its concentration and on the duration of exposure [1] and is influenced by sulfur status in plants [8]. The low dose of sulfur even can be useful to plants since sulfur is important for plants, and can help to alleviate other nutrient deficiencies in plants, such as that of Fe [9]. Sulfur is also a structural component of amino acids, proteins, vitamins and chlorophyll [8,10,11]. Sulfur enhances the development of nodules and nitrogen fixation, and also affects carbohydrate metabolism. However, exposure to high doses can lead to leaf chlorosis and necrosis, growth inhibition and plant death [12]. The concentration of SO₂ in the atmosphere has increased in many countries during the past few decades. It is estimated that parts of global plants exposed to SO₂ have prompted widespread attempts to evaluate defense responses.

The overall toxicity of these hazardous gases CO, NOₓ, and SO₂ is derived from toxic molecular species such as those from the combustion of CO₂ to CO, NOₓ to NO₂ as well as O₃, sulphite (SO₃²⁻) and bisulphite (HSO₃⁻). The detoxification reaction of these compounds further leads to the production of reactive oxygen species, such as hydroxyl radical (OH·) and hydrogen peroxide. Increased production of ROS can attack bio macromolecules and results in oxidative damage to nucleic acids, proteins and lipids [13,14]. However, plants can scavenge excess ROS by invoking the antioxidant defense system to avoid oxidative damage. Many studies have shown that some antioxidant enzymes are involved in plant response under various abiotic stresses. The induction of antioxidant enzymes is thought to be a protective reaction of plants against abiotic stress, but the exact defense mechanism is not clear.

Strawberry plants are the richest grown plants in greenhouses which are often fertilized by direct CO₂ enrichments fertilizers for heating. The CO₂ boilers/direct heating systems used in greenhouse however, often lead to incomplete combustion which results in formation of hazardous gases such as carbon monoxide (CO), nitrogen oxide (NOₓ) and sulfur dioxide (SO₂) and other hydrocarbons. In the present study, we selected hazardous gases to conduct an investigation of alterations of ROS production, photosynthetic measurements and the ascorbate-gluathione pathway in strawberry plants exposed to different concentrations of CO, NOₓ and SO₂, assessing strawberry plants as a hyper-accumulator of these gasses.

Methods

Plant material and treatments

Young strawberry (Fragaria × anansa) plants were propagated on rock–wool cubes (10 × 10 × 6.5 cm) after germination at 25°C under fluorescent light (300 μmol m⁻² s⁻¹ of photosynthetic photon flux density) for three weeks. The rooted strawberry plants were grown on the cubes with a hydroponic nutrient solution containing (m mole per litter for the macro elements): N 9.5, P 1.5, K 5.5, Ca 5.0, S 1.5, P 1.5 Mg 1.5 and (μmole per litter for the micro elements): Fe 33, Mn 22, B 10, Zn 5, Cu 1.0, Mo 0.5. The nutrient solution supplied was regularly determined for its electrical conductivity (EC) (1.0 ds m⁻¹) and pH was maintained at 6.5–7.0. Fluorescent light was supplied at 150 μmol m⁻² s⁻¹ at the canopy height for 16 h day⁻¹. Six-week-old plants were transferred to air tight glass bottles (3 l) and were supplied with 133 (low dose), 267 (medium dose) and 533 (high dose) ppm of carbon monoxide (CO), 25 (low dose), 50 (medium dose) and 199 (high dose) ppm of nitrooxide (NOₓ), and sulfur dioxide (SO₂). Upon 24 h of treatment, the leaves were separated from the order of antigenic appearance and young leaves were used for the study. Plant samples were immediately frozen in liquid N₂ and were stored in a deep–freezer (−80°C) prior to further analysis.

