

**Impact of reactive oxygen species (ROS) and reactive
nitrogen species (RNS) on photorespiratory enzyme
components in leaves of *Pisum sativum* and
*Arabidopsis thaliana***

*A thesis submitted to the University of Hyderabad
for the award of Ph. D. degree in
Plant Sciences*

By

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December 2022



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DECLARATION

I, Deepak Saini, hereby declare that this thesis entitled “**Impact of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on photorespiratory enzyme components in leaves of *Pisum sativum* and *Arabidopsis thaliana***” submitted by me under the supervision of Professor A. S. Raghavendra in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled " **Impact of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on photorespiratory enzyme components in leaves of *Pisum sativum* and *Arabidopsis thaliana*** " Submitted by **Deepak Saini** bearing registration number **16LPPH02** in partial fulfilment of the requirements for award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by his under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or full to this or any other University or Institution for award of any degree or diploma.

Parts of this thesis have been:

A. Published in the following publications:

1. **Saini, D.**, Bapatla, R. B., Pandey, J., Aswani, V., Sunil, B., Gahir, S., Bharath, P., Subramanyam, R., Raghavendra, A. S. (2022). Modulation by S-nitrosoglutathione, a natural nitric oxide donor, of photochemical components of pea (*Pisum sativum*) leaves, as revealed by chlorophyll fluorescence patterns: Aggravation of nitric oxide-effects by light. *Plant Science Today* (Accepted)
2. Bapatla, R. B.,[†] **Saini, D.**,[†] Aswani, V., Rajsheel, P., Sunil, B., Timm, S., Raghavendra, A. S. (2021). Modulation of photorespiratory enzymes by oxidative and photo-oxidative stress induced by menadione in leaves of pea (*Pisum sativum*). *Plants*, 10, 97 († = Equal contribution).

B. Presented in the following conferences:

1. **Physiological and Molecular Markers for Abiotic Stress Tolerance in Plants**, University of Calicut, Kerala. Oct 26-28th, 2022 (Category: International).
2. **8th International conference on Photosynthesis and Hydrogen Energy Research for Sustainability**, University of Hyderabad, Hyderabad. 30th Oct – 4th Nov, 2017 (Category: International).

Further, the student has passed the following courses towards fulfillment of coursework requirement for Ph.D.

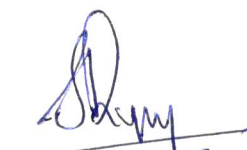
Course	Code	Name	Credits	Pass/Fail
1.	PL801	Analytical Techniques	4	Pass
2.	PL802	Research ethics, Data analysis and Biostatistics	3	Pass
3.	PL803	Lab work and Seminar	5	Pass


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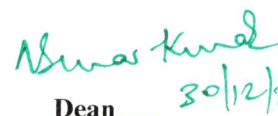

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Acknowledgements

I express my gratitude to my supervisor, **Prof. A.S. Raghavendra**, for his suggestions, encouragement, guidance and support. I am deeply indebted to his continuous support in every scientific aspect like writing, communication, and presentation skills, which enabled me to complete this Ph.D. program.

I would like to thank **Prof. S. Rajagopal**, Head, Department of Plant Sciences and **Prof. N. Siva kumar**, Dean, School of Life Sciences, for providing necessary facilities for my research. I extend my thanks to former Heads of Department **Prof. G. Padmaja**, **Prof. Ch Venkata Ramana** and the former Deans, **Prof. S. Dayananda**, **Prof. KVA Ramaiah** and **Prof P. Reddanna**. I am also thankful to all the faculty members of **Dept. of Plant Sciences/ School of Life Sciences**.

I am highly thankful to my doctoral committee members **Prof. S Rajagopal** and **Prof. Saradadevi Tetali** for their helpful suggestions.

I thank to **Dr. Stefan Timm**, Plant Physiology Department, University of Rostock, Germany for metabolite sample analysis. I also thank **Prof. S. Rajagopal** for allowing me to use his lab facilities like nitrogen gas and Dual PAM-100 for measuring photosynthetic parameters and **Jayendra Pandey** for helping in analysis.

I sincerely thank **Prof. Yoshiyuki Murata** and Japan-Asian Youth Exchange Program in Science (SAKURA Exchange Program) for my visit to Okayama University, Japan conducted by JSTA in 2017.

I am incredibly grateful to my teachers, who taught me just not science but also ethics and morals in life.

I would like to thank **Prof. Robert Fluhr** and **Prof. Veronica G Maurino** for helpful discussions during their visit to our laboratory. I thank **Dr. Jagadish Gupta Kapuganti**, National Institute of Plant Genome Research, New Delhi for tyrosine nitration experiment and his student **Dr. Chandra** for helping in analysis.

I am thankful to my my former labmates, **Dr. Sunil**, **Dr. Rajsheel**, **Dr. Bindu**, **Dr. Gayatri**, **Dr. Srinivas**, **Dr. Aswani** and **Ramesh** and present lab mates, **Dr. Easwar**, **Shashibhushan** and **Bharath**; Project students, **Priyanka**, **Hitesh**, **Kamal**, **Sabir**, **Princy**, **Ranveer** and **Abhilasha** for the

help and enjoyable company; **Venu, Balakrishna, Joshua and Sandhya** for help in lab.

I am also thankful to my friends my friends **Ranay, Munendra, Rohit, Qadir, Saurabh, Jayendra, Arpita, Anusha, Divya, Prodosh, Neera, Mahati and Santosh** for unconditional support and love they provided me all these years.

I am thankful to all my loving friends and colleagues in **School of Life Sciences** for their affection and moral support which will remain fresh forever in my memory. I am also thankful to all the non-teaching staff members of school of life sciences.

I gratefully acknowledge the financial assistance for me from the **CSIR**. I also acknowledge funding from **UGC, CSIR, DBT, ICAR-NAIP, DST-DERB, DST-DFG, DST-JC Bose** projects to the laboratory of **Prof. A. S. Raghavendra** as well as **DST-FIST, DBT-CREBB and UGC SAP-CAS** (for funding to Department and School). I am thankful to the **Department of Plant Sciences, University of Hyderabad** for providing me an opportunity to pursue my Ph.D.

Words fail to express my heartfelt gratitude to my parents and all my family members including my brothers, sisters, brothers-in-law and all my niece and nephews to whom I'm forever indebted for their love, support and endless patience. I respect my **Parents** for their constant affection and encouragement. I finally thank **Almighty GOD** for looking after me, through good and trying times.

Deepak Saini

Abbreviations

AOA	: Aminooxyacetic acid
<i>aox1a</i>	: Alternative oxidase 1a lacking mutant of Arabidopsis
APX	: Ascorbate peroxidase
ASA	: Ascorbic acid
CAT	: Catalase
cPTIO	: 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt
DAB	: 3, 3'-Diaminobenzidine
F1,6BP	: Fructose 1,6-bisphosphatase
GK	: Glycerate kinase
GO	: Glycolate oxidase
GR	: Glutathione reductase
GSH	: Glutathione reduced
GSNO	: S-nitrosoglutathione
H ₂ O ₂	: Hydrogen peroxide
HL	: High light
HPR	: Hydroxypyruvate reductase
MD	: Menadione
ML	: Moderate light
<i>nadp-mdh</i>	: NADP-MDH lacking mutant of Arabidopsis
NBT	: Nitroblue tetrazolium chloride
O ₂ ⁻	: Superoxide
PGLP	: Phosphoglycolate phosphatase
PSI	: Photosystem I
PSII	: Photosystem II
RNS	: Reactive nitrogen species
ROS	: Reactive oxygen species
SNP	: Sodium nitroprusside
SOD	: Superoxide dismutase
<i>vtc</i>	: Ascorbate deficient mutant of Arabidopsis

All the remaining abbreviations are standard ones and as indicated by the journal *Plant Physiology* on their website: <http://www.aspb.org>. under 'Instructions For Authors'.

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Chapter 1

Introduction and Review of Literature

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

Photorespiration

Photorespiration was discovered in the 1960s (Ogren, 1984). Photorespiration was earlier considered a wasteful and inefficient process. But, currently photorespiration is recognized as an essential component of carbon metabolism and provide tolerance towards abiotic stress in plant cells (Eisenhut et al., 2019; Shi and Bloom, 2021). The core photorespiratory pathways in plant cells involve enzymatic reactions that convert 2-phosphoglycolate to 3-phosphoglycerate (3-PGA) and located over different compartments: chloroplast, peroxisome, mitochondria and cytoplasm (Timm and Hagemann, 2020; Shi and Bloom, 2021; Fu and Walker, 2022).

When RuBP fixes CO₂, it produces 2 molecules of 3-PGA, while photorespiration starts when RuBP fixes O₂ inspite of CO₂, produce one 3PGA and one 2PG molecule. 2PG inhibits photosynthetic carbon fixation and should be rapidly degraded through photorespiratory metabolism. In chloroplast, 2PG is generated by RuBP. Phosphoglycolate phosphatase (PGLP) convert 2PG into glycolate. Glycolate enters the peroxisome from the chloroplast. Glycolate oxidase (GO) uses O₂ and converts glycolate into glyoxylate, that gives rise to H₂O₂, which is scavenged by catalase, liberating H₂O and O₂. In the peroxisome, glycolate is converted to glycine using glutamate: glyoxylate aminotransferase (GGAT). Glycine entered mitochondria, and got converted in to serine, ammonia, and CO₂ by glycine decarboxylase. In the peroxisome, serine: glyoxylate aminotransferase

(SGAT) converts the serine, which is generated in the mitochondria, into hydroxypyruvate. Hydroxypyruvate reductase (HPR), reduces hydroxypyruvate to glycerate. Glycerate was phosphorylated by glycerate-3-kinase (GK) to generate 3PGA, that regenerated ribulose 1,5-bisphosphatase (Hagemann and Bauwe, 2016; Timm and Hagemann, 2020; Eisenhut et al., 2019).

Significance of photorespiration under stress condition

Photorespiration plays a central role in supporting photosynthetic CO₂ fixation and proposed to play a critical part against various stress situations as drought, high light or supraoptimal temperature (Wingler et al., 2000; Voss et al., 2013; Saji et al., 2017). Photorespiration acts as an efficient energy sink by regenerating ADP, NADP and other reducing equivalents (Voss et al., 2013; Sunil et al., 2019). Therefore, strong photorespiratory flux could aid in preventing chloroplastidal electron transport chain from over-reduction and finally photoinhibition of the (Kozaki and Takeba, 1996; Huang et al., 2019).

Photorespiration acts as an excellent sink for the consumption of ATP/NADPH. As a result, photorespiration aided in reducing the levels of ROS and offers defence against oxidative damage brought on by various forms of abiotic stress (Sunil et al., 2013). Photorespiratory processes can directly dissipate reducing equivalents and energy, or they can do so indirectly by using an alternate oxidase (Voss et al., 2013). Active photorespiration helped to sustain photosynthesis and plant growth and also increase tolerance to abiotic conditions like drought or high light (Cui et al., 2016; Timm et al., 2019). Expression of photorespiratory genes was altered by prolonged stress. It is therefore not

surprising that photorespiratory mutants were more vulnerable to stressful situations (Hodges et al., 2016).

Under high light, photorespiration was enhanced in *Chlorella sorokiniana* and in *Nicotiana tobaccum* (Xie et al., 2016; Huang et al., 2019). In *Pisum sativum*, HL elevated photorespiratory enzyme activity (Bapatla et al., 2021). Under fluctuating light in tomato, HL-plants showed higher photorespiration which enhanced the PSI electron sink downstream (Shi et al., 2022). Enhanced CO₂ decreases the photorespiration in *Coffea arabica* (Marcal et al., 2021) while In *Arabidopsis thaliana*, low CO₂ boosted photorespiration (Eisenhut et al., 2017). High PGLP and GOX activities greatly increased *Arabidopsis* and rice's ability to withstand abiotic challenges (Cui et al., 2016; Timm et al., 2019).

Reactive Oxygen Species (ROS)

The levels of not only reactive oxygen species (ROS) but also reactive nitrogen species (RNS) increases whenever plants are exposed to biotic and abiotic stresses. These two substances, ROS and RNS, have an impact on fundamental metabolic processes like photosynthesis, respiration, and even resulted in cell death (Mittler, 2017; Gong et al., 2020; Kaya et al., 2020; Sychta et al., 2021).

ROS regarded as harmful byproducts of aerobic metabolism that cannot be avoided and are involved in plant cell signalling (Apel and Hirt, 2004). The levels of ROS need to be at optimal levels. Antioxidants and antioxidant enzymes (APX, CAT and SOD) were considered as ROS scavengers (Lee et al., 2007). The antioxidant compounds include ascorbate (ASC), glutathione (GSH), and

tocopherols (Szarka et al., 2012).

Bioenergetic electron transport system in mitochondria and chloroplasts generate ROS. Peroxisomes also form another major site besides the plasma membrane. ROS include form such as singlet oxygen ($^1\text{O}_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\text{OH}^\cdot$) (Mansoor et al., 2022).

When chlorophyll is excited by light to reach triplet state to O_2 in the antenna system, singlet oxygen is generated. Singlet oxygen can damage PSI and PSII, proteins, pigments, nucleic acids, etc. The half-life of singlet oxygen is about 3 μs .

Generation of H_2O_2 in normal metabolism includes photorespiration in peroxisomes, mitochondrial electron transport, and meyer reactions in chloroplasts. Abiotic and biotic stress increase the H_2O_2 generation via these routes or through plasma membrane-localized NADPH oxidase and peroxisomal glycolate oxidase. Lower concentration of H_2O_2 acts as a signal for photosynthesis and photorespiration. H_2O_2 can traverse membranes and, as a result, have oxidative effects far from the site of its formation since it has a longer half-life than superoxide (Neill et al., 2002; Noctor et al., 2002; Sharma et al., 2012; Sehar et al., 2021; Anjum et al., 2022).

Superoxide radical production also occurs during the electron transport chain in thylakoids as well as mitochondria. It is typically converted into other ROS types like singlet oxygen, H_2O_2 , superoxide, etc. Hydroxyl radical is another of the ROS family members that are extremely reactive and harmful. It is produced as a result of an H_2O_2 and O_2 reaction that iron (Fe^{2+} , Fe^{3+}) catalyses at neutral pH.

It can damage protein and also lead to the destruction of the membrane. This radical cannot be salvaged by an enzymatic system. The overproduction of hydroxyl radicals results in cellular death (Pinto et al., 2003).

Antioxidant defense machinery

Antioxidant defence machinery, which aids in reducing stress-related damage, was a ROS defence component. Enzymatic and non-enzymatic parts make up the antioxidant machinery.

Non-enzymatic components

Non-enzymatic components included ascorbate, glutathione, proline, tocopherol, carotenoids, and flavonoids. These antioxidants are generally enhanced under diverse abiotic stresses. One of the necessary substrates to scavenge H_2O_2 is ascorbate. Ascorbate acts as a cofactor and is responsible for the formation of tocopherol. Ascorbate helps plants cope better with abiotic challenges by improving their water status, nutritional status, antioxidant capacity, and overall photosynthetic efficiency (Hasanuzzaman et al., 2019).

Glutathione was considered a strong non-enzymatic antioxidant. It was found in vacuoles, mitochondria, ER, and nuclei. It acts as a raw material for various peroxidases and also plays a crucial part in ROS scavenging. Under various abiotic stress conditions, it enhanced the tolerance to plants. Glutathione peroxidase or glutathione S-transferase uses it as a substrate for the reaction. In addition to that, under stressful conditions, it also reduced proteins oxidative denaturation by defending their thiol groups (Cheng et al., 2015; Hasanuzzaman et al., 2017).

Proline is recognised as one of the most powerful osmoprotectants and signaling molecules. Additionally, it makes a considerable contribution to primary metabolism as a component of proteins and free amino acids (Singh et al., 2015). Proline is an essential solute of the physiological reactions of plant species to various stresses and gets accumulated without damaging cellular structures. Proline is also being investigated for its abilities as a powerful antioxidant defence molecule, protein stabiliser, metal chelator, ROS scavenger etc (Dar et al., 2016; Adejumo et al., 2021).

The scavenging of ROS and lipid radicals is carried out by the lipophilic antioxidants known as tocopherols. Photosynthetic organisms only produce tocopherols found in green tissues of plants. Lipids and other chloroplast membrane structures, such as the PSII, can be protected by tocopherols (Kamal-Eldin, 1996; Hollander-Czytko et al., 2005; Das and Roychoudhury, 2014). Carotenoids are mostly found in photosynthetic as well as non-photosynthetic tissues and are lipid soluble. An increased concentration of this aids in developing resistance to various abiotic stressors. Carotenoids in chloroplasts function mainly as photosynthetic pigments and photoprotectors (Nisar et al., 2015; Sun et al., 2018).

Flavonoids provide blue, red, and purple pigmentation in seeds, flowers, leaves, and fruits. Additionally, flavonoids are essential for neutralising ROS and defending plants from diverse abiotic stresses (Winkel-Shirley, 2002; Fini et al., 2011; Petrucci et al., 2013)

Antioxidant enzyme components

A member of the metalloenzymes family found in all aerobic organisms, superoxide dismutase (SOD) is a powerful antioxidant. SOD dismutates O_2^- into H_2O_2 and O_2 . Three SOD isoforms are known: Mn-SOD, Cu/Zn-SOD, and Fe-SOD, localized in different compartments of the plant cell (Mittler, 2002; Pilon et al., 2011).

Ascorbate peroxidase (APX) scavenges H_2O_2 to H_2O and DHA via ascorbate-glutathione (ASC-GSH). The five isoforms of APX are located in different organelles, i.e., mitochondrial, peroxisomal, cytosolic, and chloroplastid (stromal and thylakoidal). APX was more effective at scavenging H_2O_2 in stressful situations than CAT because it has a stronger affinity for H_2O_2 (Sharma and Dubey, 2004)

Glutathione reductase, an oxidoreductase flavoprotein, convert GSSG to GSH. To regenerate ascorbic acid (AsA) from MDHA and DHA, reduced glutathione (GSH) is depleted and is subsequently changed into its oxidised form (GSSG). ASC-GSH cycle enzyme GR catalyses the production of a disulfide bond, which keeps the cellular ratio of GSH/GSSG high. Smaller amounts are also found in the cytosol and mitochondria, although the majority of it is present in chloroplasts (Szarka et al., 2012).

Guaiacol peroxidase (GPX) eliminated extra H_2O_2 both under stress and during regular metabolism. As electron donors, GPX favoured aromatic substances like pyrogallol and guaiacol. GPX was regarded as a crucial enzyme for the elimination of H_2O_2 due to its presence in both intracellularly (cytosol and

vacuole) and extracellularly (in the cell wall) (Asada, 1999).

Dehydroascorbate reductase uses reduced glutathione to convert dehydroascorbate (DHA) into ascorbic acid (AsA). DHAR is widely distributed in the roots, seeds, green and etiolated shoots of plants (Chen and Gallie, 2006; Eltayeb et al., 2007).

Monodehydroascorbate reductase regenerate ascorbic acid (AsA) from the reduction of monodehydroascorbate (MDHA), using NADPH as a reductant. MDHAR is crucial for a plant's ability to respond to oxidative stress due to its ability to keep the intracellular ascorbate redox state primarily in the reduced state (Mittler, 2002; Yeh et al., 2019).

Reactive nitrogen species: Focus on nitric oxide (NO)

In plants, nitric oxide (NO) is a typical signaling molecule. The protonation of nitrogen-containing compounds, which results in the creation of reactive chemical species or free radicals, occurs in the reactive species, or RNS. S-nitrosothiols (SNOs), S-nitroglutathione (GSNO), and peroxynitrite (ONOO) are additional radicals (Del Rio et al., 2006; Zhou et al., 2022). NO is a multifaceted free radical found in plants that can modulate various physiological processes (Verma et al., 2020). There are contrasting claims that NO can be harmful or beneficial for plant cells against stress (Arasimowicz and Floryszak-Wieczorek, 2007; Sami et al., 2018; Bhardwaj et al., 2021; Wani et al., 2021).

In biological tissues, NO has a half-life of less than 6 seconds. Due to its short half-life, NO displays its extremely reactive character, reacting both directly and indirectly with DNA, lipids, and proteins as reactive nitrogen species and

other radicals (Wink and Mitchell, 1998; Thomas et al., 2001). The enzymes found in many subcellular compartments can undergo post-translational modifications (PTMs) through the use of NO and associated compounds like peroxynitrite and GSNO.

Crosstalk between NO and ROS

Under abiotic or biotic stress conditions, ROS and NO interaction decides a cell's fate (Rodriguez-Serrano et al., 2009). When NO reacts with superoxide, a powerful and harmful oxidant, peroxynitrite, is formed (Verma et al., 2020). ROS and NO are considered essential components for PCD in plants (Janicka et al., 2022). Previous reports showed that in maize, H_2O_2 was implicated in NO-mediated PCD (Kong et al., 2013). Another study found that in soybean cells, cell death was caused by an imbalance between ROS (H_2O_2 and superoxide) and NO (Delldonne et al., 2001).

Post-translational modifications: Another possibility of meeting the challenges of stress

Besides the help from antioxidants and antioxidant enzymes, plant cells use PTM's of important enzymes as another approach. These modifications such as S-nitrosation, tyrosine nitration, phosphorylation, methylation, and glycosylation (Dai Vu et al., 2018; Arefian et al., 2021; Wang et al., 2021; Gough and Sadanandom, 2021). Peroxynitrite, GSNO, and other similar compounds that are members of the RNS family can mediate post-translational changes (PTMs). S-nitrosation and tyrosine nitration was the most frequent PTMs that can modify the functionality of target proteins (Gupta et al., 2020; Corpas et al., 2021). Some

proteins in plants undergo S-nitrosation or tyrosine nitration, such as catalase, glycolate oxidase, hydroxypyruvate reductase, malate dehydrogenase etc (Ortega-Galisteo et al., 2012; Corpas et al., 2019).

S-nitrosation and tyrosine nitration

S-nitrosation, or S-nitrosylation, is a covalent process in which thiol groups are oxidised by one electron. S-nitrosation involves binding the NO group to cysteine residues (Ortega-Galisteo et al., 2012; Begara-Morales et al., 2018). When *Arabidopsis* cells were exposed to GSNO, S-nitrosation of 63 and 46 candidates were found (Lindermayr et al., 2005; Fares et al., 2011).

A NO-based post-translational modification is tyrosine nitration (PTM), which can be considered as a marker of nitrosative stress (Corpas et al., 2007; Bartesaghi and Radhi, 2018; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2019). There are two mechanisms involved in tyrosine nitration: first, peroxynitrite is formed when superoxide and NO react ($O_2^{\cdot-}$). The second method makes use of $\bullet NO_2$, which is produced when hemoperoxidase and hydrogen peroxide (H_2O_2) react with NO_2 (Alvarez and Radi, 2003; Ferrer-Sueta et al., 2018).

Key enzyme components of photorespiratory metabolism

Photorespiratory metabolism enzymes are found in different subcellular organelles, such as phosphoglycolate phosphatase (PGLP) and glycerate kinase, located in chloroplast, while glycolate oxidase (GO), catalase (CAT), and hydroxypyruvate reductase (HPR) located in peroxisomes were studied. Here, we have discussed these enzymes in brief.

Phosphoglycolate phosphatase (PGLP) was detected and purified from higher plants (Baldy et al., 1989; Richardson and Tolbert, 1961; Belanger and Ogren, 1987). PGLP converts 2-PG to form glycolate, which produces the final product of 3-PGA via the photorespiratory pathway and reenters the Calvin cycle (Liu et al., 2022)

Glycolate oxidase (GOX) was detected to be localized in leaf peroxisomes (Tolbert et al., 1968). Glycolate is converted to glyoxylate and H_2O_2 in peroxisomes by GOX, which uses oxygen as a substrate to complete the reaction. GOX1 and GOX2 isoforms of glycolate oxidase are mostly involved in photorespiratory metabolism, while GOX3 performs L-Lactate metabolism in roots (Esser et al., 2014; Dellerio et al., 2016; Engqvist et al., 2015).

Catalase (CAT) converted H_2O_2 into H_2O and O_2 (Mhamdi et al., 2012; Halliwell and Gutteridge, 2015; Palma et al., 2020). Different isoforms of catalase include CAT1, CAT2, and CAT3. Among these isoforms, leaves contain CAT2, and CAT3 was in vascular tissues but not found in leaves (Frugoli et al., 1996; Mhamdi et al., 2010).

The existence of hydroxypyruvate reductase (HPR) was initially reported by Stafford et al. (1954), and the location in the peroxisome was identified by Tolbert et al. (1970). The conversion of hydroxypyruvate to glycerate was catalyzed by Hydroxypyruvate reductase. There are other isoforms of HPR located in other organelles, like HPR2 in the cytoplasm and HPR3, whose location was unknown (Timm et al., 2008, 2011; Wang et al., 2022).

Glycerate kinase (GLYK) was first done from the leaves of spinach (Kleczkowski et al., 1985; Chaguturu, 1985) and later from the pea as well as rye (Schmitt and Edwards, 1983). GLYK is an essential enzyme for photosynthetic carbon assimilation in plants. GLYK is classified into three phylogenetically separate groups. Class II GKs was believed to generate glycerate 2-phosphate, but class I GKs and class III GK (GLYK) could make glycerate 3-phosphate (Bartsch et al., 2008).

Role of primary metabolites during oxidative stress

Plants accumulate ROS when they are subjected to numerous abiotic stressors, which affects their metabolism because they are sessile. ROS are essential signalling molecules that plants need to function and develop at the cellular level. Excessive ROS production causes oxidative stress, which harms cells and triggers programmed metabolic processes that cause cell death (Mittler et al., 2017; Waszczak et al., 2018). Plants detoxify ROS by highly developed metabolic systems to deal with oxidative stress. Plants contain scavenging systems that prevent ROS accumulation and synthesize necessary metabolites in the presence of excessive ROS production. Compatible solutes and antioxidants are known as anti-stress compounds. Some metabolic responses are common to stresses, while certain responses could be specific to a few stress (Apel and Hirt, 2004; Lehmann et al., 2009; Noctor et al., 2015).

Sugars and its Derivatives

Sugars like glucose, sucrose, fructose, and raffinose generally increase on exposure to stress. Raffinose protects plant cells from oxidative damage and acts

as an osmoprotectant (Nishizawa et al., 2008). Sucrose is a main transported sugar in plants. Exposure to H₂O₂, there was a rapid buildup of sucrose and fructose and a subsequent rise in glucose levels in *Arabidopsis* (Morelli et al., 2003; Van den Ende et al., 2009; Chen and Hoehenwarter, 2015).

TCA cycle metabolites

As substrates for numerous biosynthetic pathways, tricarboxylic acid cycle intermediates (TCA cycle) exhibit a highly dynamic nature in their levels, which reflects the processes of metabolite production and consumption. Although it has been demonstrated that the levels of TCA cycle metabolites are increased when respiration is stimulated and that plant cells under oxidative stress experience the typical drop in the TCA cycle metabolites pool (Dastogeer et al., 2017; Obata et al., 2011; Dumont and Rivoal, 2019; Ishikawa et al., 2010). Modulation of TCA cycle enzyme activity played a substantial role in the metabolic profile modification under these circumstances. Among the TCA cycle enzymes, aconitase, 2-oxoglutarate-dehydrogenase, and pyruvate-dehydrogenase was only a few inhibited by oxidative stress.

Table 1. The table represents the enzymes that are involved in dark mitochondrial respiration, Calvin-Benson cycle or photorespiration, which are located in the different subcellular compartment, their principles, and standard references used for estimation.

Compartment	Enzyme	Principle	Reference
Chloroplast	Phosphoglycolate phosphatase	Release of inorganic phosphate via formation of phosphomolybdenum blue	Somerville and Ogren, 1979
	Glycerate Kinase	Formation of 3-PGA and ADP by coupling via pyruvate kinase and lactate dehydrogenase	Kleczkowski and Randall, 1985
	Fructose 1,6-bisphosphatase	Fructose 1,6-bisphosphate is hydrolyzed and dephosphorylated to produce fructose-6-phosphate and inorganic phosphate.	Lee and Hahn, 2003
Peroxisome	Glycolate oxidase	Formation of glyoxylate from glycolate	Yamaguchi and Nishimura, 2000
	Catalase	Decomposition of H_2O_2 to H_2O and O_2	Patterson et al., 1984
	Hydroxypyruvate reductase	NADH utilization to convert hydroxypyruvate to glycerate	Timm et al., 2008
Mitochondria	Aconitase	Isomerization of citrate to isocitrate via cis-aconitase	Lehmann et al., 2009

Amino acids

Secondary metabolites synthesis uses amino acids served as substrates. It was revealed that oxidative stress significantly affected the amino acid profile in plants (Chen and Hoehenwarter, 2015; Lehmann et al., 2009; Lehmann et al., 2012). Wide range of amino acids, including glutamine, proline, gamma-aminobutyric acid (GABA), etc. produced due to stress and can be used as appropriate osmolytes or as precursors for secondary metabolites. In contrast to that, low-abundance amino acids are not produced, but instead can build up as a result of accelerated protein breakdown. (Ishikawa et al., 2010; Lehmann et al., 2009; Hildebrandt, 2018). A reduction in glycine and a rise in PGA and HPR after MD treatment suggest that the photorespiratory pathway has been activated (Baxter et al., 2007).

Approaches to induce oxidative and nitrosative stress

ROS and NO are generally elevated under stress conditions. At low quantities, ROS and NO served as helpful signaling molecules, but at higher concentrations, they harmed cellular components or even killed cells (Mandal et al., 2022; Zhou et al., 2022). Menadione (MD), a strong oxidant, and two NO donors, S-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) were used in the current investigation to mimic the effects of stress. MD generates ROS by competing with ubiquinone and generating superoxide at cytochrome c, while the exact location of NO generation is not clear.

MD is used as an oxidant to generate ROS, particularly in mitochondria. To examine oxidative stress in plants, researchers used MD, a redox-active

vitamin K3 molecule (Sun et al., 1999). They may act as electrophiles or oxidants that draw in electrons, and they may also act as nucleophiles or reducers in this scenario by donating electrons. The concentration and circumstances of chemical and cellular exposure had a significant impact on the extent to which these features contribute to total toxicity (Castro et al., 2007). In addition, MD acts as a strong priming agent to increase resistance to salt and freezing stress (Borges et al., 2014). Before soaking, menadione was administered to Arabidopsis seeds to minimise salt stress deleterious effects (Jiménez-Arias et al., 2015).

The most popular NO donor was shown to be sodium nitroprusside (SNP) due to its comparatively inexpensive cost, well-documented use, and continuous, prolonged NO generation when compared to other NO donors (Ederli et al., 2009; Zandonadi et al., 2010). SNP emitted both NO and CN^- ion, especially when exposed to light. The amount of CN^- released from SNP is fortunately far less than the amount of NO released into the medium (Arnold et al., 1984; Leavesly et al., 2008).

Due to its reputation as a more physiological NO donor, GSNO may be used (Corpas et al., 2013b). GSNO serves as a NO storage and vehicle of NO throughout the cell. NO is produced by the interaction of nitrous acid with GSH (Broniowska et al., 2013; Corpas et al., 2013b). Earlier it was shown in pea leaves that the subcellular localization of GSNO by immunogold particles and electron microscopy immuno- cytochemistry in mitochondria, chloroplasts, and peroxisomes (Barroso et al., 2013).

Approaches to restrict photorespiration

We have used aminooxyacetate and low oxygen to restrict photorespiration. Aminooxyacetate is known to block the glycine degradation to serine or ammonia and inhibit the photorespiratory pathway, decreasing photosynthetic efficiency (Kleczowski et al., 1987; Gonzalez-Moro et al., 1993; Han et al., 2018). Nitrogen gas was used to purge the incubation medium, lowering the O₂ concentration (Riazunnisa et al., 2006). The oxygen concentration in the incubation media was around 230 nmol ml⁻¹ (21%), but after purging with N₂, it was reduced to 75 nmol ml⁻¹ (7%).

Use of *Arabidopsis* mutants with the disrupted redox state

A little flowering plant called *Arabidopsis thaliana* is related to numerous agricultural plants, including broccoli, cauliflower, and cabbage. The genome of *Arabidopsis* is small and well-characterized. *Arabidopsis* was chosen due to its short life cycle, simplicity of genetic modification, and the decoding of its full genome (Meinke et al., 1998; Garcia-Hernandez et al., 2002). Numerous mutants, transgenics, and established methods are readily available, providing enormous potential for physiological and biochemical research (Hayashi and Nishimura, 2006).

We used mutants that lack redox-balancing enzymes in our research. Mutants such as *nadp-mdh*, *aox1a*, and *vtc* lack the necessary redox-balancing enzymes. *Arabidopsis* Biological Resource Center (ABRC) was used to obtain the mutants. The *nadp-mdh* mutants are deficient in NADP-dependent malate dehydrogenase (Hebbelmann et al., 2012). Chloroplastic NADP-MDH, an enzyme

with redox regulation, is active in light and inactive in darkness. Oxaloacetate is converted to malate by NADP-MDH (Scheibe, 2004; Beeler et al., 2014). Mutant *aox1a* lacks mitochondrial alternate oxidase and directly connects the ubiquinol oxidation with the reduction of O₂ to H₂O. (Strodtkotter et al., 2009; Vanlerberghe, 2013). A GDP-mannose pyrophosphorylase (GMPase) gene mutation causes *vtc* to lack ascorbate. The ascorbate biosynthetic route includes the enzyme GMPase. The growth of the *vtc* mutant is slow and has a stunted appearance. The sensitivity of *vtc* mutant was more in HL or in salinity due to only 30 % of ascorbate of the WT (Conklin et al., 1999; Pastori et al., 2003; Gao and Zhang, 2008).

Doubts or gaps in our knowledge

Despite the considerable progress in our knowledge of photorespiration, several aspects need further attention:

1. It is well established that the modulation of metabolic pathways under stress conditions is due to the dynamic changes in the levels of ROS and NO. It is not clear if ROS or NO has an equal role or if one of them dominates the other.
2. Changes in ROS or NO can occur in different compartments of the plant cell. Since both are mobile, ROS can be expected to travel across the cell and regulate the events in compartments other than the site of production. However, there is no evidence so far for such effect.
3. The specific effects of oxidative or nitrosative stress on the components of the photorespiratory enzymes are less well understood.
4. Once the photorespiratory flux has been better characterized, it will be

easier to ascertain how altered photorespiration impacts secondary plant-functional pathways and other portions of the central carbon metabolism. Metabolite analysis is one of the approaches. It is necessary to examine the metabolite patterns when the photorespiratory pathway is modulated, particularly under biotic or abiotic stress responses.

5. It is possible to restrict photorespiration by elevating CO₂ or decreasing O₂ or using suitable inhibitors. It is unclear how the overall photorespiratory enzyme components are modulated under restricted photorespiration. It would be interesting to know the consequences on ROS levels too, under restricted photorespiratory conditions.
6. Despite the fact that several research teams employ mutants deficient in redox balancing elements, the majority of them observed the response to high light or drought stress. In this context, it would be quite interesting to study mutants that are compromised in redox-balancing components.
7. PTMs mediated by ROS and RNS affect the proteins, resulting in inhibition or activation or no change of proteins, which shows the ambiguous nature at varying stress conditions. Studying the comparative effects of PTMs triggered by ROS or RNS on the photorespiratory and nitrogen metabolism-related proteins would be interesting.
8. Recent initiatives to improve photorespiration or utilize photorespiratory shunts yielded encouraging outcomes. Eventually, these ideas will have to be used to apply to target crops from model organisms and tested in a range of applicable agricultural scenarios, along with potentially superior

alternatives that may be made possible by synthetic biology.

Some of the above points mentioned above were chosen for further studies, as indicated in the next chapter (Objectives and Approach).

Chapter 2

Objectives and Approach

Chapter 2

OBJECTIVES AND APPROACH

Whenever the plants are subjected to abiotic stress like salinity, drought, or high light, the levels of ROS and RNS (mainly NO) increase, affecting the physiological processes. One of the consequences of oxidative stress is the upregulation of photorespiration as an attempt to minimize ROS levels. The driving signal for modulating photorespiration under abiotic stress can be ROS or NO, or both. The impact of elevated ROS on photorespiratory metabolism is well known, but the studies of elevated NO on photorespiratory metabolism are quite limited.

Existing gaps/unanswered questions

Despite the considerable progress in our knowledge of photorespiration, several aspects need further attention:

Several stresses frequently cause a rise in ROS and RNS (particularly, NO). The effects of photo-oxidative stress on the photorespiratory enzyme components, when a particular compartment (mitochondria) is selectively targeted to raise the levels of ROS, have not been adequately investigated. Similarly, the impact of NO on the photorespiratory enzyme components is not well studied. So, it would be interesting to check the effects of different NO donors (SNP and GSNO) on the photorespiratory enzyme components, photosynthetic performance, or PTMs, particularly under HL.

Many reports show the modulation of photorespiration under salinity, drought, etc., but modulation under restricted photorespiration would be interesting to know the consequences on ROS levels and photorespiratory

enzyme components. Though different research groups used mutants majorly looked at overall response to high light/drought stress. Many research groups well study the effect of oxidant/drought on the metabolites. But it would be interesting to study the impact of photo-oxidative stress on the metabolites, which is not well studied.

PTMs mediated by ROS and RNS affect the proteins, resulting in inhibition or activation or no change of proteins, which shows the ambiguous nature at varying stress conditions. Studying the comparative effects of PTMs triggered by ROS and RNS on the photorespiratory and nitrogen metabolism-related enzymes or proteins would also be interesting. Studies on the response of photorespiratory metabolite flux to changing environmental conditions and the possible role of metabolites would help us to understand compartmentation transporters.

Overall Objectives:

1. Examine the changes in photorespiratory metabolism in pea leaf discs under oxidative and photo-oxidative stress induced by menadione and/or high light, an oxidant targeting mitochondria.
2. Study the changes caused by NO donors on photorespiratory metabolism and photosynthetic characteristics in leaf discs of a pea.
3. Examine the changes in the photorespiratory metabolism in *Arabidopsis thaliana* after the suppression of photorespiration by either low O₂ (~7 %) or aminooxyacetic acid (AOA).
4. Investigate the status of photorespiratory metabolism in *Arabidopsis* mutants lacking genes involved in antioxidant defense systems.

5. Determine the levels of primary metabolites in *Arabidopsis thaliana* after exposure to menadione-induced oxidative stress.

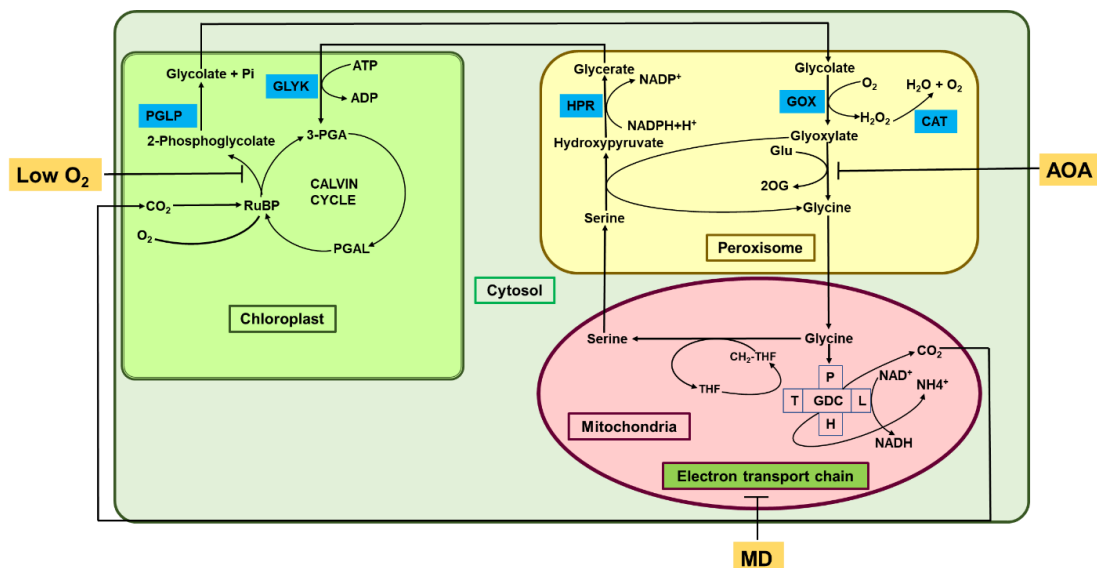


Figure 2.1. A simplified photorespiratory scheme highlighting the enzymes studied and modulators attempted in this work. Some of the cofactors of enzyme reactions are not shown for the sake of simplicity (Modified from Mishra, 2014).

We have used two plants, i.e., *Pisum sativum* (pea) and *Arabidopsis thaliana*, for our studies. We have checked the effect of oxidant (MD) on the photorespiratory enzyme components in pea. After that, we shifted our experiments to *Arabidopsis* wild type and their mutants (*nadp-mdh*, *aox1a*, and *vtc*) lacking key redox components to confirm the effect of MD on photorespiratory enzyme components that were already done in pea. *Arabidopsis* was used as a model plant because of the availability of a large number of mutants. The specific experimental approaches are as follows:

Plant material

Pea (*Pisum sativum*) and *Arabidopsis thaliana*. Both have their own advantages and limitations.

Pea (Pisum sativum)

Pea is a C3 winter-season crop belonging to the Fabaceae family. It is easy to grow pea plants and has a short life cycle. Due to the thin and delicate leaves, it will be simple to treat with chemicals. The plants were grown in a greenhouse at a natural 12 h light/dark and temperature of 23±2°C. We will be used leaf discs in most of our experiments, but leaves will be used in a few studies.

Arabidopsis thaliana

We will use *Arabidopsis thaliana*, a model C3 plant that belongs to the Brassicaceae family. *Arabidopsis* leaves are also thin and tender and can be easily treated with test compounds. The plants will be raised in controlled growth rooms, with 8/16 h light/dark and an average temperature of 21°C. *Arabidopsis* is an excellent source of mutants with desired deficiencies. We will be chosen following mutants for our studies: (i) *aox1a*, lacking mitochondrial AOX, (ii) *nadp-mdh*, without chloroplastic NADP-MDH, (ii) *vtc*, reduced ascorbate content.

Treatments, oxidants, NO donors, and photorespiratory inhibitors

Leaf discs from pea leaves are chosen to resemble *in vivo* conditions. Leaf discs will be prepared using a paper punch by submerging the leaf in water to minimize mechanical stress. For pea, leaf discs will be used, and leaves for *Arabidopsis*. The discs/leaves will be exposed to test compounds

(oxidants, NO donors, and photorespiratory inhibitors). In the case of pea, all the experimental treatments would be 3 h in dark, moderate ($300 \mu\text{E m}^{-2} \text{s}^{-1}$), or high light ($1200 \mu\text{E m}^{-2} \text{s}^{-1}$). In the case of *Arabidopsis*, the leaves would be kept for 3 h. The ML and HL are 150 and 600. External agents that increase ROS (namely MD) or NO (SNP and GSNO) would be used. Photorespiration would be restricted by either lowering O_2 or the addition of AOA, an inhibitor of glycine/serine formation. Menadione interferes with oxidative electron transport, triggering ROS production primarily in mitochondria. The mechanism of MD action is indicated in Figure 2.2, while the exact location of NO production is still unclear. We have described the results and discussion as five chapters, which are related to the five objectives specified earlier.

An oxidant (menadione) can be used to initiate oxidative stress in different organelles. For e.g., MD is a redox-active quinone that produces superoxide radicals due to the inhibition of mitochondrial electron transport at the ubiquinone site (Figure 2.2). Subsequent effects on key photorespiratory enzymes located in chloroplasts or peroxisomes would be examined. The specific role of superoxide and H_2O_2 would be assessed by employing the scavengers of superoxide and H_2O_2 . Two NO-donors (SNP and GSNO) would be used to check their effects on photorespiratory metabolism. Both SNP and GSNO elevated the NO production in plant cells. GSNO would be considered specific to NO, while the SNP could release both the NO and cyanide. SNP should therefore be used with caution. To validate the elevated NO, examine the role of NO by using cPTIO would be employed as the NO scavenger and to ensure NO-specific effects on photosynthesis and photorespiration.

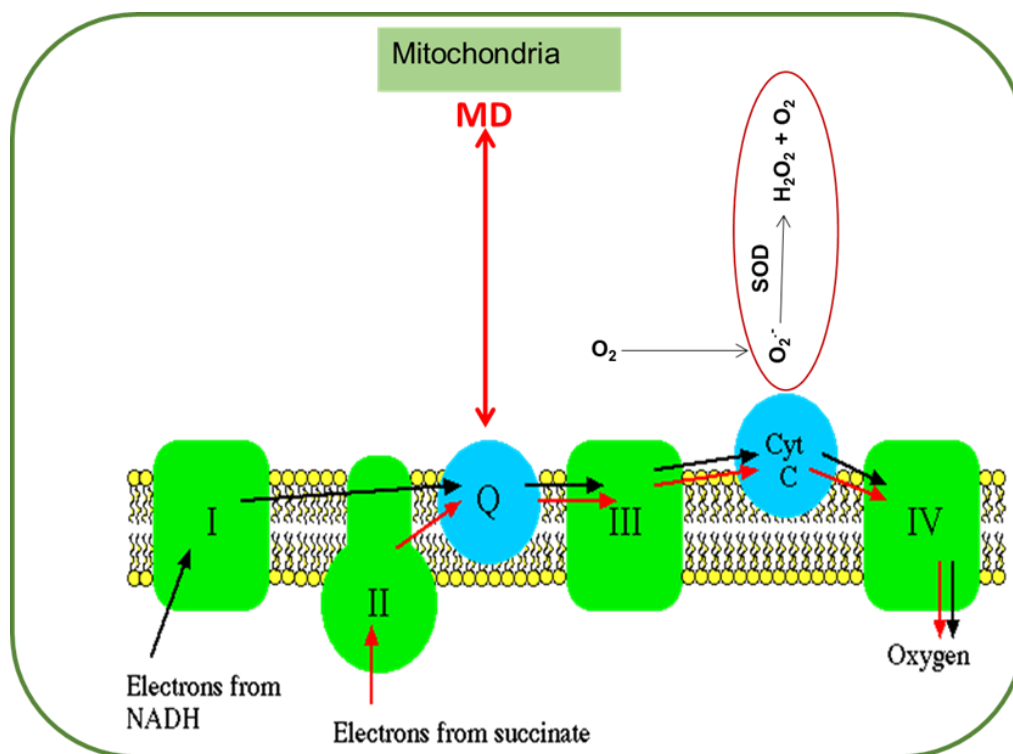


Figure 2.2. In mitochondria, MD competes with the ubiquinone pool for electrons and elevates ROS (particularly superoxide). Superoxide gets converted into hydrogen peroxide (H₂O₂) and oxygen by superoxide dismutase (Modified from Castro et al., 2008).

Photorespiration will be restricted by using low O₂ or AOA, a photorespiratory inhibitor. The concentration of O₂ will be reduced in the incubation medium by purging it with N₂ gas. AOA inhibits the conversion of glyoxylate to glycine, resulting in the overflow of glyoxylate and the inhibition of photorespiration. Mutants of *Arabidopsis* will be a good model to validate the role of a particular gene or system. Thus, *Arabidopsis* mutants deficient in antioxidants or related systems will be checked. Changes in metabolites indicate the extent of oxidative stress under given conditions. The metabolites analysis will be done by using LC-MS/MS under oxidative stress created by MD in leaves of *Arabidopsis thaliana*. An overall

view of photorespiratory metabolism and the targeted enzymes in the present study is shown in Figure 2.1. Further, the attempts to modulate photorespiration by low O₂ or interfering with electron transport, specifically in mitochondria, are also shown.

Chapter 3

Materials and Methods

Chapter 3

MATERIALS AND METHODS

Plant Material

Pea seeds *Pisum sativum* L., cv. Arkel (Durga seeds, Chandigarh, India) were sterilized with 4% sodium hypochlorite and soaked overnight in water. The seeds were then washed and germinated in a tray. The seedlings were moved to trays that contained soil and manure in a ratio of 3:1. The plants were kept in a greenhouse where the average day/night temperatures were 30 °C and 20 °C.

The Ohio State University's Arabidopsis Biological Resource Center (ABRC, Columbus, Ohio; website: <https://www.arabidopsis.org>) provided the seeds of *Arabidopsis* [(Col-0, Columbia wild type) and mutants]. Seeds were sown on a soilrite-mix in disposable plastic cups and kept in the dark for 48 hours at 4°C to break seed dormancy. Disposable cups were then moved to a growth room to allow for germination. The plants were raised in an airconditioned room with 21°C and 8/16 h of light/darkness. Once a week, a nutrient medium (Table 3.1) was provided to the *Arabidopsis* plants. We conducted our experiments using leaves from plants that were 7 to 8 weeks old.

Leaf discs preparation and treatment

About 0.25 cm² of leaf discs were made with a paper puncher while submerged in water. The leaf discs (28 equivalent to 100 mg) were incubated in a medium (1 mM CaCl₂, 1 mM KCl and 2 mM potassium phosphate buffer pH 6.5), with or without NO donor compounds, SNP (2.5 mM) or GSNO (1 mM), with or without menadione (10 µM), with or without PR inhibitors (1 mM

Table 3.1 The composition of nutrient medium used for *Arabidopsis* (Adapted from Somerville, 1982).

Macronutrients	Volume/L
Micronutrient mix	1 ml
Fe- EDTA	2.5 ml
1 M Ca (NO ₃) ₂	2.0 ml
1 M MgSO ₄	2.0 ml
1 M KH ₂ PO ₄ (pH 5.6)	2.5 ml
1 M KNO ₃	5.0 ml

***Pisum sativum* (Arkel variety)**

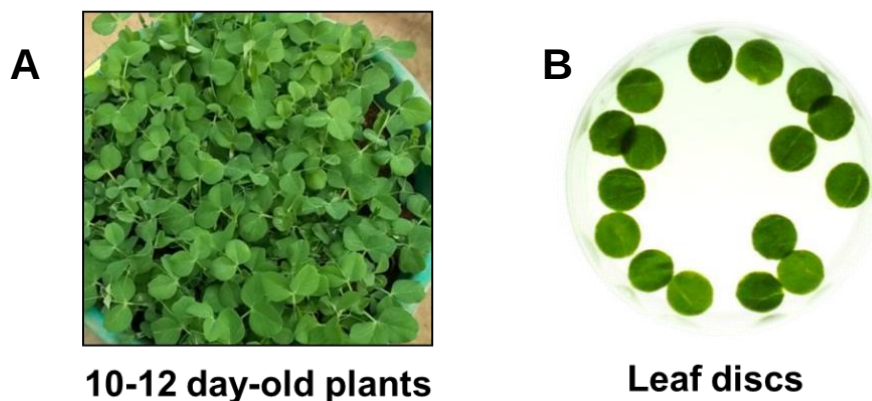


Figure 3.1. Plants of pea (*Pisum sativum*, variety Arkel) (A) in a greenhouse. Leaf discs, ca. 0.25 cm² (B) prepared with a sharp paper punch.