Determination of H₂O₂ and O₂⁻⁻⁻⁻¹

H₂O₂ concentration was measured calorimetrically as described by Lin and Kao [15] using titanium sulfate. H₂O₂ concentration was calculated using the extinction coefficient 0.28 mM⁻¹ cm⁻¹ and was expressed as nmol g⁻¹ tissue fresh weight. For O₂⁻⁻⁻⁻¹ determination fresh tissue was extracted in phosphate buffer (pH 7.5) and centrifuged at 10,000g for 5 min. The resultant extract was added to 1 ml of 10 mM NH₄OH–HCl and phosphate buffer (pH 7.8) and incubated at 25°C for 1 h. After incubation the mixture was added with 0.5 ml of 17 mM alpha aminobenzene sulphonic acid and 0.5 ml of 7 mM alpha naphthylamine solution and was incubated again for 20 min at 25°C and absorbance was recorded at 530 nm.

Measurement of net photosynthetic activity and Fₖ/Fₘ ratio

Photosynthetic rate was measured using a portable photosynthesis measurement system (LI-6400XT, LI-COR, Inc., Lincoln, NE). Chlorophyll fluorescence (Fₖ/Fₘ) was measured by using a chlorophyll fluorescence meter (PAM 2000; Heinz Walz GmbH, Effeltrich, Germany). The leaves were adapted to dark conditions for 30 min before the measurement. The maximum fluorescence (Fₘ) and minimum fluorescence (F₀) were determined by applying a saturating light pulse (20 kHz) of 1100 μmol m⁻² s⁻¹ PPFD for 3 μs. The maximum PS II quantum yield (Fₖ/Fₘ) was calculated as Fₖ/Fₘ=(Fₖ−F₀)/Fₘ for 24 h at different concentrations of CO, NOₓ and SO₂.

Photosynthetic pigments and stomatal observation

The content of chlorophyll and carotenoid was estimated by the method of Hiscox and Israclstam [16]. The fresh leaves were collected in glass vials, to which 10 ml dimethyl sulfoxide (DMSO) was added, after which samples were kept in an oven at 65°C for complete leeching of pigments for 1 h. Optical density was recorded at 480, 645, 520 and 663 nm. The content of total chlorophyll and carotenoid was calculated using the formulae given by Arnon [17].

For stomatal observation, thin layers of leaf tissues were carefully cut and were laid on glass slides, covered with cover slips by adding few drops of water, and were observed under a light microscope (Leica DM4000 M) at 40 × magnification.

Rubisco determination by SDS-PAGE

Leaf tissues were homogenized at 4°C in 100 mM Tris buffer (pH 7.5) containing 5 mM of DTT, 2 mM iodoacetate and 5% (v/v) glycerol at a leaf; buffer ratio of 1:5–10 (g:ml). For this extraction, a buffer without sodium or potassium ion was recommended for SDS–PAGE analysis because those cations reduce the solubility of DS (dodecyl sulfate). Before centrifugation, a TritonX100 (25%, v/v) was added to a portion of leaf homogenate to make a final concentration of 0.1% (v/v). An addition of TritonX100 was effective for the extraction of Rubisco bound to the membrane fraction [18]. The homogenates were centrifuged at 5000g for 3 min at 4°C. A lithium DS solution (25% w/v) and 2-mercaptoethanol were added to the supernatant fluid to a final concentration of 1.0%
(w/v) and 1% (v/v), respectively. This preparation was immediately treated at 100 °C for 1 min, and was then stored at −30 °C until the analysis of SDS-PAGE. The samples containing 2–10 μg RubisCO were loaded onto 12% polyacrylamide gel. After electrophoresis, the gels were stained in 0.25% (w/v) CBB-R. The stained bands corresponding to larger and smaller subunits of RubisCO were cut out of the gels with a razor blade and were eluted in 1–2.5 ml of formamide in a stoppered amber test tube at 50 °C for 5 h with shaking. The absorbance of the resultant solution was read at 595 nm with a spectrophotometer. RuBisCO content was determined by using the standard curve calculated from the absorbance of a known amount of purified RuBisCO.