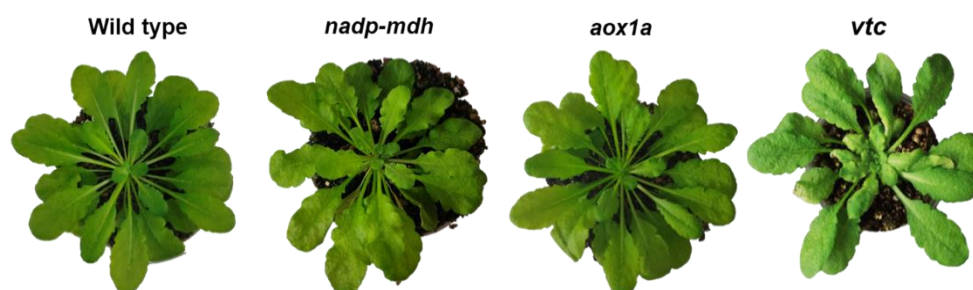


Figure 3.2. Wild type (Col-0) and mutants (*nadp-mdh*, *aox 1a*, and *vtc*) of *Arabidopsis thaliana* were grown under controlled environments with 8 h (120 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 16 h light/dark. The plants above are 7–8 weeks old.

AOA and 7% O₂). In the case of pea, the leaves were kept for 3 h in darkness or light: moderate (ML, 300 $\mu\text{E m}^{-2} \text{s}^{-1}$) or high (HL, 1200 $\mu\text{E m}^{-2} \text{s}^{-1}$). For *Arabidopsis*, the ML and HL were 150 and 600. When required, the oxidant, menadione (MD), or NO-donors (SNP or GSNO) were present in the incubation medium. Similarly, scavengers of ROS (catalase and tiron) and NO (cPTIO) were also present.

Accumulation of ROS: Hydrogen peroxide (H₂O₂) and superoxide (O₂⁻)

ROS accumulation was monitored by using either 3,3'-diaminobenzidine (DAB) or nitroblue tetrazolium chloride (NBT), which stains H₂O₂ and superoxide, respectively. The method used by Kwon et al. (2013) and Bapatla et al. (2021) was modified for this process. NBT and DAB (1 mg/ml each) were infiltrated into pea leaf discs for 5 minutes, followed by 3 minutes of the test compound using a vacuum pump, while in *Arabidopsis* leaves, it was 3 minutes for DAB or NBT filtration and 2 minutes for test compound, respectively. The leaf discs/leaves were then incubated under the required conditions. Following each treatment, leaf discs were left in a 3:1:1 (v/v/v) mix of ethanol, lactic acid, and glycerol. Methanol was used to remove the pigments from leaves, and the cleaned leaf discs were then photographed. H₂O₂ was visualized with brown color, while superoxide appeared as blue (formazan).

The leaves stained with NBT and DAB was crushed in liquid N₂ and then extracted in a solution of 2 M KOH - DMSO (1/1.6) or 0.2 M HClO₄ and (v/v). The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4 °C. The absorbance of the supernatant was determined at 630 nm and 450 nm,

for superoxide and H_2O_2 , respectively. Standard curves prepared with NBT (in KOH-DMSO) or H_2O_2 (in 0.2 M HClO_4 -DAB) were used for calculations.

NO content

The levels of NO in leaf discs were determined by Griess reagent (Zhou et al., 2005). After the required experiment, 400 mg of leaf discs were ground in 3 ml of chilled 50 mM acetic acid (pH 3.6). The homogenate was cleared by centrifugation at 10,000 g for 15 min at 4°C. The supernatant was set aside. The pellet was centrifuged as before after being rinsed twice with 1 ml of extraction buffer. The supernatants were combined, and 100 mg of charcoal was added. The filtrate was cleared again by passing through a syringe filter. One ml of Griess reagent was added to 1 ml of cleared extract, and the absorbance was determined at 540 nm, after 30 min at room temperature (Zhou et al., 2005).

Antioxidants: Ascorbate (AsA) and Glutathione (GSH)

Leaf discs (approximately 100 mg) ground in liquid N_2 after respective treatments. The total ascorbate content was estimated as per the method of Gillespie and Ainsworth (2007). Homogenization of tissue was done by adding 2 mL of 6% TCA. Centrifuged the extract at 13,000 g at 4°C for 5 min. Throughout the assay and preparation, all solutions, including extracts and reagents, were kept on ice.

From each sample (or standards or blanks) 100 μL of 50 mM phosphate buffer was added to duplicate aliquots (each 200 μL) of the supernatant. To reduce ASA, 100 μL of 10 mM DTT was added to the above aliquots and left for 10 min at room temperature. Then, 0.5% N-ethylmaleimide (NEM) of 100 μL was added to remove the excess DTT. To the

other aliquot of a sample, 200 μ L of water and 100 μ l of NEM were added. To both aliquots, the following mixture was added: 10% TCA (500 μ l), 4% α - α -bipyridyl (400 μ l), 43% H_3PO_4 (400 μ l), and 3% FeCl_3 (200 μ l). The contents were vortexed and incubated for 1 hour at 37 °C, and the absorbance was measured at 525 nm. The regression between the ASA standards and respective blanks were used to determine reduced and total ASA. The difference between the total pool and the oxidized AsA was used for calculating oxidised ASA.

After the appropriate treatments, leaf discs (approximately 100 mg) frozen in liquid N_2 were pulverised in 1 ml of 0.2 N HCl. The extract was centrifuged at 14,000 rpm and 4°C for 10 minutes, and the supernatant was neutralised using 0.2 N NaOH. The total, oxidised, and reduced GSH were determined as per Griffith (1980). 2 ml of assay mixture contained the neutralised extract (100 μ l), 1 unit of GR, 6.3 mM DTNB, 2 mM EDTA, 5 mM NADPH, and 100 mM phosphate buffer (pH 7.5). The absorbance at A_{412} was determined. Results were computed GSH equivalents with the aid of using a standard curve made from reduced glutathione (GSH). In order to estimate GSSG (oxidised), 10 μ l of 2-vinyl pyridine (2 VP) was added to 0.5 ml of neutralised extract, and the absorbance was measured at 412 nm. The extraction medium was neutralised and used as a blank. A GSH standard curve was used to calculate total GSH.

Photorespiratory enzymes

After treatment, the leaf discs (approximately 100 mg) were left in liquid N_2 . When ready, the frozen material was homogenised in 100 mM HEPES-KOH (pH 7.2), 10 mM 2-mercaptoethanol, and 1 mM EDTA. After centrifuging

the extracts, the supernatant was utilised for the GO and HPR assays at 10,000 g for 10 min. Chlorophyll was determined before centrifugation (Arnon, 1949).

Yamaguchi and Nishimura's (2000) method, with minor adjustments, was used to determine glycolate oxidase (GO) activity spectrophotometrically. The reaction mixture (1 ml) contained the following ingredients: 100 mM triethanolamine, pH-7.8), 3 mM EDTA, 0.75 mM oxidised GSSH, 4 mM phenyl hydrazine, 2.3 mM sodium glycolate, and the leaf extract equivalent to 12.5 µg chlorophyll. After adding sodium glycolate, the reaction's medium absorbance at 324 nm was followed for five minutes. The extinction coefficient of phenyl hydrazine was used to calculate the activity ($16.8 \text{ mM}^{-1} \text{ cm}^{-1}$).

The method described by Yamaguchi and Nishimura (2000) was used to measure hydroxypyruvate reductase (HPR). One ml of medium contained 50 mM phosphate buffer (pH-7.5), 5 mM lithium hydroxypyruvate, 85 µM NADH, and the extract equivalent to 12.5 µg of chlorophyll. The reaction was measured by the following absorbance at 340 nm for 5 minutes. The enzyme activity was calculated based on NADH extinction coefficient ($6.2 \text{ mM}^{-1} \text{ cm}^{-1}$).

PGLP activity was assessed in accordance with Somerville and Ogren (1979). One ml of reaction mixture contained 40 mM sodium cacodylate, 5 mM citrate, 5 mM ZnSO₄, and 0.5 mM EDTA, 5 mM HEPES (pH 6.3), and the chlorophyll equivalent to 12.5 µg. 2 mM 2PG was used to start the reaction at 25°C and incubated for 5 minutes. To stop enzymatic activity, 1 mL aliquots were combined with 700 µL of the acid molybdate reagent (1:6 mixture of 10% (w/v) ascorbate and 0.42% (w/v) ammonium molybdenum in 1 N sulfuric

acid). After 45°C for 20 minutes, the phosphate released was measured at 820 nm.

Glycerate kinase (GK) was determined according to Kleczkowski and Randall (1985). The extraction medium contained 40 mM tricine (pH 7.8), 2 mM MgCl₂, 1 mM EDTA, and 100 mM 2-mercaptoethanol. One ml of assay mixture contained 0.1 M Tricine (pH 7.8), 0.2 mM NADH, 5 mM ATP, 5 mM glycerate, 10 mM MgCl₂, 5 units of PGA-PK and GAP-DH, and the extract equivalent to 12.5 µg of chlorophyll. Glycerate was used to initiate the reaction, and after 5 minutes, the absorbance at 340 nm was recorded. The NADH extinction coefficient (6.2 mM⁻¹ cm⁻¹) was used to determine the enzyme activity

Other enzymes

100 mg of leaves were extracted the aconitase activity. The extraction buffer contained 0.1% ascorbate, 0.05% b-mercaptoethanol, 50 mM potassium phosphate (pH 7.8), and 10 mM EDTA. The mixture was centrifuged at 14,000 g and 4°C for 10 min. The assay combination included 2.5 mM NADP, 5 mM MnCl₂, 8 mM cis-aconitate, two units of isocitrate dehydrogenase, 50 mM HEPES buffer (pH 7.5), and the enzyme extract equivalent to 12.5 µg of chlorophyll. A spike in absorbance at 340 nm, a sign of NADPH generation, was utilised to measure aconitase activity. (Lehmann et al., 2009)

Fructose 1,6 bisphosphatase in 100 mg of leaves were determined by Lee and Hahn (2003). 100 mg of pea leaf discs were mixed with homogenized in a medium (100 mM sodium phosphate, pH 7.5, 2 mM EDTA), and centrifuged at 3,000 g for 20 minutes. The reaction mixture (1 ml) contained:

0.5 mM EDTA, 10 mM MgCl_2 , 0.3 mM NADP^+ , 1.2 U phosphoglucose isomerase, 0.6 U glucose-6-phosphate dehydrogenase, 100 mM Tris-HCl (pH 8.7), and extract corresponding to 12.5 μg of chlorophyll. After using fructose 1,6 biphosphatase to initiate the reaction, an increase in absorbance at 340 nm was noticed. The NADP^+ extinction coefficient was used to determine the enzyme activity.

Antioxidative enzymes

Leaf discs (approximately 100 mg) were collected after the appropriate treatment and crushed into a powder under liquid N_2 . The homogenised samples were mixed with a 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged for 10 min at 10,000 g. The CAT, APX, and GR were assayed using the supernatant. The Bradford method was used to determine the protein concentration using bovine serum albumin (BSA) as a reference.

The method of Patterson et al. (1984) was modified to measure catalase (CAT) activity. The oxidation of H_2O_2 was monitored at 240 nm. In a final volume of 1 ml, the reaction mixture contained 25 μg of chlorophyll, 20 mM H_2O_2 , and 50 mM phosphate buffer (pH 7.0). H_2O_2 extinction value at 240 nm, used to estimate enzyme activity, was $43.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

The activity of ascorbate peroxidase (APX) activity was determined by a slight modification of Nakano and Asada's (1981). The reaction mixture included 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ASA, 20 mM H_2O_2 , and the enzyme extract equivalent to 25 μg of chlorophyll. The activity was monitored as a 3- min drop in absorbance. Using extinction

coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$, the amount of AsA that was oxidised was determined.

The method of Jiang and Zhang (2001) was used to determine the glutathione reductase activity. The reaction mixture, which had a total volume of 1 ml, having 10 mM oxidised glutathione (GSSG), 3 mM MgCl_2 , 1 mM NADPH, and 25 mM sodium phosphate buffer (pH 7.5). 100 μg of protein extract was added to initiate the reaction. The oxidation of NADPH, as shown by a decrease in A340 nm and was used to monitor GR activity using the extinction coefficient of NADPH ($6.2 \text{ mM}^{-1} \text{ cm}^{-1}$).

For superoxide dismutase (SOD), samples were collected, ground in liquid N_2 , and then homogenised in a 50 mM phosphate buffer at pH 7.8. Then, centrifuged at 12,000 g for 10 minutes, and supernatant was utilized for SOD. Using Beyer and Fridovich's (1987), SOD activity was determined. The reaction mixture consisted of 27 ml of sodium phosphate buffer (pH 7.8), 1.5 ml of methionine (30 mg ml^{-1}), 1 ml of NBT (1.44 mg ml^{-1}), 0.75 ml of Triton X-100, and 1.5 ml of 2 mM EDTA. In 1 ml of this reaction mixture, an enzyme extract containing 50 μg of chlorophyll and 10 μl of riboflavin (4.4 mg per 100 ml) was added. The reaction mixture was then exposed to light using comptaloux bulbs for 7 minutes at 25°C using a water bath. As a blank, a tube containing enzyme extract is kept in the dark; as a control, a tube having no enzyme extract is kept in the light. The activity of SOD was determined by the difference between NBT reduction measured at 560 nm under light with or without enzyme extract. One unit of activity is the amount of protein required to stop a 50% decline in NBT when exposed to light.

Chlorophyll and Protein estimation

For chlorophyll estimation, 12.5 µl of leaf extract was added to 5 ml of 80% (v/v) acetone and mixed well with a cyclo-mixer. Chlorophyll was calculated in leaf extract using the following formula, based on Arnon (1949).

$$\text{Chl (mg/ml of leaf extract)} = (A_{652} - A_{710}) \times 11.11$$

For protein estimation, 10 µl supernatant was incubated with 1.2 ml of Bradford reagent and kept in the dark for 15 minutes. The absorbance was taken at 595 nm. The protein concentration was determined based on a standard curve made with a known concentration of BSA (Bradford, 1976).

Western blotting

Following the appropriate treatment, 100 mg of leaf discs were ground to a powder in liquid N₂, using the extraction medium 5% (w/v) sodium dodecyl sulphate (SDS), 1 mM PMSF and 125 mM Tris-HCl (pH 6.8). The extract was centrifuged at 1,000 g for 1 minute, and the supernatant was collected. The Bradford et al. (1976) method was used to estimate protein.

SDS-PAGE was carried out using small gels of 7.2 x 8.6 cm of dimensions (Laemmli, 1970). Following the SDS-PAGE, the proteins were electrophoretically transferred from the gel onto PVDF membranes. A power source of 30 V was used at 4°C for 4 hours (Towbin et al., 1979). Skim milk (5%) diluted in 1X TBST was used to block the PVDF membrane, and a primary antibody treatment of 3 hours was then applied. The PVDF membrane washed 3 times for 5 min each with 1X TBST and antibodies (Agrisera AB, Sweden) for CAT and GO (1: 1,000), HPR (1:10,000), and MnSOD (1:2,000). Next, alkaline phosphatase-conjugated secondary

antibodies at a 1: 2,000 dilution were added. The PVDF membrane was washed three times for five minutes, then one ml of substrate (BCIP/NBT) was used to produce the blot.

Transcripts

Extraction of RNA

Spectrum™ Plant Total RNA Kits (Sigma-Aldrich, USA), were used for RNA extraction. The procedure to extract RNA was from the manufacturer's instruction. Leaf sample of 100 mg were ground in liquid N₂ to a fine powder. Lysis solution (1000 µl/100 mg and BME 10µl/ml) was added, homogenized and incubated for 5 minutes at 56°C in a dry bath. The samples were centrifuged for 5 minutes at 14,000 rpm at room temperature. Supernatant was transferred into the filtration column (blue column) and placed in a new tube. The eluate was collected by centrifugation. 750 µl of binding buffer was added to the supernatant and mixed well.

Next, the solution was transferred into the binding column (red column) in a fresh tube and centrifuged for 1 min at 14,000 rpm. The supernatant was discarded. The red column was kept in a fresh tube and washed first with 500 µl of wash solution 1, and then with wash solution 2, centrifuged for 1 min, and this step was repeated again. After that, 30 µl of elution buffer was added to the column, which was kept in a new tube, and let it sit for 2-3 minutes while keeping it on ice. The tube containing the red column was centrifuged for 1.5 min at 14,000 rpm. The column was discarded, and the tube was placed on ice. The RNA concentration and quality (260/280) was measured using nanodrop.

cDNA synthesis

The cDNA synthesis protocol followed the manufacturer's recommendations (RevertAid cDNA synthesis kit, Fermentas). 5 µg of total RNA was mixed with oligo(dT) primer (1 µl), cocktail (8 µl): 5X reaction buffer (4 µl), RiboLock (1 µl) RNase inhibitor (1 µl), 10 mM dNTP (2 µl) and Reverse Transcriptase (1 µl). The final volume was made up to 20 µl with RNase-free water. The sample was thoroughly mixed, heated to 65 °C for 5 minutes in a thermal cycler, then frozen for 2 minutes in ice before a quick spin. The synthesized cDNA was used as the RT-PCR analysis template.

RT-PCR

Semi-quantitative reverse transcriptase-PCR (RT-PCR) was used to examine the gene expression patterns. *Pisum sativum* does not yet have a full genome (Kulaeva et al., 2017). The cool-season food legume database's ESTs were used to design the primers (<https://www.coolseasonfoodlegume.org>). In the case of pea, the primer sequences displayed 75% sequence identity with the enzymes in *Arabidopsis* when they were tested through BLAST (Aswani et al., 2019). For *Arabidopsis*, primers were designed based on known sequences (<http://www.ncbi.nlm.nih.gov>) (Rozen and Skaletsky, 2000; Bapatla et al., 2021). For *Arabidopsis thaliana*, Actin 8 was employed as an internal (constitutive) control, and Actin 2 for pea. Image J software was used to measure band intensities following electrophoresis and standardize them to band intensities of Actin 2 and Actin 8. The primer sequences of pea and *Arabidopsis* were shown in Table 3.2 and 3.3, respectively.

Nitrosothiols estimation

Saville-Griess test was used to determine the amount of S-nitrosothiols (SNO) with some modifications (Puyaubert et al., 2014). Leaf discs were powdered in liquid N₂ and homogenized in 600 µl of homogenization buffer (50 mM Tris-HCL, pH 8.0, and 150 mM NaCl) along with 1 mM PMSF. The extract was incubated for 20 minutes on ice and centrifuged (10,000 g, 15 min, 4°C). The supernatant (300 µl) was mixed with 1% sulfanilamide (300 µl) with or without the addition of 0.2% (w/v) HgCl₂ and incubated in the dark for 20 min. Then 0.02% NED (600 µl) was mixed and incubated for 5 min. The levels of SNO were measured photometrically at 540 nm. A GSNO standard curve was utilised to determine the SNO concentration

Tyrosine nitration

The frozen leaf sample was ground in an extraction solution containing 5 mM Tris-HCL (pH 6.8), 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 10 mM DTT, 1% Protease inhibitor, 150 mM NaCl, 1 x potassium phosphate buffer pH 7.5 and 1 mM PMSF at 4°C. The extract was centrifuged at 14,000g for 15 min, and the supernatant was subjected to further analysis. Protein in the supernatant was determined using Bradford's method

Proteins were separated by SDS-PAGE using 15 % gels. The proteins were moved over to PVDF membranes. The membrane was blocked for one hour at 25 °C with 5% skim milk before being rinsed 3 times with TBST (0.1% Tween 20 in 1X TBS). After being incubated with the primary antibody, anti-tyronitrosine antibody (1:2000 dilution, Molecular Probes) was applied for 3 h at 4 °C. Following three TBST washes (10 minutes each), Goat anti-

rabbit IgG coupled with horseradish peroxidase (1:10,000 dilution, Bangalore GeNeiTM) was used as the secondary antibody for two hours. TBST was then used to rinse the membrane three times for a total of ten minutes each. Thermo Scientific's SuperSignal® West Pico chemiluminescent substrate was used to produce the blot, which was then examined with ChemiDoc (Bio-Rad). Image J software was used to measure the band intensity and normalized it to Rubisco's levels (Vishwakarma et al., 2018).

Chlorophyll and carotenoid estimation

Chlorophyll and carotenoid pigments were extracted into 80% (v/v) acetone. The leaf extracts were cleared by centrifugation, and their absorbance was measured at 645 nm, 663 nm, 480 nm, and 510 nm. The levels of chlorophyll and carotenoids were calculated as per Arnon (1949). The formula below was used to compute the amount of chlorophyll and carotenoid present in the samples.

$$\text{Chlorophyll a} = (12.7 \times A_{663}) - (2.6 \times A_{645}) \times \text{ml acetone mg}^{-1} \text{ leaf}$$

$$\text{Chlorophyll b} = (22.9 \times A_{645}) - (4.68 \times A_{663}) \times \text{ml acetone mg}^{-1} \text{ leaf}$$

$$\text{Carotenoids} = (7.6 \times A_{480}) - (1.49 \times A_{510})$$

Monitoring photosynthesis and respiration

Photosynthesis and respiration rates were measured with a leaf disc electrode system (LD-2, Hansatech Instruments, UK) at a temperature of 25 °C. The light was provided by light-emitting diodes. 1 M bicarbonate buffer (pH 9.0, 200 µl) were used to moisten the uppermost capillary matting, creating a gaseous environment in the chamber of 5% (v/v) CO₂. On this matting, three rings of one, six, and twelve-leaf discs were symmetrically arranged. The

oxygen level in the chamber for each sample was calibrated in line with the directions from the manufacturer (Talla et al., 2011).

Determination of PSII and PSI efficiency

A Dual-PAM 100 was used to assess the efficiency and components of PSII and PSI (Walz, Germany). Before measurement, leaves were exposed to the respective treatment in the dark for 30 minutes (Schreiber, 2004; Kramer et al., 2004; Schreiber and Klughammer, 2008). The software in the equipment determined the following parameters of PSII: (Demmig-Adams, 1990; Kramer et al., 2004). Maximum quantum yield: $F_v/F_m = (F_m - F_o)/F_m$; Quantum yield of photochemical energy conversion: $Y(II) = (F_m' - F_t)/F_m'$; Quantum yield of non-regulated energy dissipation: $Y(NO) = F_t/F_m$; Quantum yield of photo-protective energy dissipation: $Y(NPQ) = (F_m' - F_t/F_m)/F_t$; Non-photochemical quenching: NPQ, qP, and qL (Schreiber, 2004).

The parameters related to PSI were determined. Quantum yield of photochemical energy conversion: $Y(I) = (P_m' - P)/P_m$; Acceptor side limitation: $Y(NA) = P_m - P_m'/P_m$; and donor side limitation: $Y(ND) = P/P_m$ (Klughammer and Schreiber, 1994). The manufacturer's manual has additional information.

Analysis of metabolites

Leaf discs were treated with MD in dark and light for 3 hours in order to analyse metabolites. After the treatment, the samples (100 mg) were frozen in liquid nitrogen. The powdered samples were mixed with 500 μ l of LC-MS buffer (150 μ l chloroform, 350 μ l methanol, 1 μ l MES (IT= internal standard), and 1 μ l carnitine (IT)). Sample was extracted using a ball mill (Retsch-mill,

30 sec at max intensity) or ground with a white stick. The samples were stored on ice until all have been proceeded. 400 µl of ice-cold water (LC-MS grade) was added to the samples. Vortexed and kept on ice. The samples were incubated for 2 h at -20°C and vortexed again. For 10 minutes, the samples were centrifuged at their highest speed, and the aqueous phase (upper, around 900 µl) was collected in a fresh eppendorf tube.

The sample was once more diluted with 400 µl of ice-cold water, vortexed, then centrifuged for 5 min at maximum speed. Aqueous phase (upper) was collected and pooled it the previous sample (~1300 to 1400 µl in total). After that, the supernatant was kept in a speedvac concentrator to completely dry the sample. Dried samples were gathered and kept in a desiccator at -20°C. We ensured that samples were not thawed throughout the extraction process. The samples were sent to Dr. Stefan Timm's lab at the University of Rostock in Rostock, Germany, for LC-MS/MS analysis.

The soluble primary metabolites were extracted, and samples were evaluated in accordance with those described by Arrivault et al. (2009, 2015), Reinholdt et al. (2019). The signals of the internal standard [(morpholino)-ethanesulfonic acid (MES), 1 mg/ml] were used to normalize the peak regions of all the metabolites for relative measurement (Timm et al., 2019).

Replications and significance

Data represented were averages ($\pm$ SE) from at least three experiments done on different days.

Table 3.2 The nucleotide sequence of gene specific primers of pea used for RT-PCR.**Pea (*Pisum Sativum*)**

Gene	Primer sequence (5'-3')
<i>APX</i>	F: GGATCCTATGGGAAAATCATACCCAAGT R: CTCGAGTCTTAGGCTTCAGCAAATCCAAG
<i>Cu/Zn SOD</i>	F: GAACAATGGTGAAGGCTGTG R: GTGACCACCTTTCCCAAGAT
<i>Mn SOD</i>	F: GGAGCAAGTTTGGTTCCATT R: AAGGTTATTCGGCCAGATTG
<i>CAT</i>	F: CGAGGTATGACCAGGTTTCGT R: AGGGCATCAATCCATCTCTG
<i>GO</i>	F: GCTTCCCTGCGTCCGAGTCTT R: GTGAGCAAGATCGACATG
<i>HPR</i>	F: CAGGTCCTC TAC TGC AGT R: TTG GTG TTA TCG GTG CTGG
<i>GK</i>	F: ATCCCTATCTTCTGCTCC R: GAC ATA CAC CGA TTT TCC
<i>PGLP</i>	F: GGCGGGGATGGT AACTGGA R: TTGATCCACACATGCCGC
<i>Actin 2</i>	F: AATGGTGAAGGCTGGATTTG R: AGCAAGATCCAAACGAAGGA

Table 3.3 The nucleotide sequence of gene specific primers *Arabidopsis thaliana* used for RT-PCR.