Total carbohydrate and sucrose content

The carbohydrate content was determined by anthrone method [19]. One hundred milligrams of dried leaf tissue were added to a boiling tube. The tissues were hydrolyzed in a boiling tube by keeping them in a water bath for 3 h with 5 ml of 2.5 N-HCl, and were then cooled at room temperature. The solution was then neutralized with solid sodium carbonate until effervescence ceased, and samples were cooled at room temperature. The solution was then heated for 8 min in a boiling water bath and absorbance at 630 nm were recorded after cooling down at room temperature. The corresponding concentration of carbohydrate was determined against a standard curve prepared by using glucose solution.

For sucrose content 0.1 g of dried leaf was taken in 10 ml of ethanol (90%). The vials were incubated at 60 °C for 1 h and volume was made up to 25 ml by adding 15 ml of 95% ethanol. A 1 ml aliquot had added to it 1 ml of 5% phenol, and the solution was thoroughly mixed. Five ml of concentrated H2SO4 was added and the solution was allowed to cool at room temperature, after which the absorbance was recorded at 485 nm using a Beckman DU 640 spectrophotometer. The corresponding concentration of sucrose was determined against a standard curve which was prepared using glucose solution.

Antioxidant enzyme assays

Superoxide dismutase (SOD) activity was determined by the method of Dhindsa et al. [20] with minor modifications. Fresh tissues (200 mg) were extracted in phosphate buffer (pH 7.3) and were then centrifuged at 15,000 g. SOD activity in the supernatant was assayed for its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) by reading the absorbance at 560 nm. One unit of enzyme activity was defined as the amount of enzyme required to inhibit 50% of the NBT photo reduction in comparison with tubes lacking the plant extract.

Catalase (CAT) activity was determined by the method of Aebi [21]. About 500 mg of grounded tissues was extracted in phosphate buffer (pH 7.3), and were then centrifuged at 15,000g for 20 min. The decrease in absorbance at 240 nm was recorded as a result of H2O2 degradation (extinction coefficient of 36 mM$^{-1}$ cm$^{-1}$). One unit of enzyme is defined as the amount necessary to decompose 1 μmol of H2O2 per min.

Glutathione reductase (GR) activity was determined by the method of Rao [22]. About 500 mg of fresh tissues was extracted in phosphate buffer (pH 7.0), and were centrifuged at 15,000g for 10 min. The supernatant was immediately assayed for GR activity through the glutathione-dependent oxidation of NADPH at 340 nm. One unit of enzyme is defined as the amount necessary to decompose 1 μmol of NADPH per min.

Ascorbate peroxidise (APX) activity was estimated by measuring the decrease in absorbance at 290 nm (extinction coefficient of absorbance 2.8 mM$^{-1}$ cm$^{-1}$ for ascorbate) according to Nokano et al. [23]. One hundred milligrams of fresh tissue were extracted in 100 mM K-phosphate (pH 7.0), and were then centrifuged at 15,000g for 10 min. One unit of enzyme was expressed as the amount necessary to decompose 1 μmol of ascorbate in 1 min.

Statistical analysis

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

Results and discussion

CO, NOx and SO2 induced ROS generation

Reactive oxygen species generation was observed by determination of hydrogen peroxide (H2O2) and singlet oxygen (O2$^{-1}$). Strawberry leaves which were exposed to CO, NOx, and SO2 gases exhibited high concentration of H2O2 and O2$^{-1}$ (Fig. 1A and B). The concentration of H2O2 and O2$^{-1}$ was observed highest by more than 90 and 80% respectively under high dose of CO, NOx, and SO2 compared to control.

In plant cells, ROS are unavoidable by-products of aerobic metabolism. Under normal growth conditions, the amount of ROS is modest, and cells experience only mild oxidative stress, whereas, many stresses enhance ROS production [13,24,25]. The results of our study clearly show that CO, NOx, and SO2 triggered the rapid induction of O2$^{-1}$ and H2O2 generation in strawberry leaves. This enhanced production of ROS under stress can pose a threat to cells and can also act as a signal to activate stress response pathways such as the ascorbate–glutathione cycle [13,26].

Photosynthetic parameters

The net photosynthetic rate in plants exposed under low doses of hazardous gases (CO, NOx, and SO2) showed a slight reduction of net photosynthesis, however, at the medium dose of gas stress the net photosynthetic rate decreased (Fig. 2A) and the reduction of photosynthesis was observed to be highest in plants under high doses of CO, NOx, and SO2.

Similarly, the ratio between Fv/Fm under CO, NOx and SO2 treatment was not different between control plants, and those exposed to a low dose of gas stress (Fig. 2B). After a medium dose of gas stress, the ratio between Fv/Fm decreased, and the lowest ratio was observed under a high dose of gas stress by 13%, 24% and 11%, respectively, when compared to the control.

We also observed that photosynthetic pigments (total chlorophyll and carotenoid) under CO, NOx, and SO2 were reduced slightly under a low dose of gas stress, whereas under a medium dose of hazardous gas stress the photosynthetic pigments reduced significantly and the highest reduction was observed in plants under a high dose of gas stress, reducing total chlorophyll by 54%, 60% and 59% (Fig. 3A) and carotenoid content by 66%, 80% and 70% (Fig. 3B) compared to control.

The present results show that photosynthetic parameters such as net-photosynthesis, Fv/Fm, ratio and photosynthetic pigments (chlorophyll and carotenoid) were reduced under CO, NOx and SO2, which represents that there might be a transfer of lesser electrons and lesser energy investment in the biochemical machinery of strawberry. The reduction of net photosynthesis and ratio between Fv/Fm has been also observed under salt stress in green algae [27] and in Vigna radiata under mineral deficiency [24,25]. The decline in net photosynthesis and Fv/Fm under hazardous gas stress might be due to the inactivation of reaction centers of
photosystems which receive an initial amount of light energy and cannot be exploited efficiently due to the presence of oxidative stress. The ineffective energy exploitation [28,29] leads to an extreme increase of dissipated energy, and therefore, decreases photosynthesis. In addition to this, the loss of carotenoids might be due to the loss of chlorophyll, because carotenoids absorb light energy for use in photosynthesis, and they protect chlorophyll from photo-damage. Such a decrease in chlorophyll content may also be attributed to a reduction of hazardous gases for the formation of precursor molecules, δ-aminolevulinic acid and protochlorophyllide [30].

Observation of stomata under microscope

Microscopic observations (40 × ) have shown that stomata did not show any change in behavior in plants under low dose of CO, NOx, or SO2 treatment (Figs. 4 and 5). With increased doses of gas exposure the stomata gradually closed and guard/subsidiary cells were damaged compared to those of the control, which was found to be exaggerated at high dose of gas exposed after 24 h.

As we know, stomata play an important regulatory role in leaf physiological processes, as the primary pathway for the exchange of gases between internal leaf surfaces and the atmosphere [31]. The closing of stomata as a result of exposure under hazardous gas has been reported in several species such as Vicia faba [32] and Pisum sativum [31] under SO2 stress. The closure of stomata under high concentrations of CO, NOx, and SO2 demonstrated that certain key enzymes involved in stomatal metabolism might have been affected, which are also involved in several other metabolic activities in plants such as malate formation and metabolism in epidermis, and turgor pressure of guard cells. The destructive damage of guard cells and density might also occur due to defoliation of leaves during growth conditions of strawberry, and indeed, due to high temperatures under hazardous gas conditions [33], which might have resulted in the induction of palisade and sponge parenchyma cell length and thickness.
RuBisCO contents

Fig. 4 shows that the RuBisCO content determined by SDS-PAGE, the RuBisCO content in plants under low dose CO, NOx or SO2 showed great change in expression, and statistically significant results, as generated by a spectrophotometer, have shown a huge change even early stages of hazardous gas treatment. With increasing hazardous gas doses the reduction of RuBisCO content was observed to be highest in plants exposed to low doses of gas stress when compared to that of the control.