Arabidopsis thaliana

Gene	Primer sequence (5'-3')
<i>CAT1</i>	F: AATCGACAACAGTGCAGACACAC R: GACCTCGAGTTCCGACAGTCA
<i>CAT2</i>	F: TGCTGGAAACTACCCTGAATGG R: TCAACACCATACGTCCAACAGG
<i>GOX1</i>	F: TCTCATTGGCAGCTGAAGGA R: GAGTGTCCCATTCTGGTGGTA
<i>GOX2</i>	F: TTTGCACTAGCTGCTGAAGGA R: ATAACCTGGGCAAATGGCGT
<i>PGLP</i>	F: GGATCCCAATGGCGCCTCAGCTTCTCTC R: GGATCCCTAGGGGGACTCCATCAGTTTTATG
<i>Actin 8</i>	F: GGAGCAAGTTTGGTTCCATT R: AAGGTTATTCGGCCAGATTG

Chapter 4

Modulation of Photorespiratory Enzymes Components by Oxidative and Photo- oxidative Stress Induced by Menadione in Leaves of Pea (*Pisum sativum*)

Chapter 4

MODULATION OF PHOTORESPIRATORY ENZYMES COMPONENTS BY OXIDATIVE AND PHOTO-OXIDATIVE STRESS INDUCED BY MENADIONE IN LEAVES OF PEA (*PISUM SATIVUM*)

Introduction

Photosynthesis is markedly modulated by biotic or abiotic stress factors, including high light (HL) or drought/chilling. Plants use a variety of strategies to protect and sustain photosynthesis against these stresses (Raghavendra and Padmasree, 2003). To optimize photosynthesis, interactions between mitochondria and chloroplasts via peroxisomes and cytosol are crucial (Gardestrom et al., 2002; Padmasree et al., 2002; Raghavendra and Padmasree, 2003; Sunil et al., 2019). Photorespiration is a prime example of how various cellular organelles are compartmentalized (Bauwe et al., 2010; Timm and Bauwe, 2013). Striking coordination between mitochondrial and peroxisomal photorespiratory metabolism was demonstrated even *in vitro* reconstructed system (Raghavendra et al., 1998). Photorespiration is now established as a crucial defence against photoinhibition in addition to dark mitochondrial respiration (Kozaki and Takeba, 1996; Wingler et al., 2000; Voss et al., 2013). Readers with interest in photorespiratory metabolism might consult comprehensive reviews that are available (Foyer et al., 2009; Bauwe et al., 2010; Hodges et al., 2016; Shi and Bloom, 2021).

On exposure to stress, the levels of ROS and even NO go up in plant cells. Under such oxidative stress, photorespiration can lower ROS levels (Raghavendra and Padmasree, 2003; Voss et al., 2013; Sunil et al., 2019).

When CO₂ is limiting, under HL, photorespiration was an effective sink for reductants and recycling ATP/ADP and NADP (Igamberdiev et al., 2001; Martins et al., 2014). Increased photorespiration in tobacco plants exposed to HL helped energy balancing and facilitated the water-water cycle (Huang et al., 2016). Additionally, the recycling of ammonia produced by mitochondria during photorespiration (Bauwe et al., 2010; Hodges et al., 2016). The adequate photorespiratory flow was necessary for nitrate uptake (Rachmilevich et al., 2004; Foyer et al., 2009; Bloom, 2015).

The present chapter describes the effect of an oxidant, MD, which primarily targets mitochondria in pea leaf discs in dark, moderate, or high light (HL). Under oxidative and photo-oxidative stress, the patterns of ROS generation, changes in photorespiratory enzymes/proteins/their transcripts, and changes in antioxidants and antioxidant enzymes were all investigated. Significant increases in ROS levels (superoxide and H₂O₂), antioxidants and their enzymes demonstrated stress induction by MD. As a result, the photorespiratory enzyme components in peroxisomes and chloroplasts were modulated. These findings indicated that oxidative stress created in mitochondria by MD effectively communicated to other cellular compartments in pea leaves.

Results

Pea leaf discs accumulated superoxide and H₂O₂ with increasing MD concentrations (Fig. 4.1) and with time (Fig. 4.2). For the subsequent studies, we used 10 µM MD and 3 h of exposure duration.

Changes in levels of ROS

The accumulation of superoxide and H₂O₂ in pea leaf discs was monitored by NBT and DAB staining, respectively. The presence of blue and brown color represented the superoxide and H₂O₂. The amount of ROS in the leaf discs was quantified and represented graphically. When exposed to MD, there was a noticeable elevation in the ROS (both superoxide and H₂O₂). The ROS levels increased in ML and further increased in HL conditions. H₂O₂ level rose by up to 40% after exposure to MD, although superoxide content rose by up to 60% above control (Fig. 4.3).

Photorespiratory enzymes: Activities, protein levels, and their transcripts

The activity of GO and CAT (peroxisomal enzymes), was higher when treated with MD and light (Fig. 4.4A and B). Activities of two chloroplastic GK and PGLP, were higher by 30% in HL (Fig. 4.4C and D). To complement these increases, the protein levels of these enzymes were checked by western blotting under a given stress. The protein levels of GO, CAT, and HPR rose when exposed to MD. Those of GK and PGLP was not measured due to the unavailability of proteins. The protein levels of GO and CAT increased in dark, ML and HL treated with MD, while the protein levels of HPR increased only marginally (Fig. 4.5).

Enzyme transcripts of GO, CAT, HPR, GK, and PGLP increased under dark, ML, and HL conditions after MD treatment. The maximum increase in the transcripts of these enzymes were noticed particularly under HL treated MD condition (Fig. 4.6). The transcripts of HPR were only marginally increased (Fig. 4.7A). The activity of aconitase which is located in

mitochondria, was reduced in samples exposed to MD compared to untreated samples (Fig. 4.7B)

Changes in the levels of antioxidants

In the dark, there was not much of a buildup of ascorbate and glutathione in pea leaf discs. Under ML and HL in presence of MD, there was a considerable rise in ascorbate and glutathione levels (Fig. 4.8A and C). In the case of dark, the ratio of reduced to total ascorbate or glutathione did not alter substantially, but in the case of ML and HL treated samples, the ratio decreased markedly (Fig. 4.8B and D).

Changes in the activities and transcripts of antioxidant enzymes

Among the antioxidant enzymes, GR and SOD significantly increased in the presence of MD under both ML and HL, while the APX activity increased with MD under HL. The activity of SOD was slightly higher even in the dark, but there was little impact on APX or GR (Fig. 4.9). Similarly, there was no significant change in transcripts of APX, GR, and SOD when kept in the dark. When treated with MD under HL, transcripts of glutathione reductase (GR), Cu/ZnSOD, and FeSOD increased. The levels of APX or MnSOD only slightly changed (Fig. 4.10).

Reversal of MD-effects by superoxide or H₂O₂ scavengers

Attempts were made to identify the exact species of ROS involved in the modulation by MD using CAT and tiron as H₂O₂ and superoxide scavengers, respectively (Willekens et al., 1997; Kwon et al., 2013). CAT was quite effective in scavenging H₂O₂ than superoxide. In contrast, the reduction in superoxide and H₂O₂ by tiron was similar (Fig. 4.11). When CAT and tiron

both were present, the extent to MD-induced changes in photorespiratory enzymes (GO, and GK) were subdued (Fig. 4.12).

Discussion starts on page no. 64.

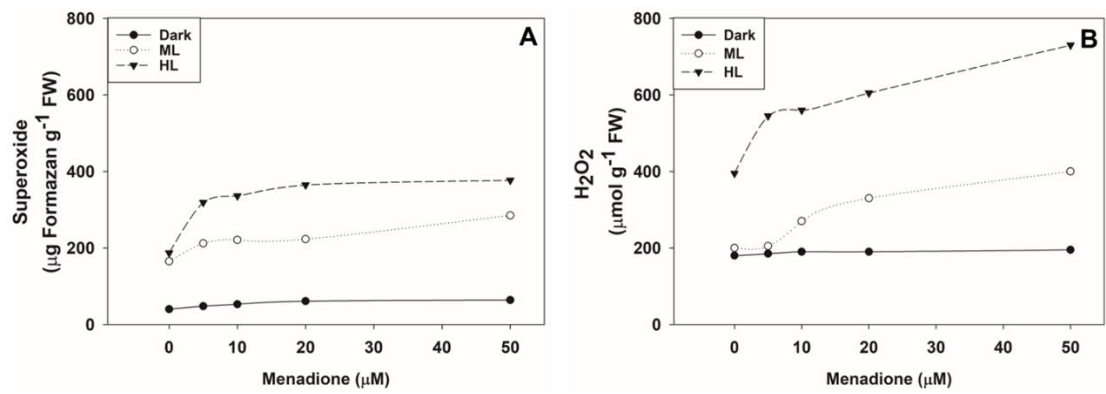


Figure 4.1. Patterns of ROS accumulation in pea leaf discs treated with MD (0 to 50 µM) in dark, ML (300 µE m⁻² s⁻¹), and HL, (1200 µE m⁻² s⁻¹). The superoxide levels were quantified as formazan formed after incubation with NBT (A), while the levels of H₂O₂ were measured with DAB (B). Data represent averages (± SE) from three experiments done on different days.

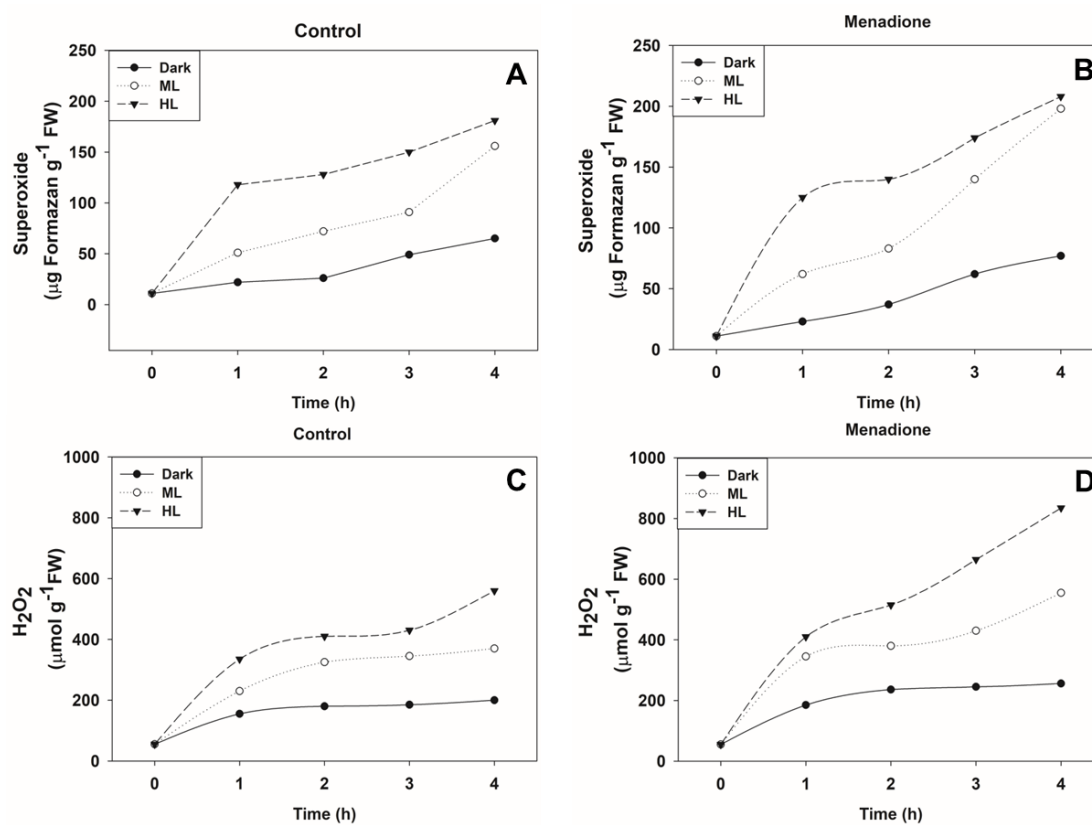


Figure 4.2. ROS levels on exposure to MD for varying periods in pea leaf discs. The treatments and averages were all as in Fig. 4.1.

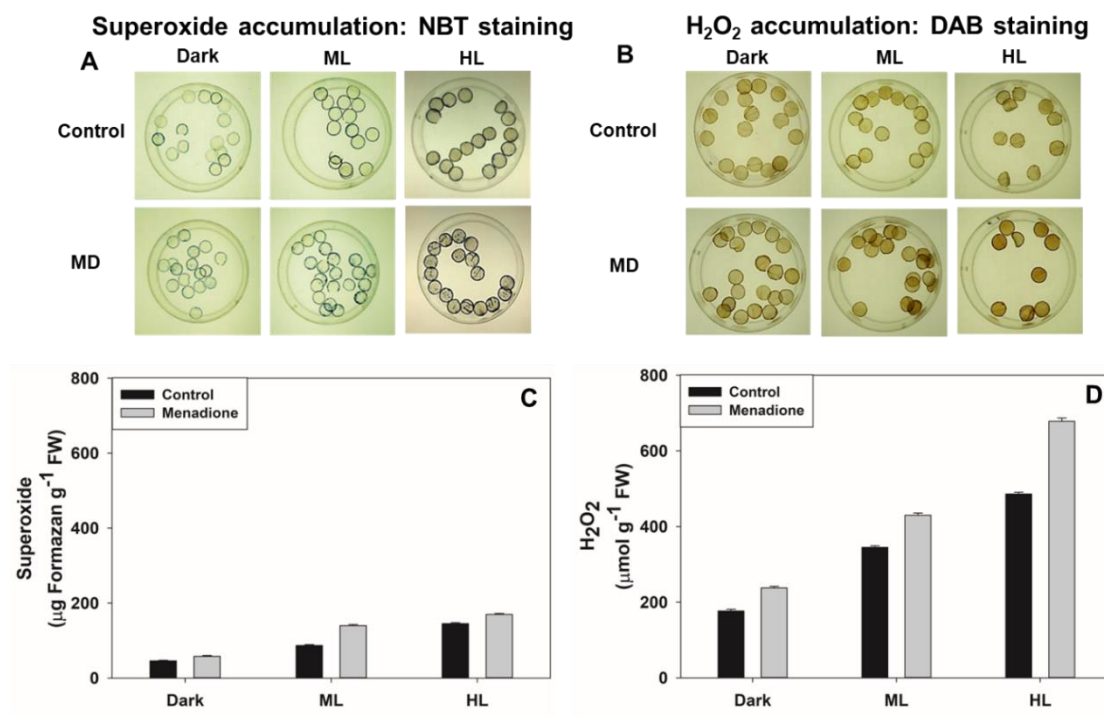


Figure 4.3. Superoxide and H₂O₂ levels, visualized by DAB and NBT staining in leaves of *Pisum sativum* upon treatment with MD. Blue color represented the superoxide upon treatment (A), while the brown color showed up the patterns of H₂O₂ (B). Superoxide and H₂O₂ were quantified from NBT and DAB-stained leaves (C and D). The treatments and averages were all as in Fig. 4.1.

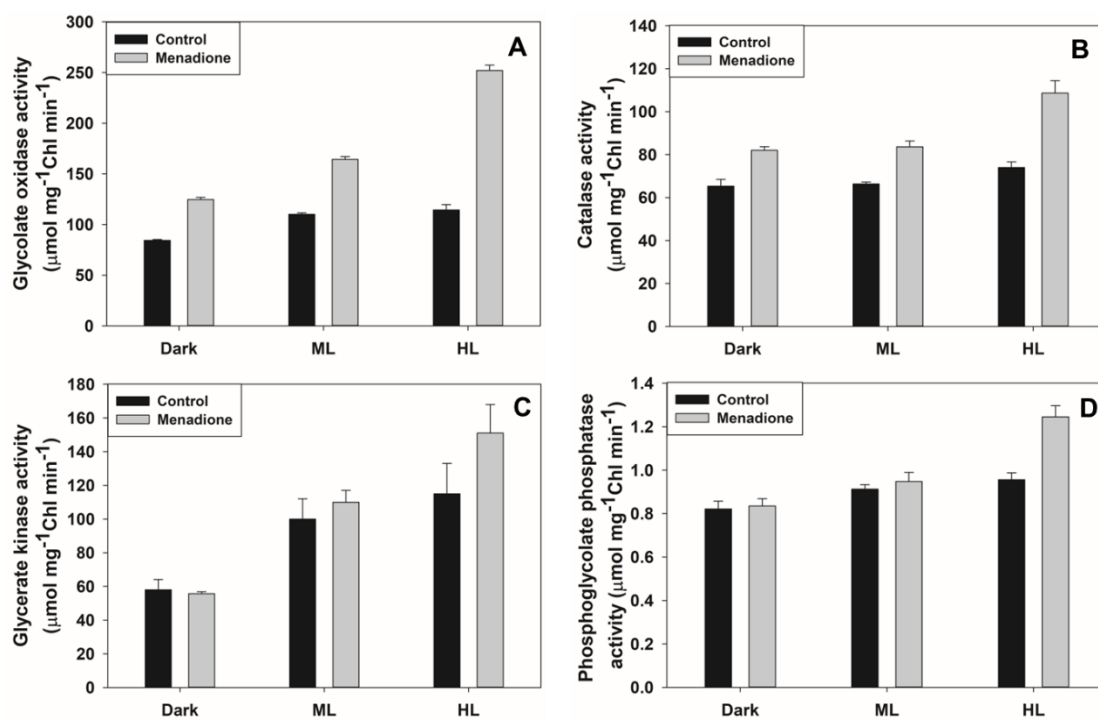


Figure 4.4. Photorespiratory enzyme activities, GO (A), CAT (B), GLYK (C), and PGLP (D) were measured. The treatments and averages were all as in Fig. 4.1.

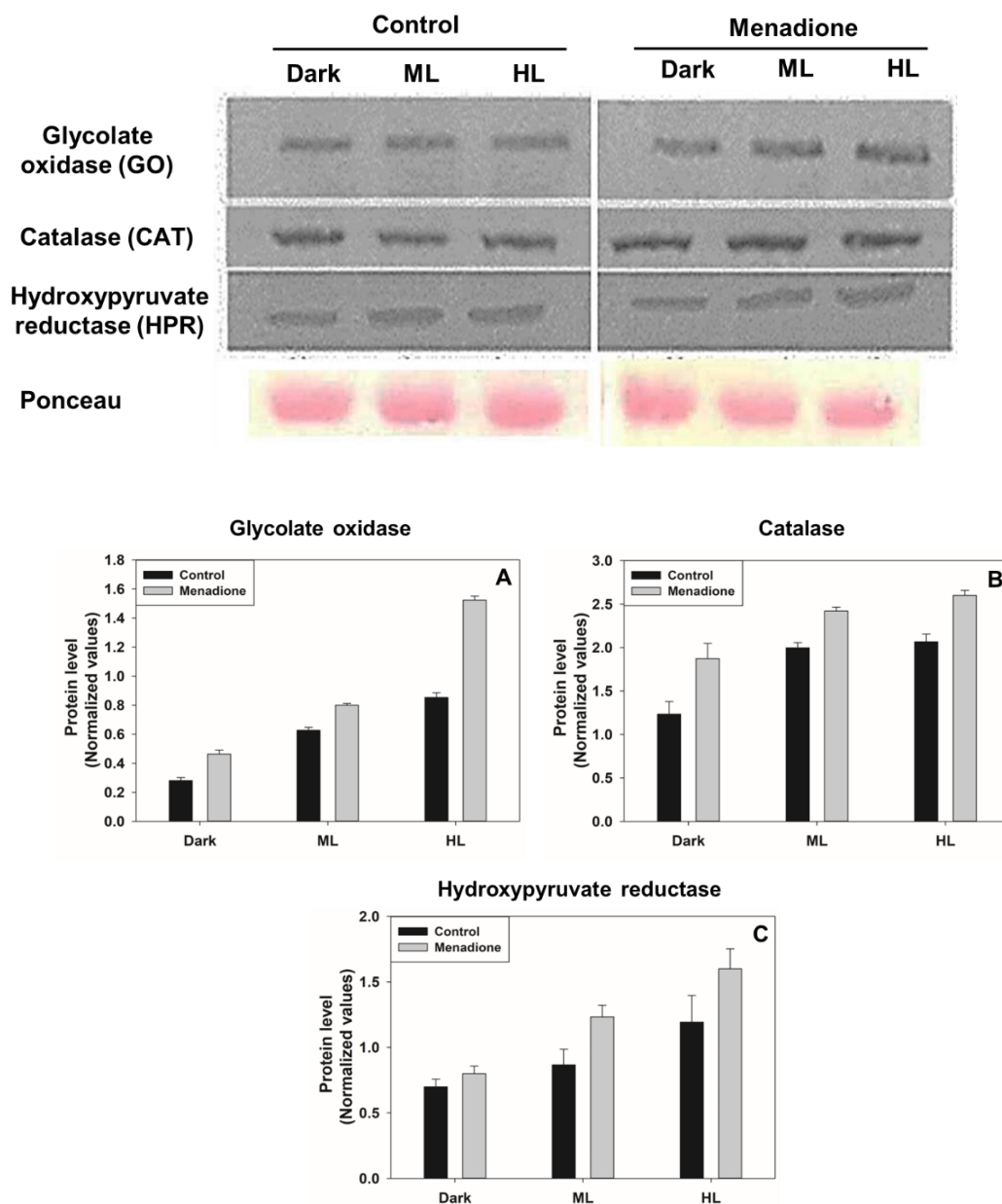


Figure 4.5. The protein levels of CAT, GO, and HPR were measured in leaves of *Pisum sativum* upon exposure to MD (Top panel). Based on image J, the ratios of GO, CAT, and HPR protein to Rubisco large subunit were computed (Bottom panel, A to C). The treatments and averages were all as in Fig. 4.1.

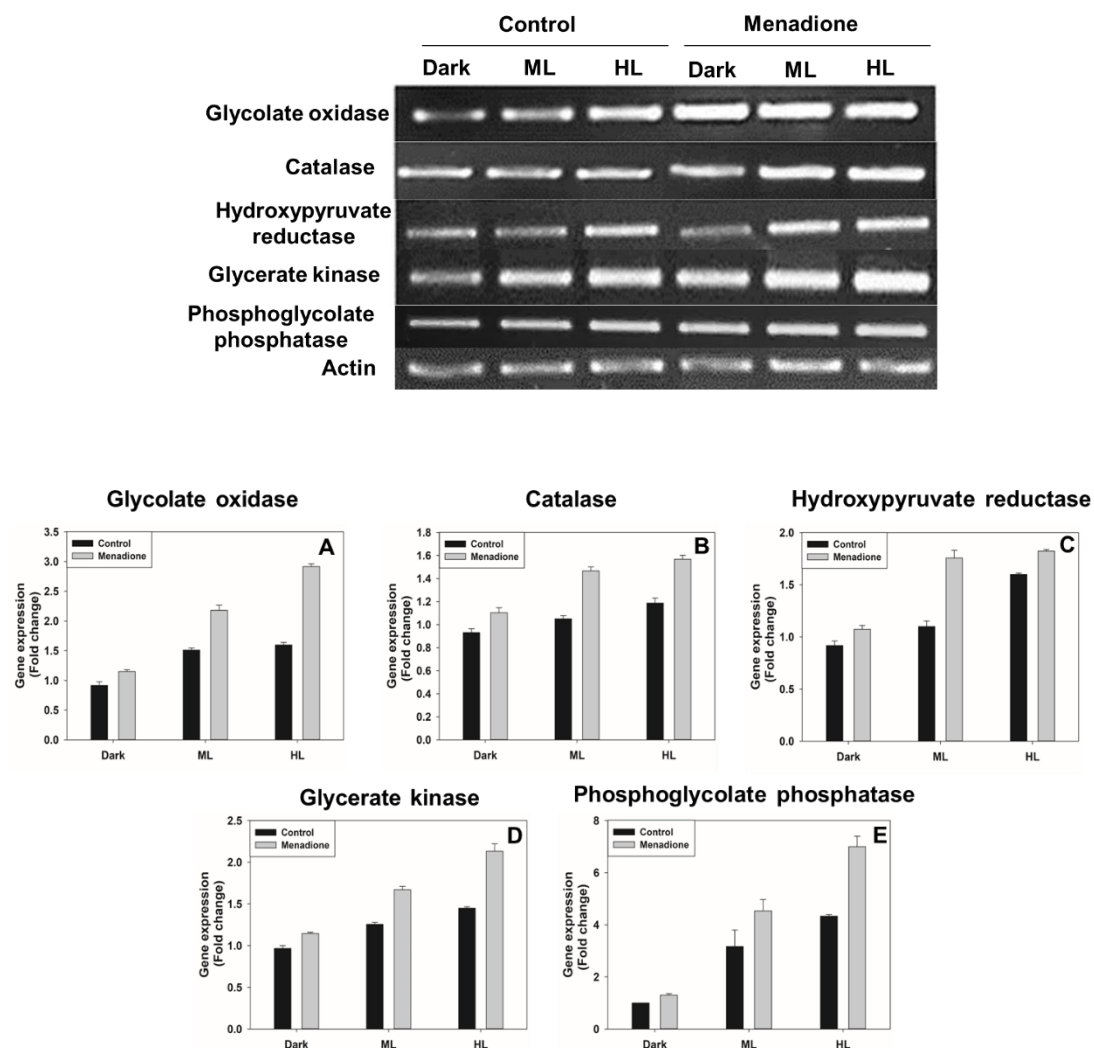


Figure 4.6. The transcripts of selected photorespiratory metabolism by semi-quantitative RT-PCR in *Pisum sativum* in leaf discs on exposure to MD. The scans are shown in Top panel. Actin 2 was the internal control. The quantified values were given in Bottom panel (A to E); GO (A), CAT (B), HPR (C), GLYK (D), and PGLP (D). The treatments and averages were all as in Fig. 4.1.