The changes in RuBisCO content in response to various abiotic stresses has been reported in many species, such as in *Trifolium alexandrinum* [34] under salt stress conditions. The maintenance of a higher RuBisCO content in plants has been correlated with a higher rate of photosynthesis in certain genotypes, and a lower content of RuBisCO is correlated with lower rates of photosynthesis under abiotic stresses [35]. In the present study, the reduction of RuBisCO content under hazardous gas stress (CO, NOx and SO2) coincides with a reduction of photosynthesis (Fig. 2). In recent studies we observed the proteomic data of *Brassica napus* under Fe-deficiency to reduce most proteins classified as photosynthetic precursors, including RuBisCO content [10]. The loss of RuBisCO protein might be due to the progressive depletion of biochemical pathways associated with signal transduction and gene regulation, and in particular, might be involved in protein synthesis [36,37], and also might be associated with an excessive production of ROS which leads to incorrect folding or assembly of proteins, and consequent protein degradation [38].

![Fig. 4. Representative images of stomata, as affected by low, medium and high doses of CO, NOx, and SO2. 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) at 40 x magnification.](image-url)
Carbohydrate and sugar content

The total carbohydrate in plants under low dose of CO, NOx or SO2 was reduced compared to control (Fig. 6A) and more reduction was observed in plants under medium dose of hazardous gas stress by 54%, 81% and 72% respectively. The reduction was observed to be highest under high dose of CO, NOx and SO2, by 65%, 87% and 70% respectively.

Sucrose content in plants under low dose of CO, NOx and SO2 showed a slight reduction, which was greater (and significant) in medium dose plants (Fig. 6B), however, a high dose of CO, NOx and SO2 reduced the sucrose content by 23%, 48% and 65%, respectively, when compared to control.

The decrease in total carbohydrates and sucrose content of damaged leaves probably corresponded with photosynthetic inhibition, or with the stimulation of a lower respiratory rate. Higher starch accumulation in damaged leaves may have resulted both in the higher resistance of their photosynthetic apparatus [39] and low starch export from the mesophyll. The negative effect of hazardous gases on carbon metabolism is a result of their possible inhibitory stress by 54%, 81% and 72% respectively. The reduction was observed in plants under medium dose of hazardous gas stress, (B) quantification of the large subunit protein band. Vertical bars indicate significantly different at \( p < 0.05 \) according to Tukey’s studentized range test.

Fig. 5. Regulation of RuBisCO larger and smaller subunits to low, medium and high doses of CO, NOx and SO2 (A) SDS-PAGE of leaf proteins from control and gas stressed plants. (B) quantification of the large subunit protein band. Vertical bars indicate ± SE by means with \( n = 3 \). Means denoted by different letters are significantly different at \( p < 0.05 \) according to Tukey’s studentized range test.

\[ \text{RuBisCO content} \]

| Cont | CO | NOx | SO2 |
|------|----|-----|-----|
| LS   |    |     |     |
| SS   |    |     |     |

\[ \text{RuBisCO content (mg g}^{-1} \text{FW)} \]

\[ \text{Low dose Medium dose High dose} \]

- Low dose: Control (Cont), CO, NOx, SO2
- Medium dose: LS
- High dose: SS

\[ \text{CO, NOx and SO2-induced antioxidative defense response} \]

We analyze the evolution of stress responses in strawberry plants exposed to low, medium and high doses of CO, NOx or SO2 for 24 h. The treatment evokes physiological changes in the form of ascorbate glutathione cycle. There was a stark contrast between the responses of all antioxidative enzymes examined in this study. CO, NOx, and SO2 increased SOD, CAT, GR and APX, activity at low and medium doses of gas stress conditions (Fig. 7A–D), while at higher doses, SOD activity was decreased by 55%, 55.1% and 78%, catalase activity was decreased by 81%, 89% and 86%, GR activity was decreased by 69%, 30% and 47% and APX activity was decreased by 32%, 62% and 25% compared with control.