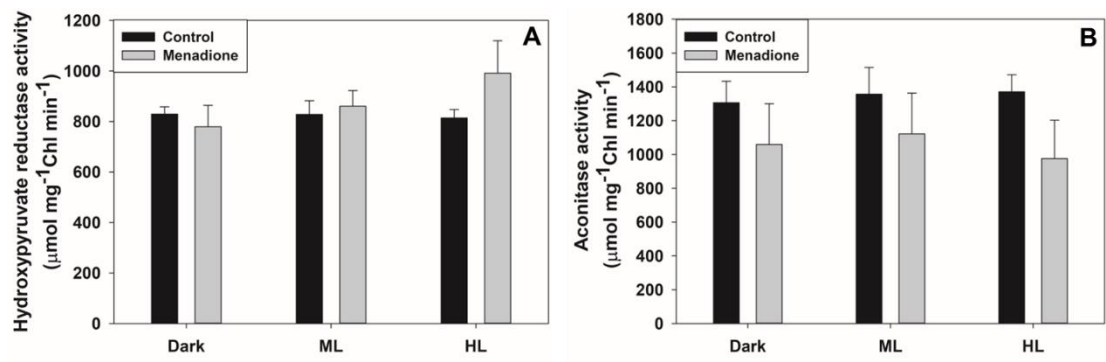


Figure 4.7. Photorespiratory enzyme activities, HPR (A), and Aconitase (B), were measured. The treatments and averages were all as in Fig. 4.1.

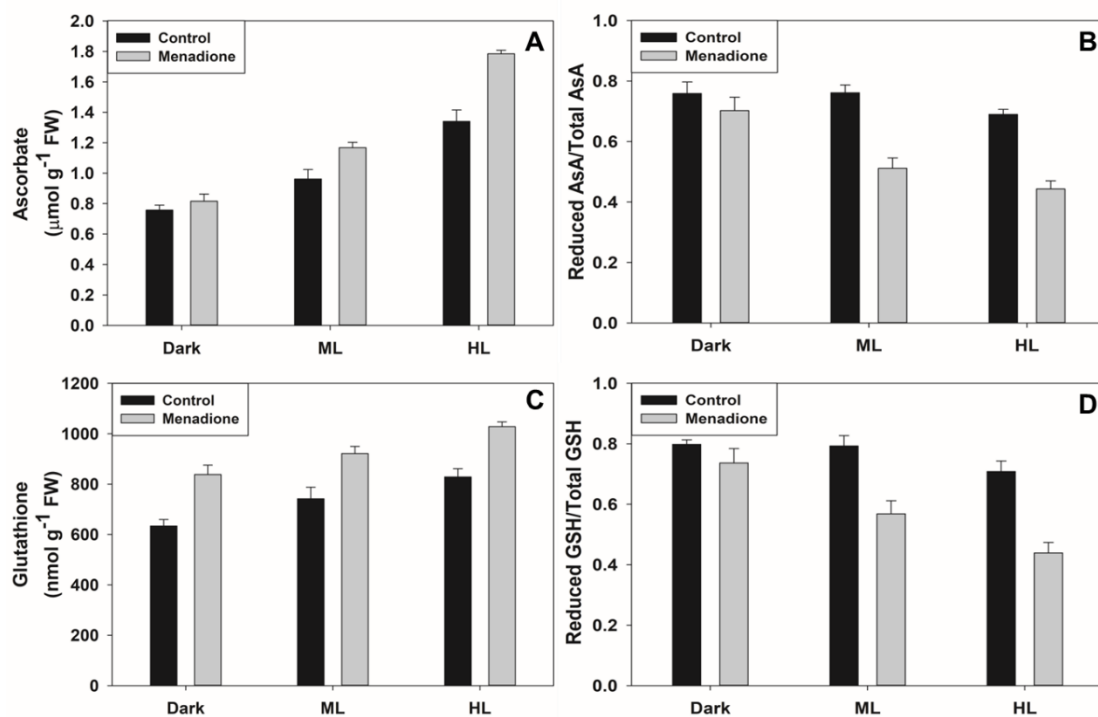


Figure 4.8. Ascorbate (A), glutathione (C), and their redox ratios (B and D) were assessed as non-enzymatic antioxidants. The treatments and averages were all as in Fig. 4.1.

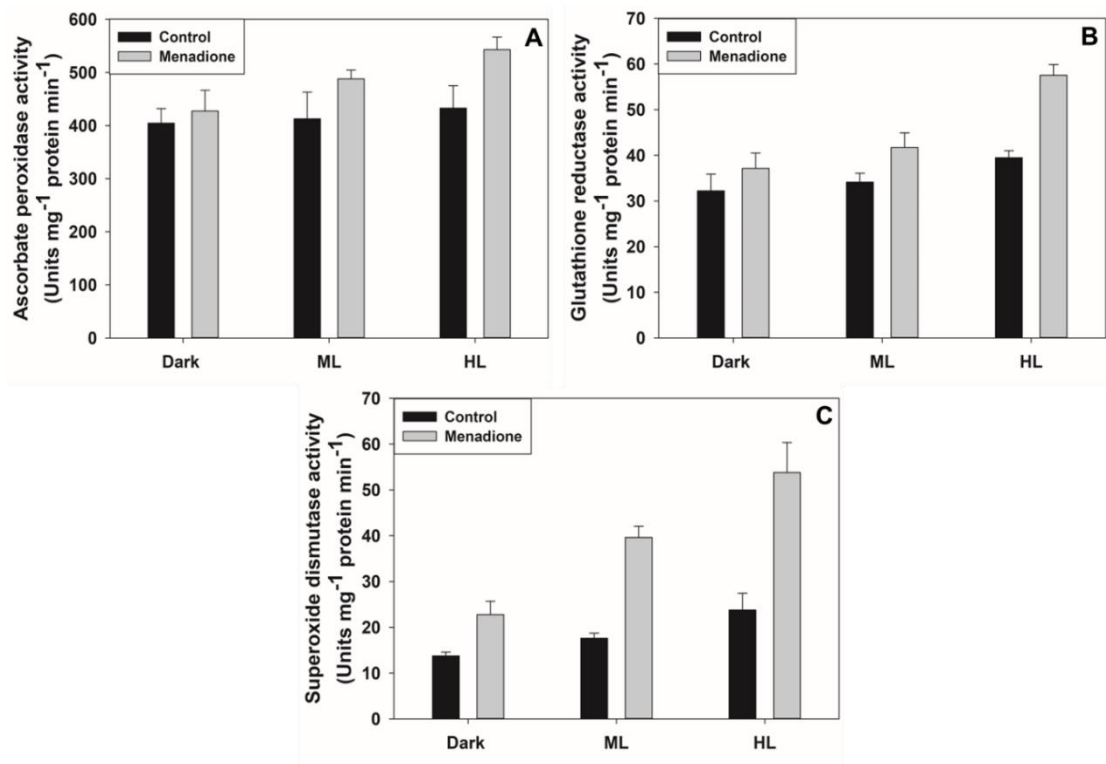


Figure 4.9. Antioxidant enzyme activities, APX (A), GR (B), and SOD (C) were measured. The treatments and averages were all as in Fig. 4.1.

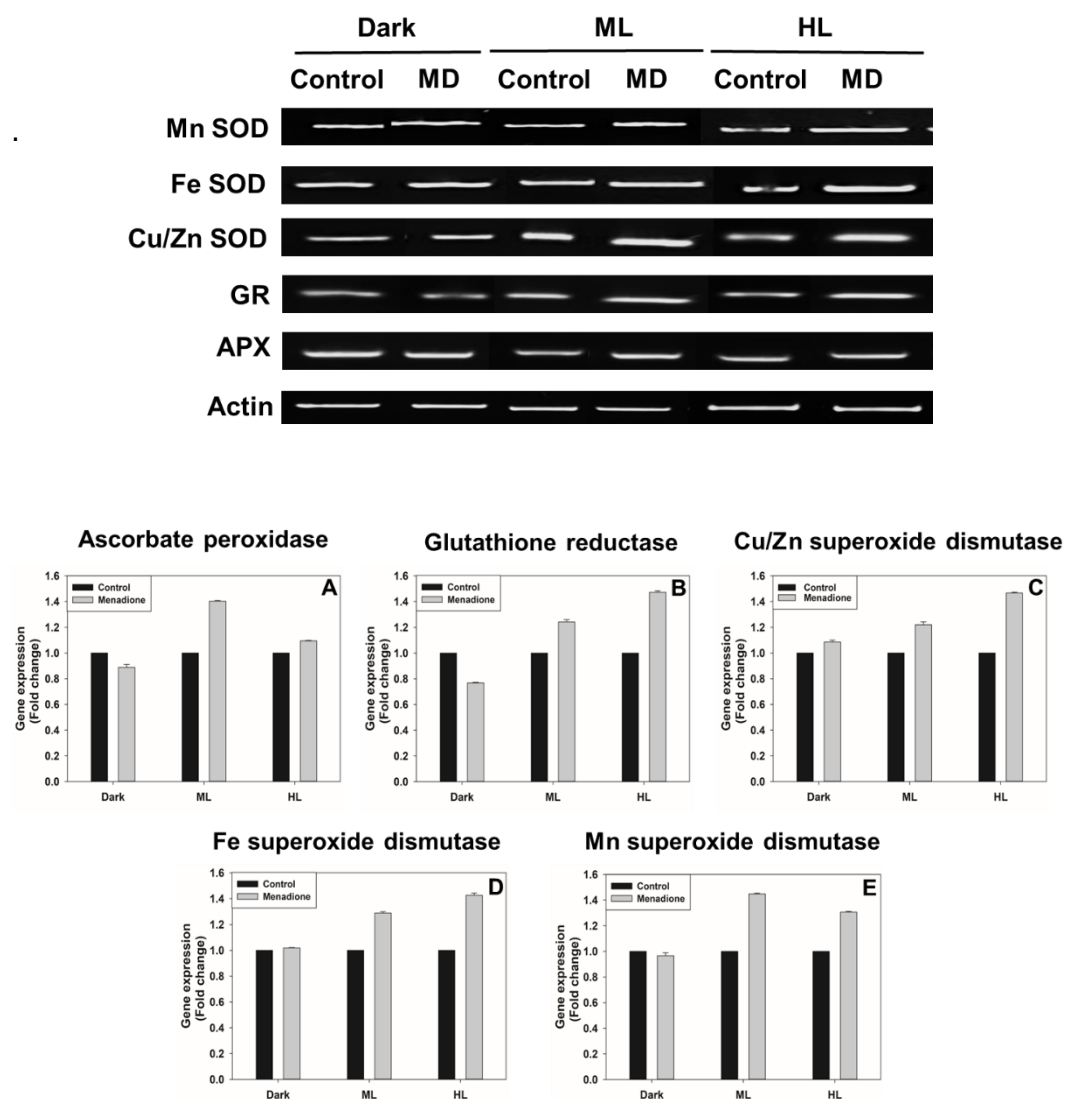


Figure 4.10. The transcripts of selected antioxidant enzymes by semi-quantitative RT-PCR in leaf discs of *Pisum sativum* on exposure to MD. The scans are shown in Top panel. Actin 2 was the internal control. The quantified values were given in Bottom panel (A to E); APX (A), GR (B), Cu/ZnSOD (C), FeSOD (D), MnSOD (E). The treatments and averages were all as in Fig. 4.1.

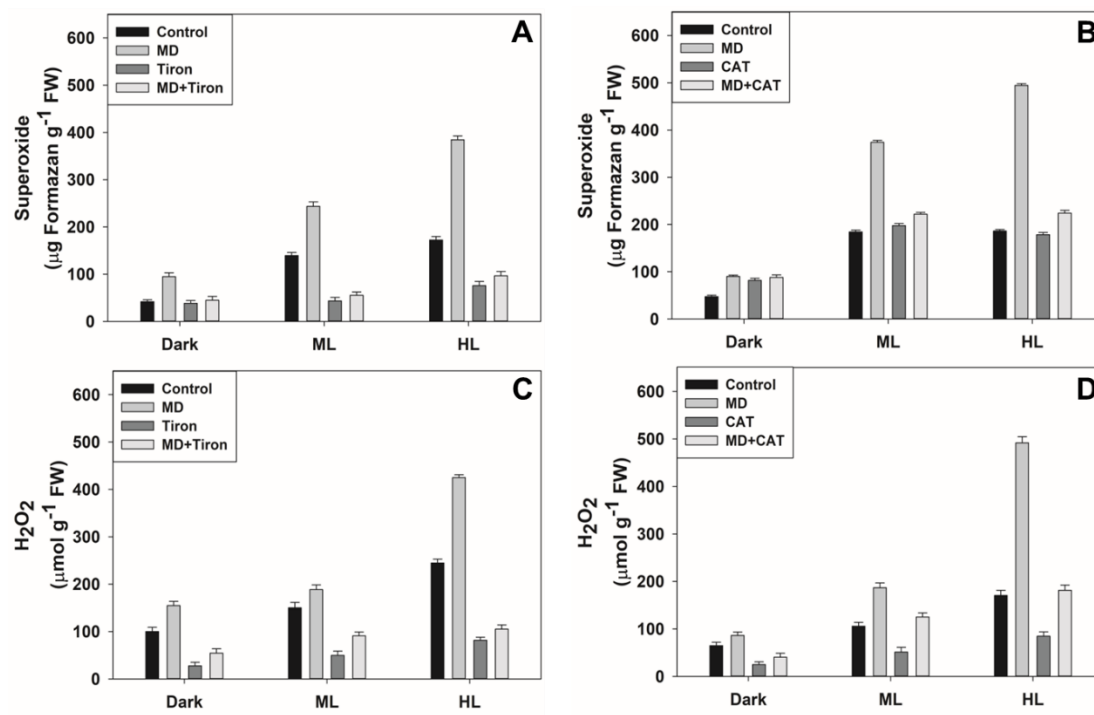


Figure 4.11. After being treated with or without MD, tiron (2.5 mM) or catalase (200 U) scavenged superoxide or H_2O_2 in pea leaf discs. The treatments and averages were all as in Fig. 4.1.

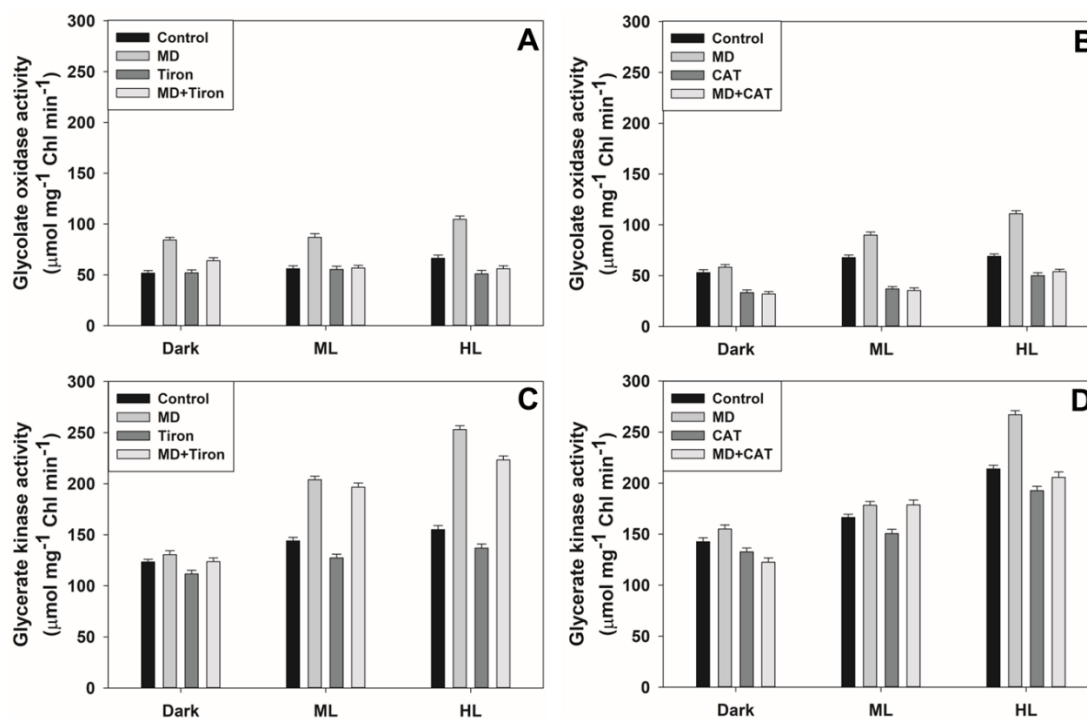


Figure 4.12. Photorespiratory enzymes; GO (A), and GLYK (B) were scavenged by tiron) or H_2O_2 by (catalase). The treatments and averages were all as in Fig. 4.1.

Discussion

Plants must adapt to varying light intensity and meet at the same time the challenges of biotic and abiotic stresses (Barczak-Brzyzek et al., 2017). High ROS concentrations triggered under stress, can harm seriously plant metabolism (Szymanska et al., 2017). Photo-oxidative stress was more harmful than oxidative stress i.e., in darkness (Aswani et al., 2019). Our findings confirmed that oxidative stress caused by MD was considerable and rather strong under HL. To minimize the stress effects, we employed a significantly lower dose (10 μ M). The marked photorespiratory enzyme modulation by HL was amplified further by MD treatment, is therefore not surprising.

Treatment with MD caused marked changes in ROS

Abiotic stress responses include the process of photorespiration, which can aid in adaptation to a variety of environmental factors (Voss et al., 2013; Timm et al., 2019; Leverne et al., 2021). Additionally, it was suggested that mitochondrial alternative oxidase and chloroplastic cyclic electron transport would complement the advantages of photorespiration (Sunil et al., 2019). Our findings highlight the signaling function of ROS in coordinating mitochondrial, chloroplastic, and peroxisomal responses. Under such a confluence of stimuli, plant adaptation is based on modulating ROS (Chaudhary et al., 2017; Cerny et al., 2018).

The levels of superoxide and H_2O_2 significantly increased upon MD treatment (Fig. 4.3), like previous reports (Obata et al., 2011; Lehmann et al., 2012; Aswani et al., 2019). ROS are crucial cues that help plants adapt to various stresses. When chlorophyll molecules are too excited and the

equilibrium between PSI and PSII reaction centres disrupted, a significant amount of superoxide was produced (Mignolet-Spruyt et al., 2016). MD is an active quinone that generates ROS by inducing oxidative stress, primarily in mitochondria (Schwarzlander et al., 2009). MD caused ROS generation in the mitochondria, which then diffuse to other compartments, according to experiments using redox-sensitive GFP (m-roGFP2) (Lehmann et al., 2009). The small but significant rise in ROS levels, even in darkness, suggested that MD can produce ROS even when plants are not illuminated. This is not unexpected, as Mor et al. (2014) found increased in ROS levels even in darkness in fluorescent (flu)-like mutants of *Arabidopsis thaliana* on treatment with Rose bengal.

Exposure to MD, a mitochondrial oxidant, altered photorespiratory enzymes in peroxisomes and chloroplasts

Under abiotic stress conditions, photorespiration was a crucial protective mechanism that shielded photosynthesis (Sunil et al., 2019). Under osmotic or HL stress, it was previously shown that photorespiratory enzymes like GO or CAT activity increased (Voss et al., 2013). Observations demonstrating the combined actions of enzymes spread across many subcellular compartments are, however, rare. Despite the predominant disruption in the mitochondria, MD treatments had an impact on photorespiratory enzymes in the peroxisomes and chloroplasts. For instance, MD-treated leaf samples from HL showed increased levels of the peroxisomal (GO/CAT) and chloroplastic enzymes (GK/PGLP) (Fig. 4.4). The activities of GO and CAT were quite higher in HL or drought conditions in several plants (Silva et al., 2015; Yuan et al., 2016; Neto et al., 2017) Similarly, after

exposure to paraquat or heavy metals, chloroplastic GK and PGLP activities were increased (Ananieva et al., 2002; Hristova and Popova, 2002; D'Alessandro et al., 2013).

The metabolism of short-term ambient CO₂ adaptation was examined using mutant *Arabidopsis* plants. The increase of photorespiration in our studies was evident in both the transcriptional and translational levels as well as the enzyme activity (Figs. 4.4 to 4.6). Aconitase activity decreased when treated with MD in light (Fig. 4.7B), similar to previous reports in *Arabidopsis* (Lehmann et al., 2009; Baxter et al., 2007). Pretreatment with MD resulted in oxidative stress that degraded mitochondrial proteins inhibited the metabolism of TCA cycle enzymes, and altered the NADPH pools (Obata et al., 2011; Sweetlove et al., 2002). As per our observations, the use of MD might be an interesting and effective tool to modify the cellular redox and photorespiratory components.

Thus, the photorespiratory components in peroxisomes and chloroplasts responded to the redox disturbance in mitochondria with MD. These disturbances could be traced to changes in ROS levels. When scavengers were in the incubation media, the increase in superoxide (tiron) or H₂O₂ (catalase) and the modulation of photorespiratory enzymes by MD were reduced (Figs. 4.11 and 4.12). The increase of both superoxide and H₂O₂ in leaves overall was clearly the cause of the photorespiratory modulations.

Increase in non-enzymatic and enzymatic antioxidants

Antioxidants were always modulated under stress conditions. In our studies also, ascorbate and glutathione content increased particularly in light and treated with MD (Fig. 4.8). ASC content was upto 3-fold under salinity stress

in *Nitraria tangutorum* (Gao et al., 2022). The increase in the ascorbate and glutathione content on exposure to MD was around 1.2-fold, particularly under HL. Under MV exposure, ascorbate and glutathione content was increased in potato plants (Hemavathi et al., 2011). Both ascorbate and glutathione content increased in pea leaf discs upon MD treatment (Aswani et al., 2019). The ratio of reduced to total ASC or GSH was reduced, particularly under HL treated with MD, which shows that accumulation of ROS induced oxidative stress lead to oxidative damage and that the reduced environment was changing to an oxidized state. Previous reports also showed a reduction in the ratio of reduced to total ASC or GSH in pea leaf discs (Aswani et al., 2019).

Under oxidative and photo-oxidative stress, antioxidant enzymes and transcripts (APX, GR, and SOD) were enhanced (Figs. 4.9 and 4.10). The maximum increase in SOD activity could be to provide a first line of defense against different forms of ROS within the plant cell. Isoforms of SOD's were found in different compartments, such as MnSOD in mitochondria, FeSOD in chloroplasts, and Cu/ZnSOD in cytosol, peroxisomes, and plastids (Huseynova et al., 2014). In the case of *Chlamydomonas reinhartii*, the increase in antioxidant enzyme activities (SOD, CAT, and APX) were noticed upon MD treatment (Sirisha et al., 2014). Similarly, the increase in the antioxidant enzyme activities were observed in *Phanerochaete chrysosporium* on treatment with MD (Tongul and Tarhan, 2014). These results were expected, as the increase in the enzymatic and non-enzymatic antioxidants shielded the plant from damage caused by ROS.

Conclusions from this chapter

1. Oxidative stress by MD triggered the accumulation of H_2O_2 and superoxide in leaf discs. Maximum increase in ROS (H_2O_2 and superoxide) levels were in HL treated with MD samples.
2. A noticeable increase in photorespiratory metabolism (activity, gene expression, and enzyme protein content) suggested that MD-mediated mitochondrial ROS generation can activate photorespiratory components in other subcellular compartments, namely peroxisomes and mitochondria.
3. Ascorbate and glutathione contents rose while the redox ratio dropped, showing that MD induced oxidative stress and creation an oxidising environment after treatment. Activity and expression of antioxidant enzymes increased as a part of the plant antioxidant defense system.
4. Suppression of ROS and photorespiratory enzymes by scavengers of specific ROS confirmed the role of both H_2O_2 and superoxide in the upregulation of photorespiration.

Chapter 5

Modulation of Photorespiratory Enzymes and Photosynthetic Characteristics by Nitric oxide in Leaves of Pea (*Pisum sativum*)

Chapter 5

MODULATION OF PHOTORESPIRATORY ENZYMES AND PHOTOSYNTHETIC CHARACTERISTICS BY NITRIC OXIDE IN LEAVES OF PEA (*PISUM SATIVUM*)

Introduction

Prolonged stress, such as high light or drought, harmed the photosynthetic system, particularly PSII, leading to photoinhibition (Wada et al., 2019; Roeber et al., 2021; Shi et al., 2022). Photosynthesis was protected from photoinhibition by several components of plant metabolism, including photorespiration and mitochondrial respiration (Talla et al., 2011; Sunil et al., 2019; Lima-Melo et al., 2019). Photorespiration optimizes photosynthesis and protects against photoinhibition, by keeping ROS levels low and promoting PSI activity (Wada et al., 2018). The crucial nature of photorespiration as a protective mechanism was emphasized by photorespiratory mutants (Wingler et al., 2000; Voss et al., 2013; Saji et al., 2017; Timm, 2020).

Along with ROS, abiotic and biotic stress also induce the production of reactive nitrogen species (RNS), mainly nitric oxide (NO). These species are produced in all the cellular compartments. However, their production is amplified when plants encounter different abiotic stresses. These ROS and RNS function as signaling molecules, when present in low concentrations, but when present in excessive concentrations, they harm cellular components (Mandal et al., 2022; Zhou et al., 2022). The free radical NO can affect a variety of physiological processes in plants (Verma et al., 2020; Lopes-Oliveira et al., 2021). There are conflicting statements on

whether NO can protect plant cells from stress or harm them (Sami et al., 2018; Bhardwaj et al., 2021; Wani et al., 2021).