In this study, the activities of antioxidative enzymes (APX, SOD, CAT and GR) were found to be increased at low and medium doses of CO, NOx and SO2 stress, whereas they were significantly decreased at high doses of gas stress. Several reviews have been published on different aspects of ascorbate and glutathione, ranging from their biosynthesis [41,42], to their roles in the transport system [43] and stress defense [44] in plants. It has been generally established that glutathione and ascorbate play a prominent role in non-enzymatic mechanisms to prevent the oxidation of cellular compounds [44], and are involved in the induction of enzymes and gene expression. SOD catalyzes the dismutation reaction of \( O_2^{-1} \) and \( H_2O_2 \) for the initial period of stress conditions, as induction of these enzymes was observed in our study, whereas, after prolonged exposure the SOD could not dismutate these ROS into \( H_2O_2 \) and \( H_2O \), which resulted in the reduction of enzyme activities of ascorbate glutathione cycle. Glutathione is involved as a substrate for glutathione peroxidase and is therefore necessary for the removal of lipid peroxidases, because GR reduces the oxidized glutathione, which is generated by glutathione peroxidase [42,45]. The observed increase in GR at initial period of stress resulted in the detoxification of oxidative damage, while a high dose of CO, NOx and SO2 resulted in a disturbance in ascorbate glutathione pathways. CAT and APX are the primary \( H_2O_2 \) scavenging enzymes in plants. In this study, CO, NOx and SO2 pronouncedly increased APX activity and CAT activity in the initial period of stress, which indicates that APX and CAT are efficient enzymes for detoxifying ROS arising from hazardous gas treatments. Moreover the increased activity of these enzymes can contribute to resistance and enhancement since they participate in many other physiological processes in plant defense reactions.

Conclusions

In conclusion, this is a systematic study which provided a specific insight into the changes in ROS production, photosynthesis
physiology and antioxidant status in strawberry plants under CO, NO, and SO₂ stresses, which contribute the mechanism of plant adaptation and promote that strawberry plant, can act as hyper-accumulator to mitigate oxidative damage. Our results suggest that CO, NO, and SO₂ caused a high level of ROS production in the form of O₂⁻⁻ and H₂O₂, as well as a reduction of photosynthetic physiology. Conversely, antioxidant enzymes of ascorbate glutathione pathways were induced to scavenge the negative effects of gas stresses. However, increased levels of lipid peroxidation products in strawberry plants exposed to CO, NO, and SO₂ for high dose demonstrated that the antioxidant defense system was no longer used to mitigate free radicals. Ascorbate glutathione cycle status may play a critical role in defense against CO, NO, and SO₂, as well as the maintenance of photosynthesis metabolism in plants, and will help to mitigate oxidative damage under abiotic stress. These results suggest that strawberry plants can act as a hyper-accumulator for detoxifying these gases.