It is known that ROS has the capacity to act as a mobile signal and modulate subcellular compartments (Mignolet-Spruyt et al., 2016; Cerny et al., 2018), but the role of NO, is not clear. Under varied stress situations, the fate of plant cells is determined by crosstalk between ROS and NO (Rodriguez-Serrano et al., 2009). The reports on the modulation by NO of photosynthesis, particularly PSI or PSII, are unclear. Studies using chlorophyll (Chl) fluorescence demonstrated that exposure to NO resulted in either an increase or decrease in PSII-related responses (Wodala et al., 2010; Wodala and Horvath, 2008). Few reports suggested protection due to the NO of PSII components under abiotic stress, such as drought or salinity (Sahay et al., 2020; Hajihashemi et al., 2021). This discrepancy in the studies might be brought on by the use of several NO-donors (Wodala et al., 2008; Ordög et al., 2013) or various monitoring tools for Chl fluorescence characteristics.

The effects of S-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP), are presented and discussed in this chapter. On exposure to two NO-donors, a significant rise in NO and ROS levels (superoxide and H₂O₂) was seen. The presence of oxidative/nitrosative stress was confirmed by the rise in antioxidant enzymes, nitosothiols, tyrosine nitrated proteins, and suppression of aconitase. There was modulation in the activities, protein, and transcripts levels of typical photorespiratory enzymes. Further, the photosynthesis and the pigments were decreased. Similarly, PSII and PSI were affected by NO-donors.

Results

Increase in NO content on exposure to NO-donors

With rising SNP and GSNO concentrations, NO accumulated in pea leaf discs. We selected 2.5 mM SNP, 1 mM GSNO, and a 3 h exposure duration for a subsequent experiment based on the kinetics of NO production in the dark, ML, and HL (Fig. 5.1). The highest release of NO occurred, with 2.5 mM SNP and 1 mM GSNO. There was not much increase in NO content on exposure to SNP in darkness. On treatment with GSNO, a 7~fold increase in NO content was noticed even in dark conditions. The increase in NO content by SNP in ML and HL ranged from 1.5 to 13-fold over control (without NO-donor). Similarly, the extent of increase in NO by GSNO ranged from 7 to 11-fold in dark, ML, and HL conditions. The NO-contents were markedly dampened in the presence of cPTIO, indicating that most of the detected NO was true (Fig. 5.2).

Changes in levels of ROS

When exposed to SNP and GSNO under ML and HL conditions, there was a noticeable rise in the ROS (both superoxide and H₂O₂) content. Superoxide concentration was elevated, especially under HL, by up to 3.3-fold by SNP and 2.3-fold by GSNO (Fig. 5.3). The H₂O₂ content in leaf discs of pea was monitored by DAB staining, which led to the precipitation of brown color in the leaf discs. When quantified, H₂O₂ levels increased up to 2.3-fold by SNP and 1.6-fold by GSNO over control (Fig. 5.3). cPTIO alone marginally increases the ROS but, when used in combination with SNP and GSNO, decreases the ROS content (both superoxide and H₂O₂) (Fig. 5.4).

Modulation of photorespiratory enzyme components

Two peroxisomal enzymes, GO and HPR, showed enhanced activity when subjected to SNP and HL. A significant increase in GO while a marginal increase in HPR was noticed with SNP, particularly under HL. There were not much changes were observed in GO, CAT, HPR and PGLP on exposure to SNP and GSNO in the dark. Exposure to GSNO decreased the GO and HPR activity in ML and HL conditions. The two chloroplastic enzymes, GK and PGLP, on the other hand, increased in response to exposure to SNP and GSNO, especially under HL conditions, while being unaffected by ML (Fig. 5.5).

In view of the significant alteration of photorespiratory enzymes, we checked the protein levels and transcripts of corresponding genes. SNP treatment of pea leaf discs resulted in increased amounts of GO, CAT, and HPR proteins and transcripts, especially under high light, while GSNO decreased GO, CAT, and HPR proteins and transcripts. The transcript levels of PGLP and GK increased on exposure to SNP and GSNO, while the protein levels of these enzymes were not measured due to the unavailability of antibodies (Figs. 5.6 to 5.9).

Aconitase was reduced by SNP in ML and HL conditions while not affected in the dark whereas it was decreased in dark, ML, and HL GSNO-treated samples compared to the control (Fig. 5.10A). The FBPase activity remained unaffected on exposure to NO-donors in dark, ML, and HL (Fig. 5.10B).

Changes in antioxidant enzyme components

The activities of CAT, APX, and SOD were all elevated in samples that had been exposed to SNP. The catalase activity increased only in HL-treated with SNP, while in dark and ML, it remained unaffected. The APX and SOD activities increased in ML and HL treated with SNP, while not much changes were observed in the dark. The catalase activity on exposure to GSNO decreased in ML and HL while remain unaffected in the dark. Similarly, APX and SOD activities were enhanced in dark, ML, and HL on exposure to GSNO (Fig. 5.11).

We then checked the protein levels and gene transcripts of corresponding genes under nitrosative stress conditions. The gel pictures demonstrate the protein and transcript levels after treatment with NO-donors. The protein levels of CAT and SOD increased while the transcripts levels of CAT, APX, and SOD, particularly CuZn SOD, were upregulated under SNP-treated HL. The protein levels of CAT decreased while SOD, particularly MnSOD, increased on exposure to GSNO. Similarly, the transcripts levels of CAT were downregulated while that of APX and SOD (particularly, MnSOD) upregulated marginally under GSNO (Figs. 5.12 to 5.14).

Increase in nitrosothiols and tyrosine nitrated proteins

Nitrosothiols content was estimated using the Saville-Griess assay. Nitrosothiol (SNO) contents increased in ML and HL on exposure to SNP, while not much changes were noticed in the dark. The SNO content was increased 2 to 5-fold when treated with SNP under ML and HL. Similarly, the

extent of increase in SNO by GSNO in dark, ML and HL ranged from 2 to 6-fold (Fig. 5.15).

The Coomassie blue stained protein pattern and the corresponding tyrosine nitration proteins were examined using an antibody against nitrotyrosine on exposure to NO-donors (SNP and GSNO). Tyrosine nitrated proteins intensity increased most dramatically in the case of GSNO in the dark, ML, and HL ranged from 1.5 to 2.6-fold. The increase in the tyrosine nitrated proteins in ML and HL was almost 1.2-fold in ML and HL SNP-treated samples as compared to the control. In control dark and SNP-treated dark, the expression of tyrosine nitrated proteins was less (Fig. 5.16).

NO inhibited both photosynthesis and respiration

Photosynthesis and respiration were severely inhibited in ML and HL conditions on exposure to SNP, while the inhibition of photosynthesis and respiration was moderate in ML and HL on treatment with GSNO (Fig. 5.17). There was not much change was noticed in the dark on treatment with SNP or GSNO. Further, the inhibitory effect of SNP on photosynthesis or respiration was stronger than that by GSNO.

Decrease in chloroplast pigments

The levels of total chlorophylls and carotenoids decreased significantly after preincubation with SNP or GSNO (Fig. 5.18). The decrease of total chlorophyll and carotenoids was more pronounced with SNP under ML and HL conditions while on treatment with GSNO, these decreased only under HL treatment and were not much affected in dark and ML. The decrease in total chlorophyll with SNP or GSNO treatment was up to 19 % over control (no

SNP or GSNO). Similarly total carotenoids decreased up to 26 % under HL compared to their respective control.

Effect of NO on photochemical components, as indicated by Chl fluorescence parameters

Chl fluorescence monitored at 800, and 1000 $\mu\text{E m}^{-2} \text{s}^{-1}$ were used for studying PSII and PSI components, respectively. Most of the PS-II related parameters, namely: the maximal PSII quantum yield (F_v/F_m), the total electron transport rate [ETR(II)], yield of photosystem II [$Y(II)$], Non-photochemical quenching (NPQ), quantum yield of regulated energy dissipation [$Y(NPQ)$], and the quantum yield of NPQ-regulated energy dissipation [$Y(NPQ)$], were all decreased in dark, ML and HL compared to their respective control on treatment with SNP and GSNO. In contrast, the quantum yield of non-regulated energy dissipation $Y(NO)$ increased. The SNP or GSNO-induced decrease in PSII related activities was maximum under HL-treatment. The decrease in SNP treated samples under HL was up to 85 %, and with GSNO up to 33 %. Similarly, the $Y(NO)$ values increased by 114 or 156 % with SNP or GSNO, again under HL (Fig. 5.19).

Among the PSI-related parameters that were monitored, the decrease in P_m (~75 %) was maximum with SNP treatment in HL. The ETR(I) decreased by 26 % and 11 % with SNP and GSNO, respectively, under HL (Fig. 4b), while $Y(ND)$ value decreased by 77 % with SNP. However, the $Y(NA)$ values increased with SNP by almost 5-fold. The trend in the pattern of $Y(NA)$ was opposite to that of $Y(ND)$ with respect to SNP or GSNO in dark, ML, and HL conditions (Fig. 5.20).

Discussion starts on page no. 97.

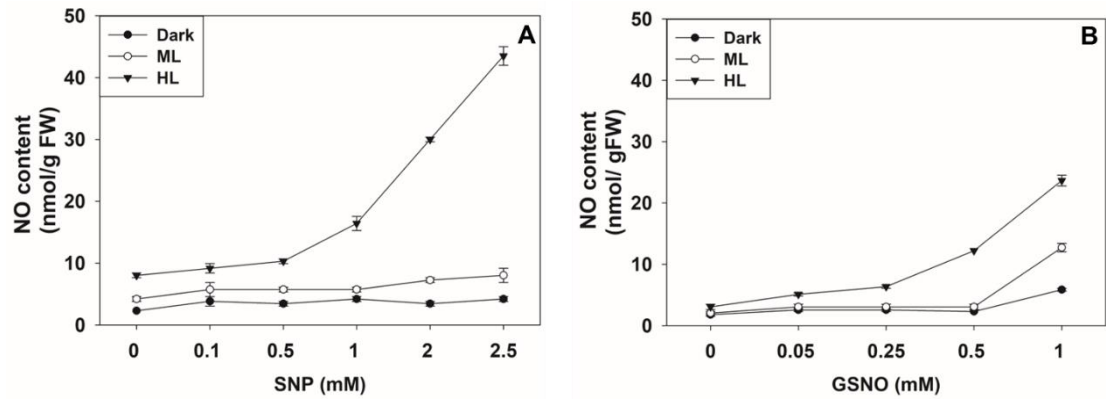


Figure 5.1. Accumulation of NO in leaf discs of pea with varying concentrations of SNP (0 to 2.5 mM), and GSNO (0 to 1 mM) in the dark, ML (300 $\mu\text{E m}^{-2} \text{s}^{-1}$) and HL, (1200 $\mu\text{E m}^{-2} \text{s}^{-1}$). Data represent averages ($\pm$ SE) from three experiments done on different days.

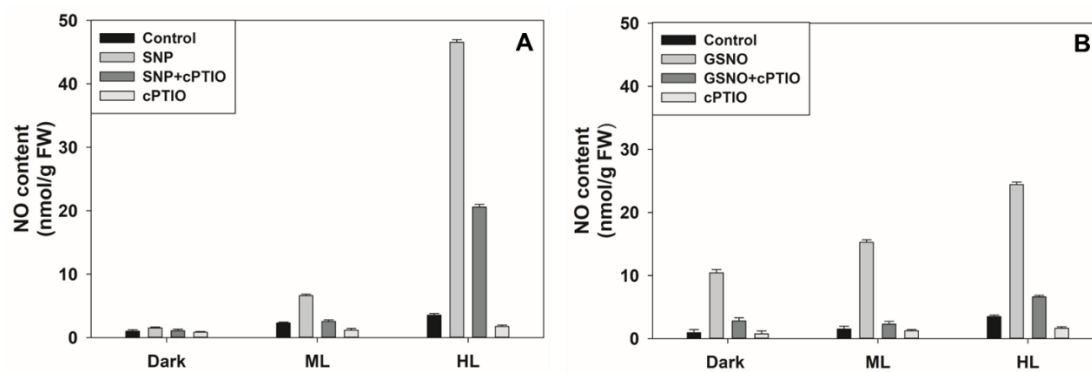


Fig. 5.2. Levels of NO in leaves of pea exposed to two NO-donors-SNP (2.5 mM) or GSNO (1 mM). The leaves were exposed to 3 h. The levels were checked in the absence or presence of cPTIO (1 mM), a scavenger of NO. The treatments and averages were all as in Fig. 5.1.

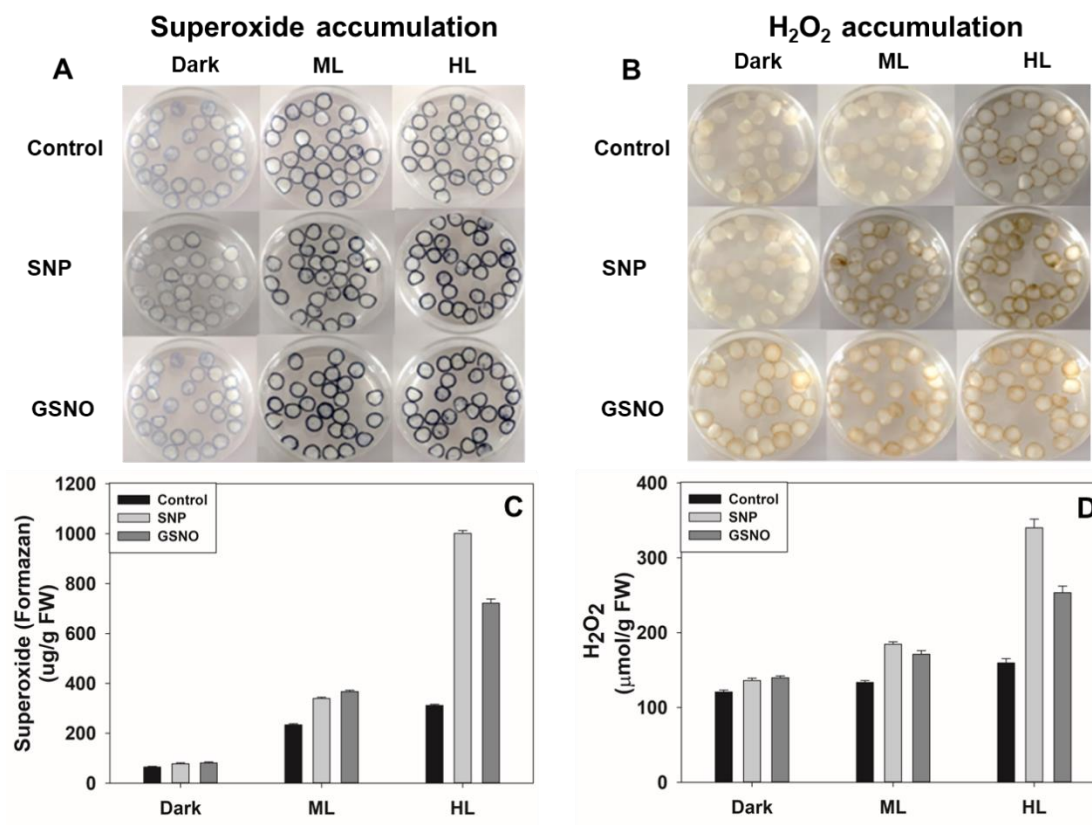


Figure 5.3. Superoxide and H₂O₂ levels, visualized by DAB and NBT staining in leaves of *Pisum sativum* upon treatment with SNP and GSNO. Blue color represented the superoxide upon treatment (A), while the brown color showed the pattern of H₂O₂ accumulated (B). Superoxide and H₂O₂ quantified from NBT and DAB-stained leaves (C and D). The treatments and averages were all as in Fig. 5.1.

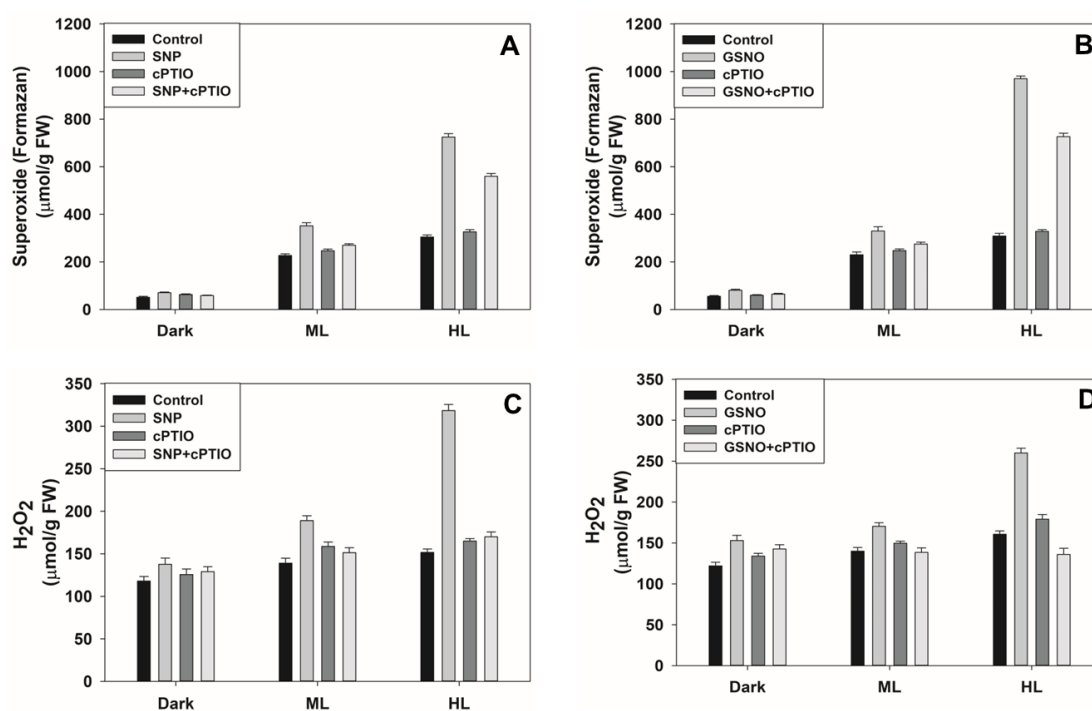


Figure 5.4. Superoxide and H_2O_2 quantified from NBT and DAB-stained leaves in leaf discs treated with GSNO (1 mM) and cPTIO (1 mM) respectively. The treatments and averages were all as in Fig. 5.1.

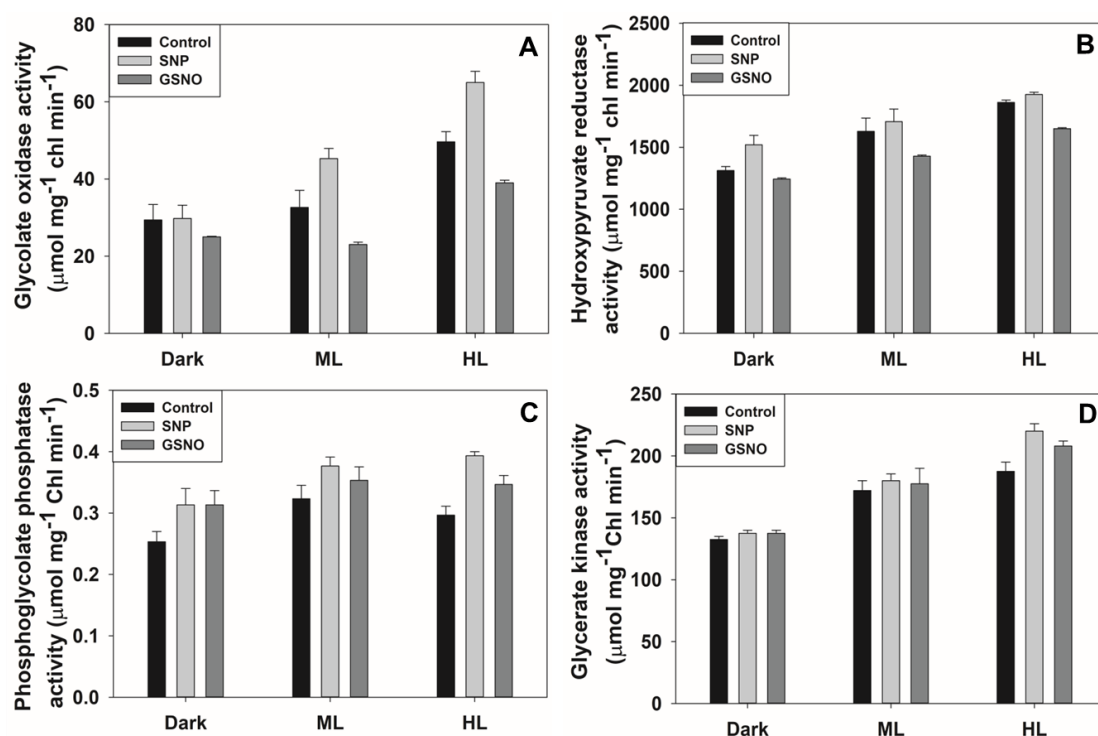


Figure 5.5. Photorespiratory enzyme activities, GO (A), HPR (B), PGLP (C), and GLYK (D) were measured. The treatments and averages were all as in Fig. 5.1.

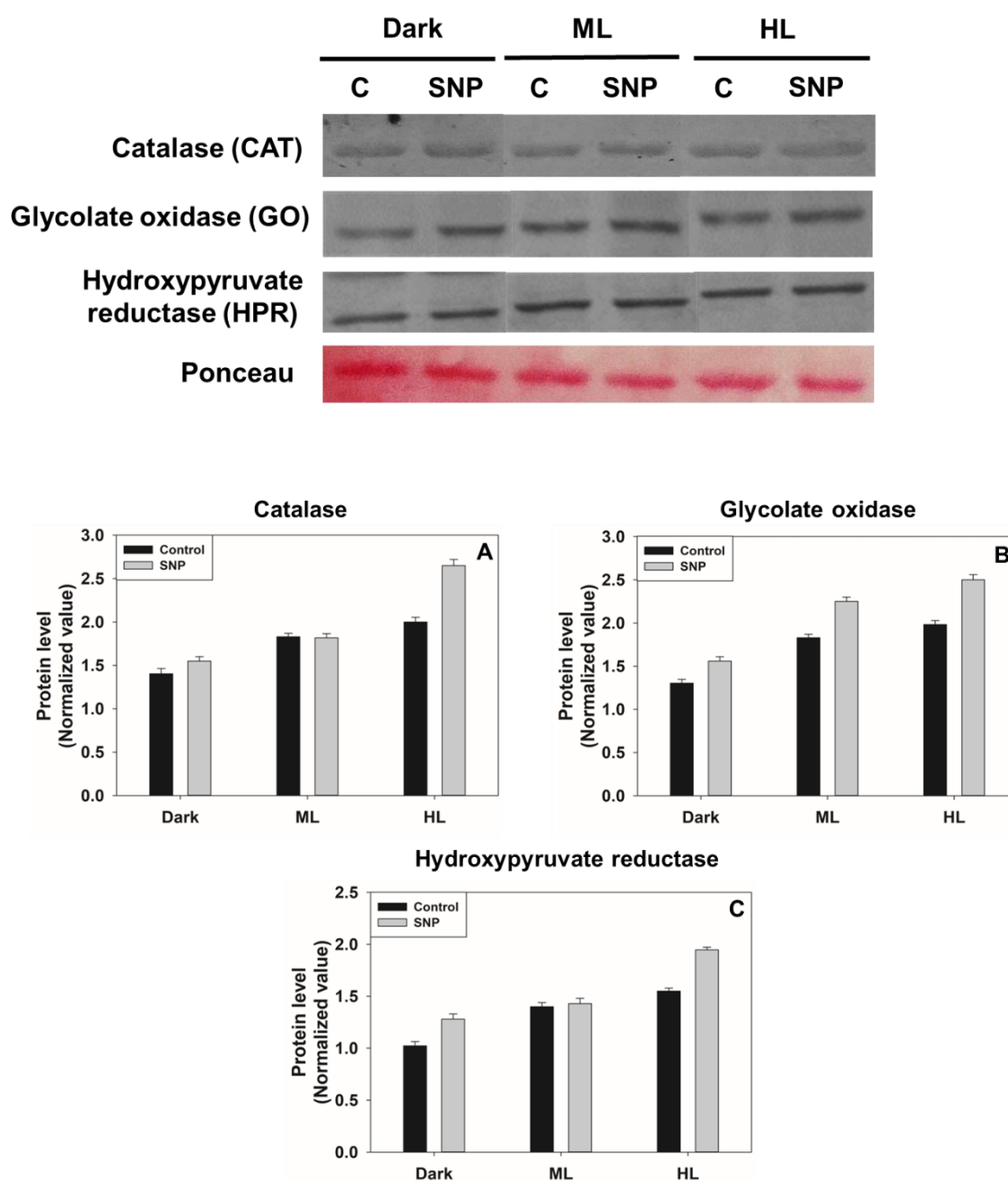


Figure 5.6. Protein levels of CAT, GO, and HPR in leaves of *Pisum sativum* upon exposure to SNP (Top panel). Based on image J, the ratios of CAT, GO, and HPR protein to Rubisco large subunit were computed (Bottom panel, A to C). The treatments and averages were all as in Fig. 5.1.