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References

[1] Long Li., Huilan Yi., Effect of sulfur dioxide on ROS production, gene expression and antioxidant enzyme activity in Arabidopsis plants, J. Plant Physiol 58 (2012) 46–53.
[2] Z.Y. Dong, W.H. Huang, D.F. Xing, H.F. Zhang, Remediation of soil co-contaminated with petroleum and heavy metals by the integration of electrokinetics and biostimulation, J. Hazard. Mater. 260 (2013) 399–408.
[3] J.Z. Wang, L.J. Cui, Y. Wang, J.L. Li, Growth, lipid peroxidation and photosynthesis in two tall fescue cultivars differing in heat tolerance, Biol. Plant 53 (2009) 237–242.
[4] R.F. Troxler, Synthesis of bile pigments in plants. Formation of carbon monoxide and phycoerythrobilin in wild-type and mutant strains of the alga, Cyanidium caldarium, Biochemistry 11 (1972) 4235–4242.
[5] T.D. Elitch, A.F. McDonagh, L.A. Palma, I.C. Lagarias, Phytochrome chlorophyll biosynthesis. treatment of tetrapyrrole-deficient Avena explants with natural and non-natural bilarienes leads to formation of spectrally active holo-proteins, J. Biol. Chem 264 (1989) 183–189.
[6] J. Xu, H. Yin, X. Liu, Nitric oxide is associated with long term zinc tolerance in Solanum nigrum, Plant Physiol 154 (3) (2010) 1319–1334.
[7] K. Bedard, K.H. Krause, The NO family of ROS generating NADPH oxidase: physiology and pathophysiology, Phys. Rev. 87 (2010) 245–313.
[8] L.J. De-Kok, Sulfor metabolism in plants exposed to atmospheric sulfur, in: M. Rennenberg, C. Brunold, L.J. De-Kok, I. Stulen (Eds.), Sulfur Nutrition and Sulfor Assimilation in Higher Plants, SPB Academic Publishing, The Hague, 1990, p. 111e130. (1990).
[9] S. Munee, B.R. Lee, D.W. Won, T.H. Kim, Changes in expression of proteins involved in alleviation of Fe deficiency by sulfur nutrition in Brassica napus L. Acta Physiol. Plant (2013), http://dx.doi.org/10.1007/s11738-013-1136-8.
[10] T.H. Maugh II, SO₂ pollution may be good for plants, Science 205 (1979) 383.
[11] L.P. Yang, I. Stulen, L.J. De-Kok, Sulfur dioxide: relevance of toxic and nutritional effects for Chinese cabbage, Environ. Exp. Bot 57 (2006) 236–245.
[12] M. Agrawal, B. Singh, M. Rajpota, F. Marshall, J.N.B. Bellis, Effect of air pollution on peri-urban agriculture: a case study, Environ. Pollut. 126 (2003) 323–329.
[13] R. Mittler, S. Vanderauwera, M. Gollery, F. Van-DeBurreme, Reactive oxygen gene network of plants, Trends Plant Sci. 9 (2004) 490–498.
[14] C.H. Foyer, G. Noctor, Oxidant and antioxidant signaling in plants: a reevaluation of the concept of oxidative stress in a physiological context, Plant Cell Environ 28 (2005) 1056–1071.
[15] C.C. Lin, C.H. Kao, Abspic acid induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings, Plant Sci 160 (2001) 323–329.
[16] J.D. Hiscox, G.F. Israelstam, A method for interaction of chlorophyll from leaf tissue without maceration, Can. J. Bot 57 (1979) 1332–1334.
[17] J.D. Arnon, G.F. Israelstam, Copper enzymes in isolated chloroplast oxidase in Beta vulgaris, Plant Physiol 42 (1979) 287–292.
[18] A. Makino, B. Osmond, Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat, Plant Physiol 96 (1991) 355–362.
[19] J.E. Hedge, B.T. Hofreiter, Carbohydrate Chemistry, in: R.L. Whistler, J.N. Be Miller (Eds.), Academic Press, New York, 1962.
[20] R.H. Dhindsa, P. Plum-Bhindsa, T.A. Thorpe, Leaf senescence correlated with increased level of membrane permeability, lipid peroxidation and decreased level of SOD and CAT, J. Exp. Bot 32 (1981) 93–101.
[21] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[22] M.V. Rao, Cellular detoxification mechanisms to determine age dependent injury in tropical plant exposed to SO₂, J. Plant Physiol 140 (1992) 733–740.
[23] M. Nakano, K. Nohota, K. Vemaraju, S.S. Tej, W.J. Skogen, B.C. Meyers, Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA, Nucleic Acids Res. 34 (2006) 731–735.
S. Muneer, T.H. Kim, M.I. Qureshi, Fe modulates Cd-induced oxidative stress and the expression of stress responsive proteins in the nodules of Vigna radiata, Plant Growth Regul. 68 (2012) 421–443.

S. Muneer, J. Ahmad, M.I. Qureshi, Involvement of Fe nutrition in modulating oxidative stress and the expression of stress responsive proteins in leaves of Vigna radiata L, Aust. J. Crop Sci 7 (9) (2013) 1333–1342.

K. Apel, H. Hirt, Reactive oxygen species: metabolism, oxidative stress, and signal transduction, Ann. Rev. Plant Biol 55 (2004) 373–399.

G. Demetriou, N. Christina, N. Eleni, K. Kiriakos, Salt stress impact on the molecular structure and function of the photosynthetic apparatus—the protective role of polyamines, Biochim. Biophys. Acta 1767 (2007) 272–280.

K. Satoh, C.M. Smith, D.C. Fork, Effects of salinity on primary processes of photosynthesis in the red alga (Porphyra perforata), Plant Physiol. 73 (1983) 643–647.

C. Lu, A. Vonshak, Effects of salinity stress on photosystem II function in cyanobacterial Spirulina platensis cells, Physiol. Plant. 114 (2002) 405–413.

H. Marschner, Mineral Nutrition in Higher Plants, Academic Press, London, 1986.

M.I. Rao, R.B. Amundson, R. Herman-Alscher, L.E. Anderson, Effect of SO2 on stomatal metabolism in Pisum sativum L, Plant Physiol. 72 (1983) 573–577.

V.J. Black, M.H. Unsworth, Effects of low concentration of sulfur dioxide on net photosynthesis and dark respiration of Vicia faba, J. Exp. Biol 30 (1979) 473–483.

V. Fernandez, T. Eichert, V. Del Rio, G. Lopez-Casado, J.A. Heredia-Guerrero, A. Anunciacion, A. Heredia, A. Abadia, Leaf structural changes associated with iron deficiency chlorosis in field-grown pear and peach: physiological implications, Plant Soil 311 (2008) 161–172.

G.M. Abogadallah, Sensitivity of Trifolium alexandrinum L to salt stress is related to the lack of long-term stress-induced gene expression, Plant Sci 178 (2010) 491–500.

C. Lu, A. Vonshak, Effects of salinity stress on photosystem II function in cyanobacterial Spirulina platensis cells, Physiol. Plant. 114 (2002) 405–413.

A. Pandey, M.K. Choudhary, D. Bhushan, A. Chattopadhyay, S. Chakraborty, A. Datta, N. Chakroborty, The nuclear proteome of chick pea (Cicer arietinum L) reveals predicted and un-expected proteins, J. Proteome Res 5 (12) (2006) 3301–3311.

M.K. Choudhary, D. Basu, A. Datta, N. Chakroborty, S. Chakroborty, Dehydration-responsive nuclear proteome of rice (Oryza sativa L) illustrates protein network, novel regulators of cellular adaptation, and evolutionary, Mol. Cell. Proteomics 8 (7) (2009) 1579–1598.

S. Luo, H. Ishida, A. Makino, T. Ma, Fe²⁺-catalyzed site specific cleavage of the large subunit of ribulose 1,5-bisphosphatecarboxylase close to the active site, J. Biol. Chem 277 (2002) 12382–12387.

E., Prokopiev Afforestation of Industrial Areas. Sofia, Zemizdat, 208 p. (In Bulg.). 1978.

M. Stiborova, M. Ditrichova, A. Brezinova, Effect of heavy metal ions on growth and biochemical characteristics of photosynthesis of barley and maize seedlings, Biol. Plant 29 (1987) 453–467.

N. Smirnoff, P.L. Conklin, F.A. Loewus, Biosynthesis of ascorbic acid in plants: a renaissance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 437.

G. Potters, L. De Cara, H. Assad, N. Horemans, Ascorbate and glutathione: guardians of the cell cycle, partners in crime? Plant Physiol. Biochem 40 (2002) 537–548.

N. Horemans, C.H. Foyer, G. Potters, H. Asard, Ascorbate functions and associated transport systems in plants, Plant Physiol. Biochem 38 (2000) 531–540.

G. Noctor, C.H. Foyer, Ascorbate and glutathione: keeping active oxygen under control, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 240–279.

M.B. Ali, E.J. Hahn, K.Y. Paek, Effects of temperature on oxidative stress defense systems, lipid peroxidation and lipoxygenase activity in Phalaenopsis, Plant Physiol. Biochem 43 (2005) 213–223.