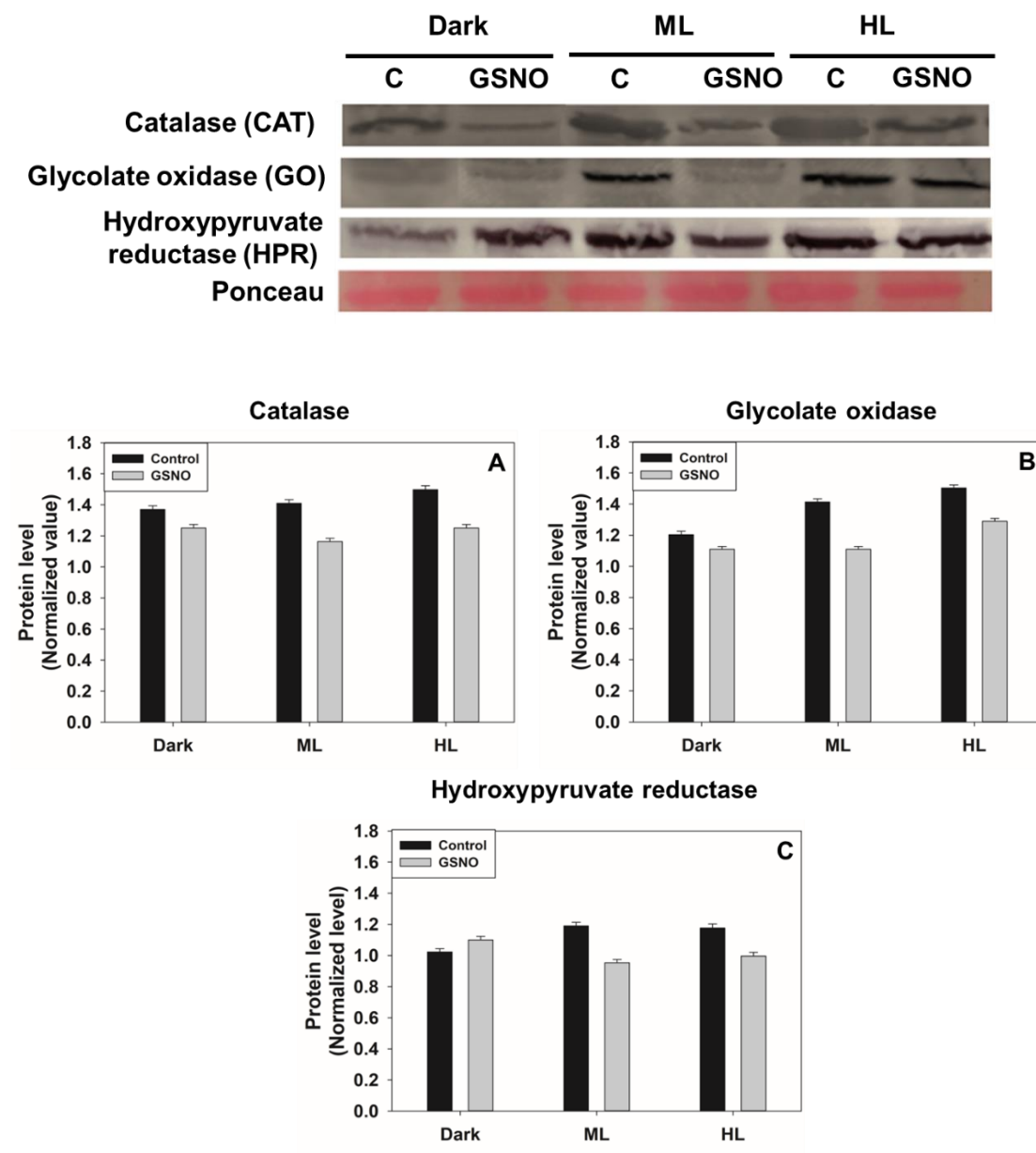


Figure 5.7. The protein levels of CAT, GO, and HPR were measured in leaves of *Pisum sativum* upon exposure to GSNO (Top panel). Based on image J, the ratios of CAT, GO, and HPR protein to Rubisco large subunit were computed (Bottom panel, A to C). The treatments and averages were all as in Fig. 5.1.

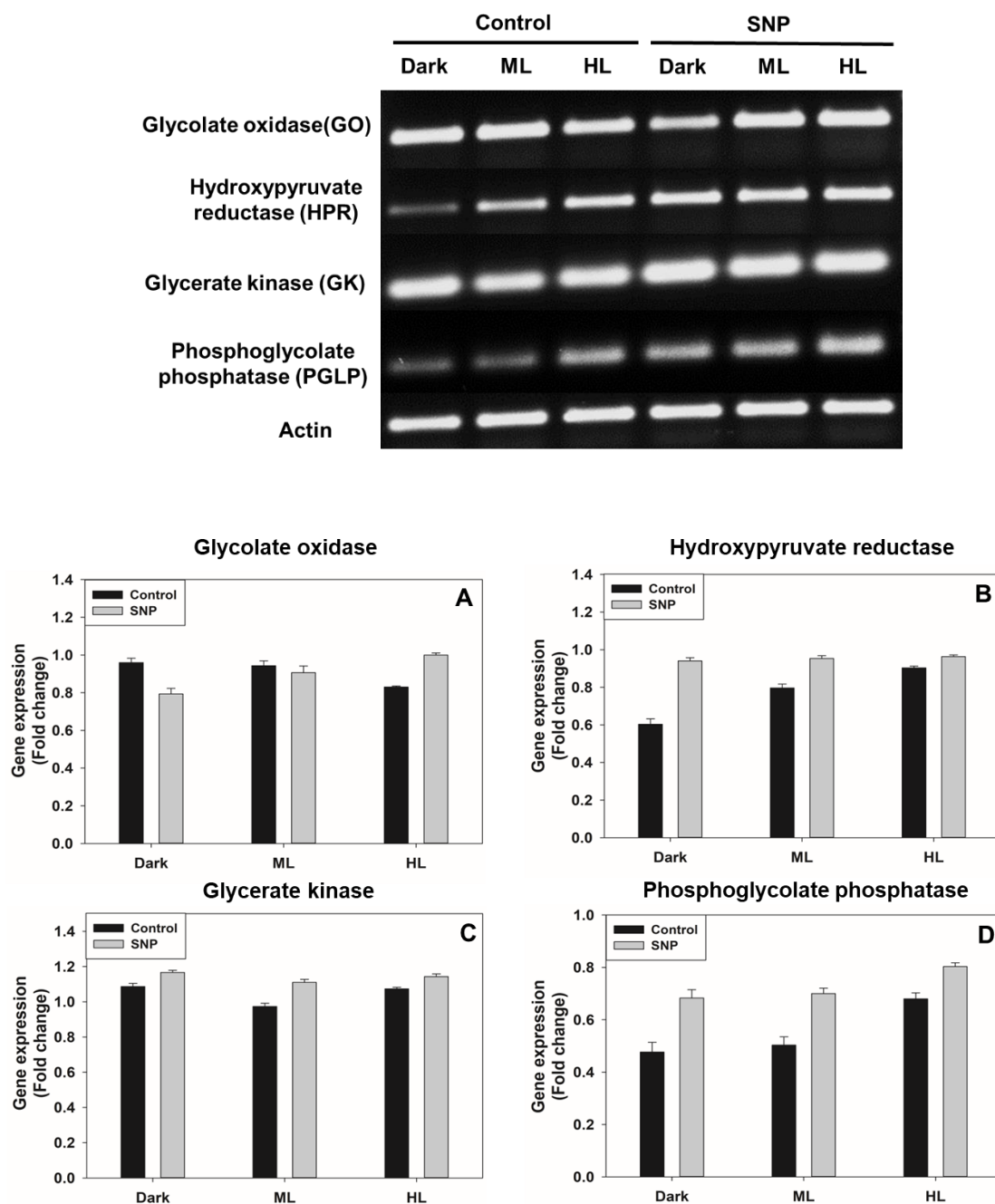


Figure 5.8. The transcripts of selected photorespiratory metabolism by semi-quantitative RT-PCR in *Pisum sativum* in leaf discs on exposure to SNP. The scans are shown in Top panel. Actin 2 was the internal control. The quantified values were given in Bottom panel (A to D); GO (A), HPR (B), GLYK (C), and PGLP (D). The treatments and averages were all as in Fig. 5.1.

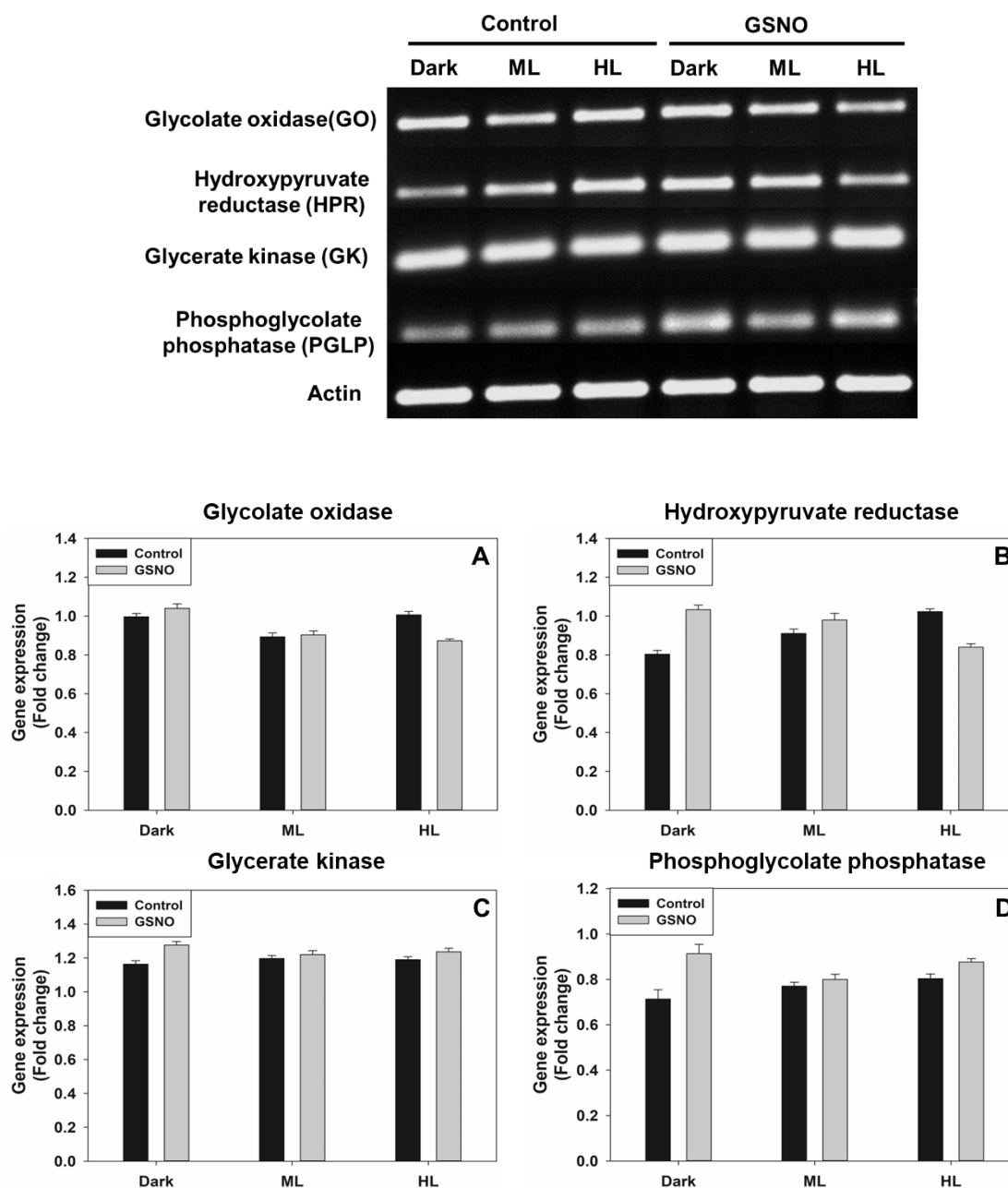


Figure 5.9. The transcripts of selected photorespiratory metabolism by semi-quantitative RT-PCR in leaf discs of *Pisum sativum* on exposure to GSNO. The scans are shown in Top panel. Actin 2 was the internal control. The quantified values were given in Bottom panel (A to D); GO (A), HPR (B), GLYK (C), and PGLP (D). The treatments and averages were all as in Fig. 5.1.

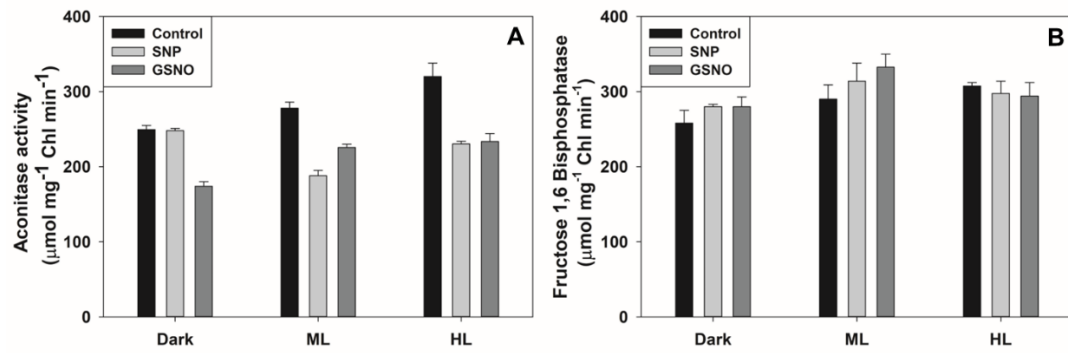


Figure 5.10. The activities of peroxisomal mitochondrial aconitase (A) and chloroplast FBPase (B) in GSNO treated samples. The treatments and averages were all as in Fig. 5.1.

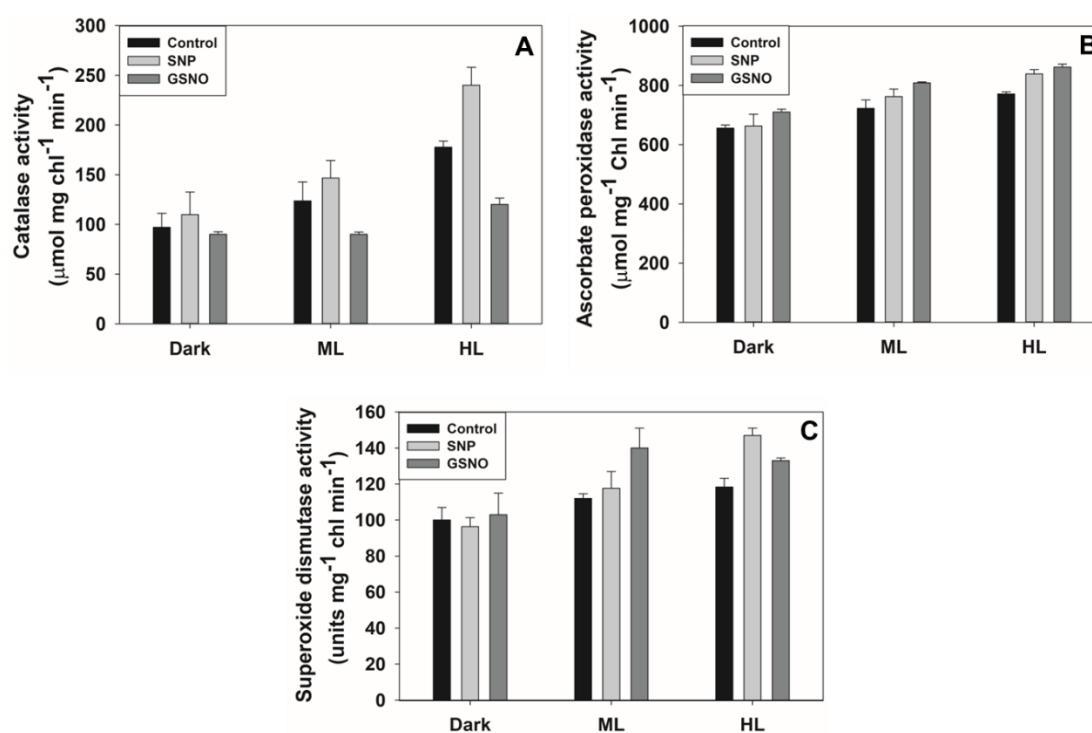


Figure 5.11. On exposure to SNP and GSNO, the antioxidant enzyme activities of CAT (A), APX (B), and SOD (C) were determined. The treatments and averages were all as in Fig. 5.1.

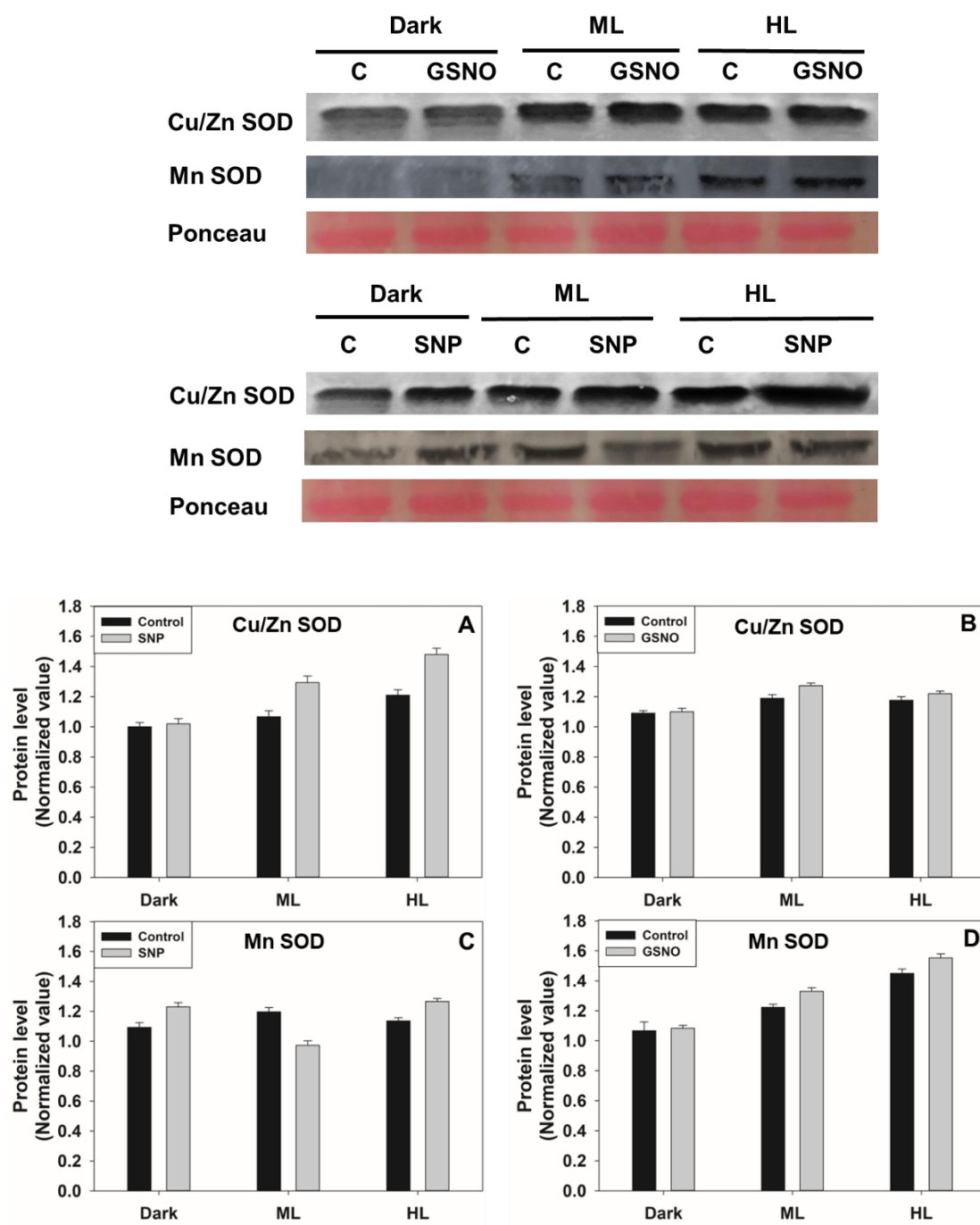


Figure 5.12. The protein levels of SOD (Cu/ZnSOD and MnSOD) were measured in leaf discs of *Pisum sativum* upon exposure to SNP and GSNO (Top panel). Based on image J, the ratios of Cu/ZnSOD and MnSOD protein to Rubisco large subunit were computed (Bottom panel, A to D). The treatments and averages were all as in Fig. 5.1.

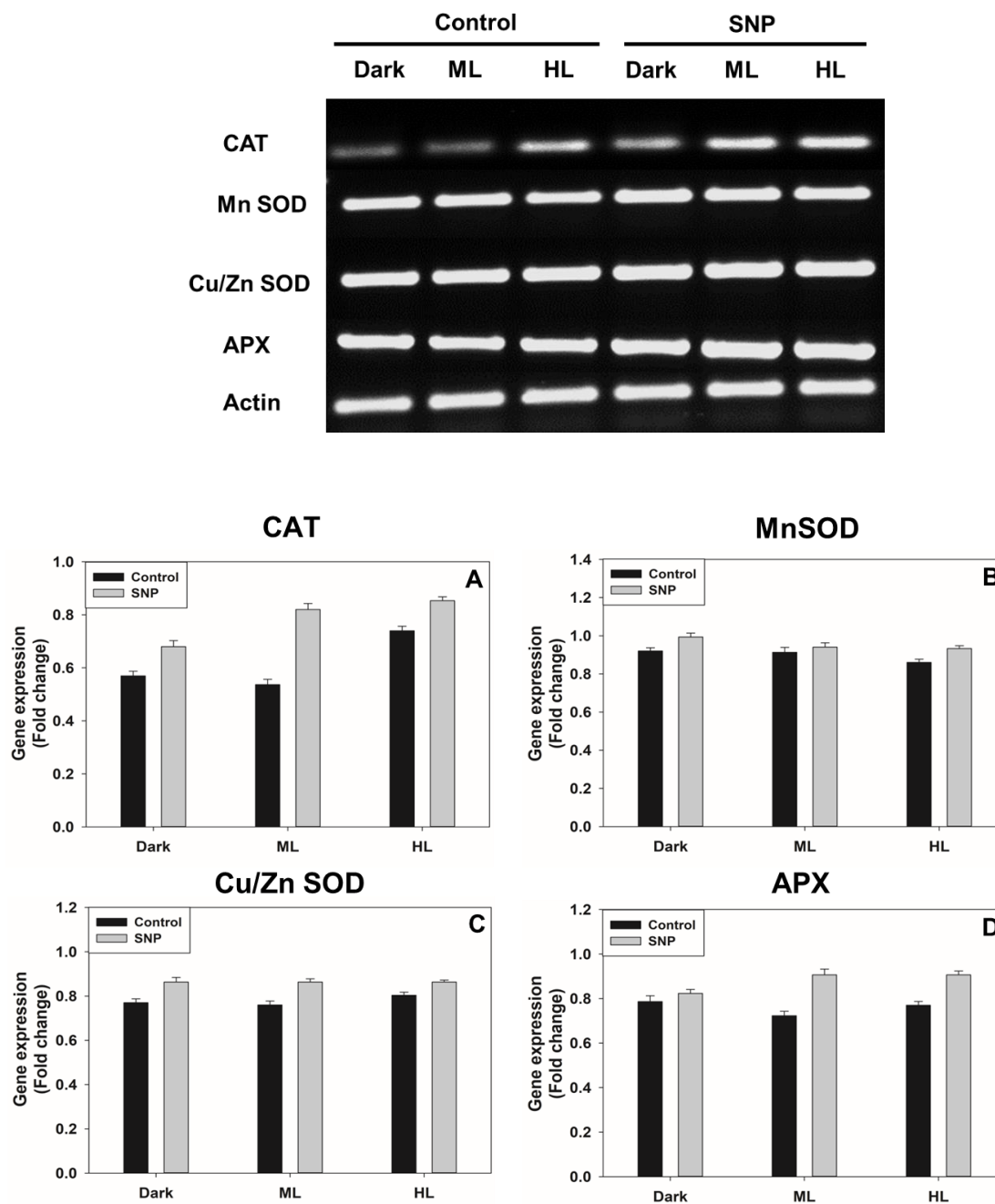


Figure 5.13. The transcripts of selected antioxidant enzymes by semi-quantitative RT-PCR in leaf discs of *Pisum sativum* on exposure to SNP. The scans are shown in Top panel. Actin 2 was the internal control. The quantified values were given Bottom panel (A to D); CAT (A), MnSOD (B), Cu/ZnSOD (C), and APX (D). The treatments and averages were all as in Fig. 5.1.

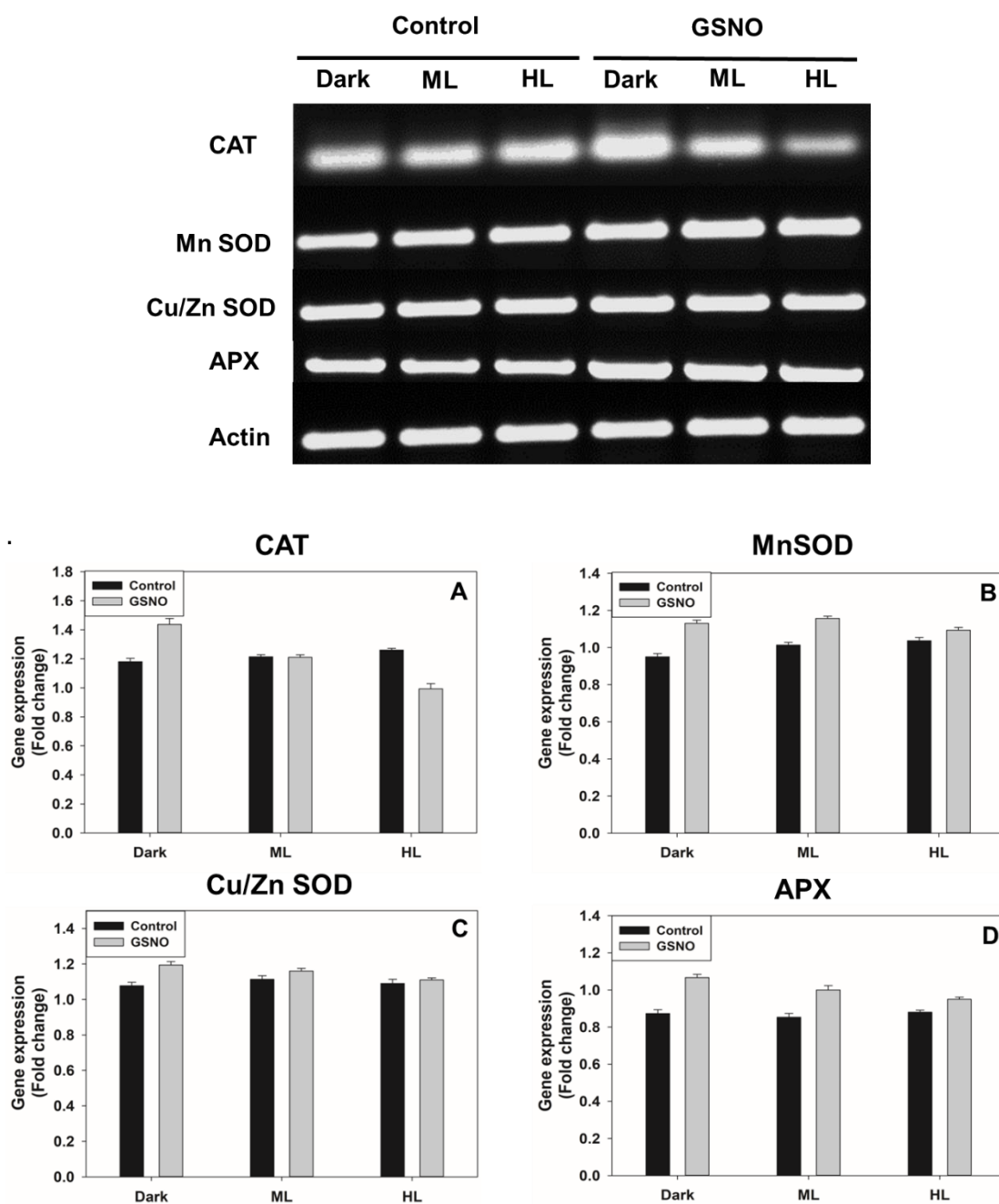


Figure 5.14. The transcripts of selected antioxidant enzymes by semi-quantitative RT-PCR in leaf discs of *Pisum sativum* on exposure to GSNO. The scans are shown in Top panel. Actin 2 was the internal control. The quantified values were given in Bottom panel (A to D); CAT (A), MnSOD (B), Cu/ZnSOD (C), and APX (D). The treatments and averages were all as in Fig. 5.1.

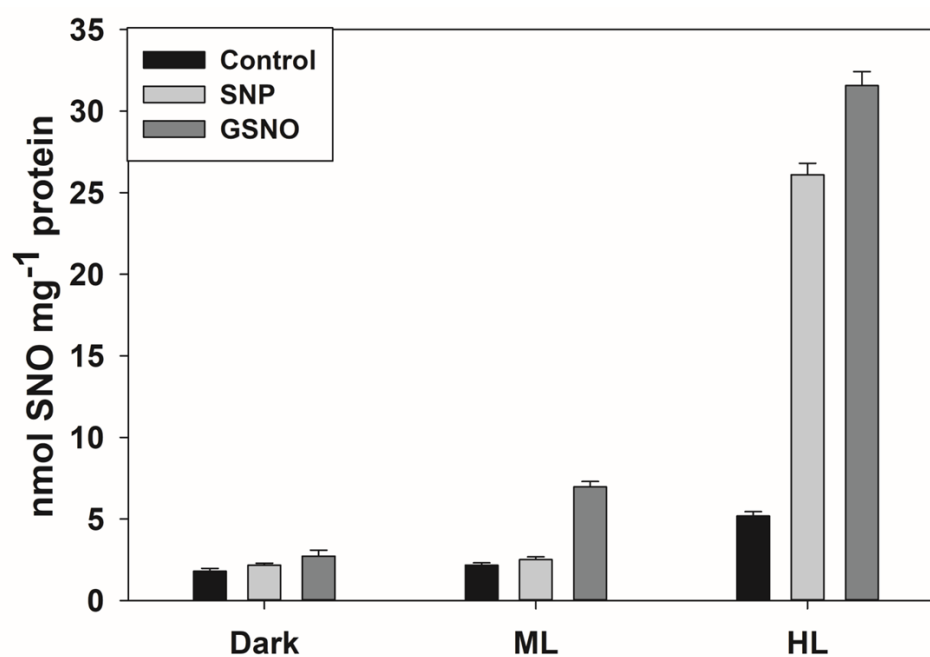


Figure 5.15. The levels of nitrosothiol on treatment with SNP and GSNO. The treatments and averages were all as in Fig. 5.1.

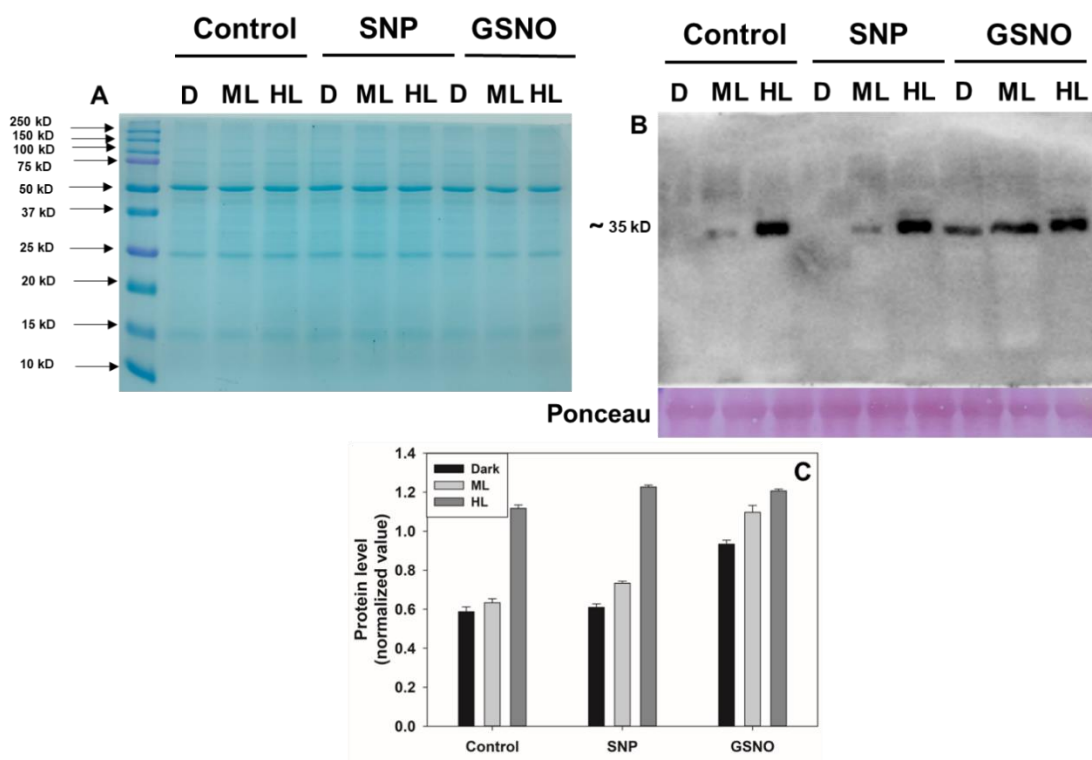


Figure 5.16. Proteins were separated by SDS-PAGE (A). The protein levels of tyrosine nitrated proteins on treatment with SNP and GSNO (B). Based on image J, the ratios of tyrosine nitrated protein to Rubisco large subunit were computed (C). The treatments and averages were all as in Fig. 5.1.

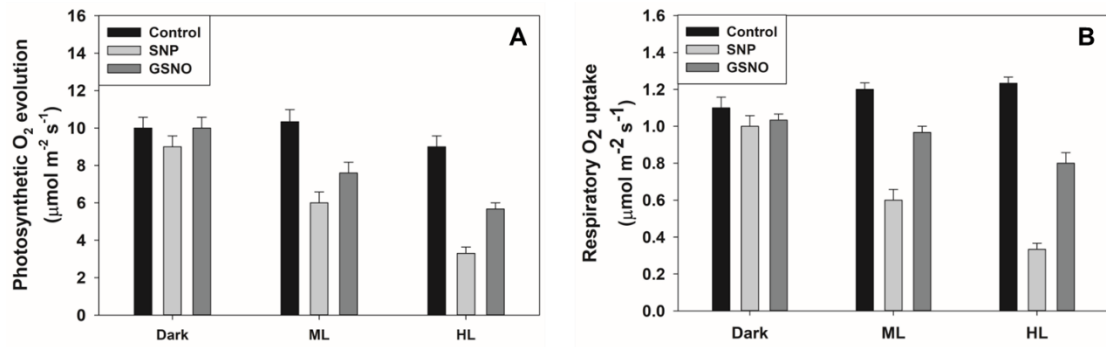


Figure 5.17. Photosynthetic O₂ evolution (A) and respiratory O₂ uptake (B) after exposure to SNP (2.5 mM) or GSNO. The treatments and averages were all as in Fig. 5.1.

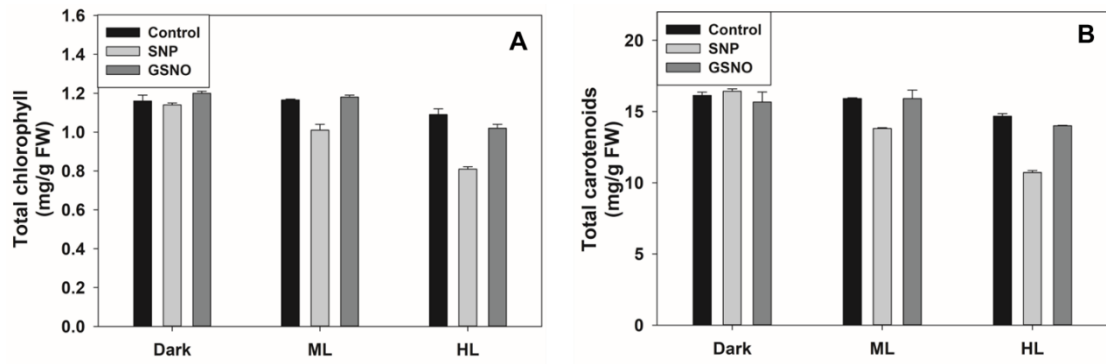


Figure 5.18. Chlorophyll (A) and carotenoid (B) contents of leaf discs in *Pisum sativum* on exposure to SNP or GSNO. The treatments and averages were all as in Fig. 5.1.

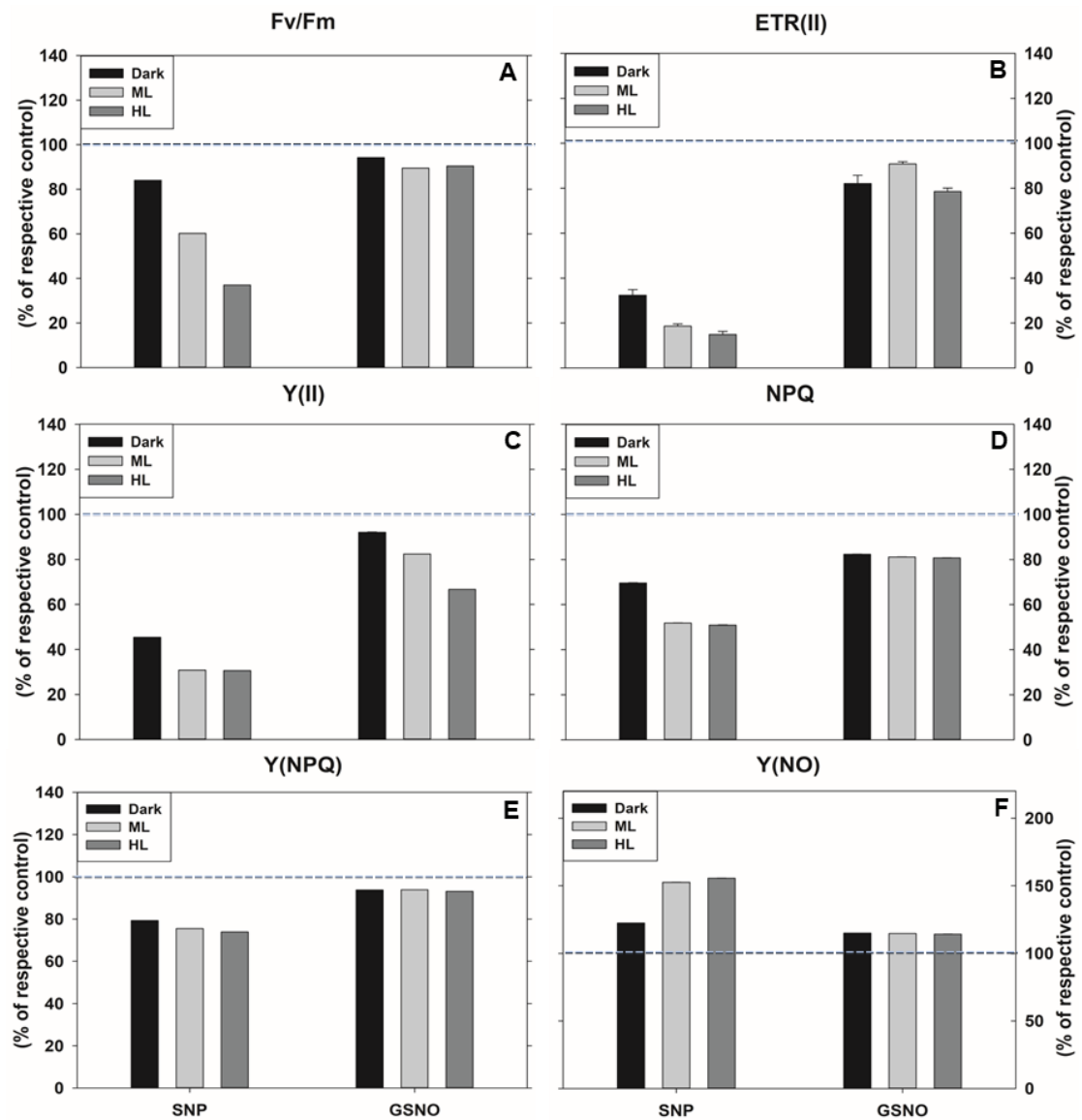


Figure 5.19. Chl fluorescence based PSII parameters in leaves of *Pisum sativum* after exposure to SNP or GSNO. These were measured at 800 PAR ($\mu\text{E m}^{-2} \text{s}^{-1}$). The treatments and averages were all as in Fig. 5.1.

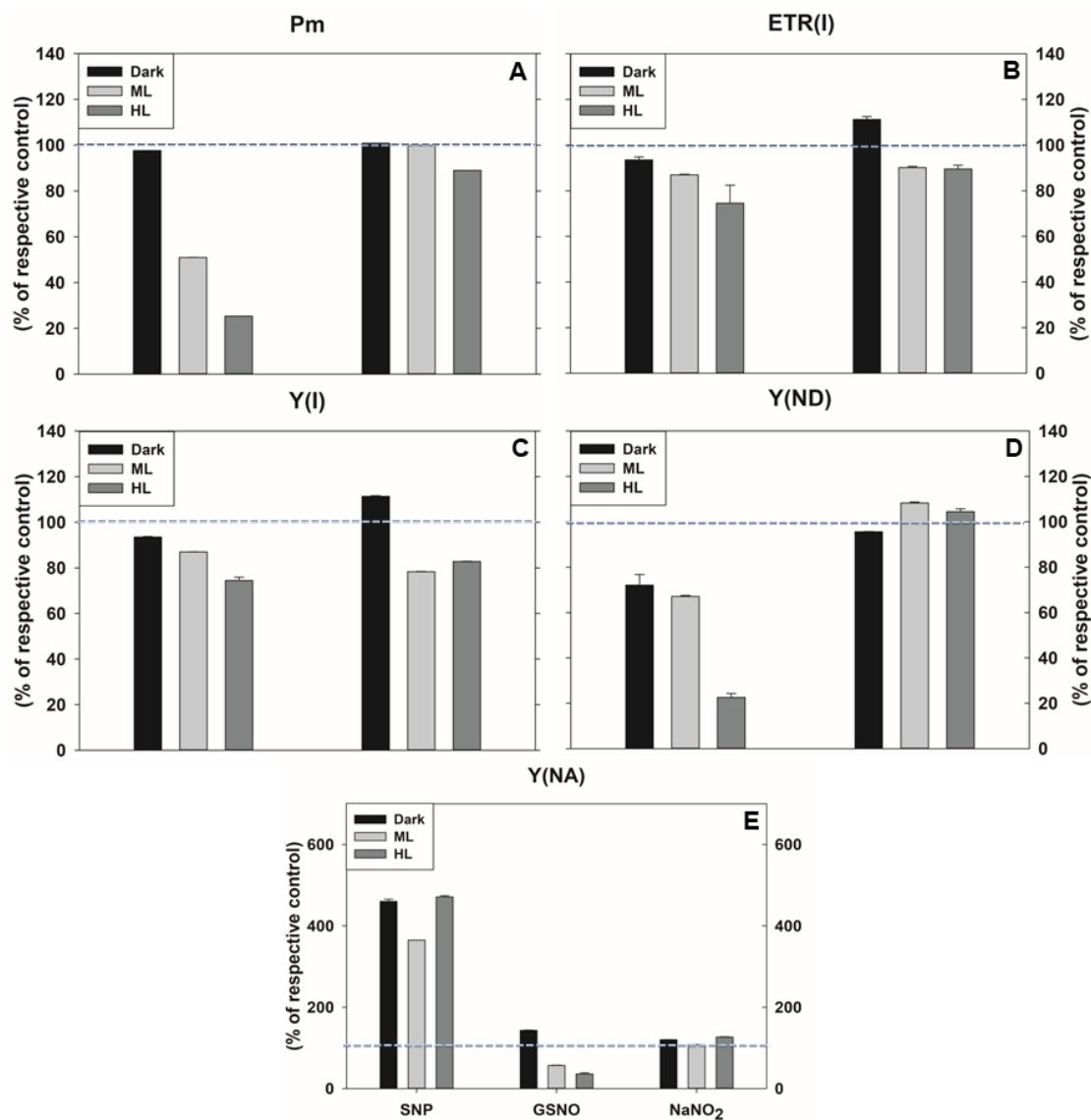


Figure 5.20. Chl fluorescence based PSII parameters in leaves of *Pisum sativum* after exposure to SNP or GSNO. These were measured at 1000 PAR ($\mu\text{E m}^{-2} \text{s}^{-1}$). The treatments and averages were all as in Fig. 5.1.

Discussion

Photo-oxidative stress caused more damage to pea leaves than oxidative stress (Aswani et al., 2019). One of the reports showed that NO induced oxidative/nitrosative stress (Lushchak and Lushchak, 2008). However, there were no previous attempts to study the modulation by SNP and GSNO of photorespiratory enzyme components. The present chapter demonstrated that SNP and GSNO modulated the photorespiratory enzyme components in cellular compartments, namely peroxisomes and chloroplasts and also the photosynthetic characteristics.

ROS and NO accumulation under nitrosative stress

We found an increase in NO and ROS when pea leaf discs were treated with SNP and GSNO (Figs. 5.2 and 5.3). Earlier workers reported that NO content enhanced on exposure to SNP and GSNO in tobacco and rice (Ederli et al., 2009; Antoniou et al., 2013; Esmail et al., 2018). Similarly, enhanced ROS (H_2O_2 and superoxide) was noticed on exposure to SNP and GSNO in tobacco, sunflower seedlings, and *Chlamydomonas reinhardtii* (Ederli et al., 2009; Singh and Bhatia, 2017; Kuo et al., 2020). NO, and ROS at low concentration act as signalling molecule, while at high concentration could be toxic (Mandal et al., 2022; Zhou et al., 2022). The combined presence of ROS and NO caused oxidative and nitrosative damage in plants (Corpas et al., 2013a; Mangal et al., 2022; Mansoor et al., 2022).

We made an effort to identify the connection between the rise in NO and ROS and the changes in photorespiratory enzymes caused by SNP and GSNO. When the NO scavenger (cPTIO) was included in the incubation

medium, the rise in NO and ROS was attenuated (Figs. 5.2 and 5.4). Thus, the changes in metabolism were assured to be due to the accumulation of NO and ROS (superoxide or H₂O₂).

Modulation of photorespiratory enzymes by SNP and GSNO in peroxisomes, chloroplasts particularly under high light

Photorespiration might shield photosynthesis from oxidative damage (Sunil et al., 2019). Key photorespiratory enzymes like GO, CAT, or HPR showed increased activity, protein levels, and transcript levels in drought, high temperatures, or photo-oxidative stress (Yuan et al., 2016; Cui et al., 2016; Bapatla et al., 2021). There were not many reports that showed how NO modulates the photorespiratory enzyme components. This work, however, is the first to demonstrate the responses of photorespiratory enzyme components located in peroxisomes and chloroplasts.

Peroxisome and chloroplast-based photorespiratory enzymes also reacted to SNP more strongly than GSNO. For e.g., SNP upregulated the key enzymes, proteins, and transcript levels of GO, CAT, and HPR. However, the modulation by NO-donors was not as pronounced as in the case of MD (**Chapter 4**). Particularly, GSNO treatment even downregulated these key enzymes, particularly under HL (Figs. 5.5 to 5.9). In comparison to GSNO, HL-mediated oxidative stress was relatively severe in modifying photorespiratory metabolism. The decrease in GO, CAT, and MDH activities by GSNO could be due to the S-nitrosylation of these proteins under physiological conditions (Ortega-Galisteo et al., 2012).

Aconitase, an enzyme located in mitochondria, responds to both oxidative as well as nitrosative stress (Lehmann et al., 2009; Gupta et al., 2012). Our results showed that both SNP and GSNO suppressed the aconitase (Fig. 5.10A). In an earlier study, exposure to NO inhibited the aconitase activity in *Arabidopsis thaliana* (Gupta et al., 2012). Similarly, other reports also showed that NO suppressed the aconitase in tobacco and yeast (Navarre et al., 2000; Lushchak and Lushchak, 2008).

The modulation of photorespiratory enzyme components by SNP and GSNO may be due to the differences in their molecular nature. SNP releases NO and cyanide (CN⁻), particularly on exposure to light (Leavesley et al., 2008; Sunil et al., 2020). The use of SNP needs caution. GSNO appeared to be ideal as it could release NO. The other reason may be due to the post-translational modifications (S-nitrosylation or tyrosine nitration) mediated by NO, which results in the activation or inhibition or no change of the enzymes (Tanou et al., 2009; Ortega-Galisteo et al., 2012; Corpas et al., 2019).

Enhanced antioxidant enzymes, nitrosothiols, and tyrosine nitrated proteins showed induction of oxidative/nitrosative stress

The activities, protein levels, and transcripts of APX and SOD were increased on exposure to SNP and GSNO (Figs. 5.11 to 5.14). The increase in the antioxidant components denoted that the plants experience a mild oxidative/nitrosative stress on exposure to NO that may be due to elevated ROS and NO or may be due to ROS and NO interaction. GSNO enhanced the SOD and CAT activities in yeast (Lushchak and Lushchak, 2008). Another report showed that SNP increased the SOD, APX, glutathione reductase (GR), and peroxidase (POD) activity in tomato (Manai et al., 2014). Similarly,

SNP and GSNO increased the APX, SOD, CAT, POD, and GR in wheat seedlings (Karpets et al., 2015; Khan et al., 2017; Silveira et al., 2017).

Nitrosothiol contents increased in ML and HL on exposure to SNP and GSNO (Fig. 5.15). SNP and GSNO increased the nitrosothiol content in tomato and *Glycine max* (Jedelska et al., 2019; Imran et al., 2021). The maximum increase in the intensity of nitrated protein was noticed in the case of GSNO, followed by SNP-treated ML and HL samples as compared to the control (Fig. 5.16). Protein nitration was demonstrated to be triggered by RNS, with peroxynitrite being one of the most reactive (Del Rio, 2015; Begara-Morales et al., 2016). Further, it is also possible to identify the nitrated proteins through MALDI-TOF, but due to the unavailability of the instrument, we could not extend the further experiment related to that.

Stresses like high light, high temperature, low temperature, and salinity or biotic increased the extent of protein nitration (Corpas et al., 2008). NO enhanced the tyrosine nitrated proteins in pepper (Chaki et al., 2015), and sunflower seedlings (Singh and Bhatia, 2017). One indicator of nitrosative stress in plants would be an increase in nitrosothiols and tyrosine protein nitration. These can be regarded as a connecting link between stress conditions and the metabolism of ROS and RNS. Therefore, the generation of NO can be considered a feedback mechanism of protection and regulation (Corpas et al., 2007, 2021).

Both Photosynthesis and respiration were sensitive to NO

The suppression of photosynthesis and respiration was severe on exposure to SNP, but was moderate in the presence of GSNO (Fig. 5.17).

The sensitivity of photosynthesis to NO is not surprising and expected (Batasheva et al. 2010; Sunil et al., 2020a, b). However, the effect of NO on respiration was ambiguous. We emphasize that SNP or GSNO affected both photosynthesis and respiration. The interference of PSII components by NO, followed by PSI, could be the reason for photosynthesis sensitivity. Since NO and its byproducts caused cytochrome c oxidase to irreversibly inhibit, the sensitivity of respiration to NO may be due to the inhibition of the cytochrome pathway (Poderoso et al., 2019; Yamasaki et al., 2001).

NO affects even the photosynthetic pigments

Carotenoids try to protect chlorophyll pigments when photosynthetic electron transport is affected, for e.g., under HL (Young, 1991; Maoka, 2020). In our study, too, NO-donors decreased the levels of both chlorophylls and carotenoids (Fig. 5.18). The loss of chloroplast pigments could be due to the enhanced NPQ. Our observations endorse the earlier reports that exogenous NO reduced the chlorophyll and carotenoid contents in soybean and tomato (Jasid et al., 2009; Hayat et al., 2010). The carotenoid contents in *Chlamydomonas reinhardtii*, by inhibiting the *de novo* carotenoid synthesis pathway was further reduced under HL (Chang et al., 2013). We believe that the interference by NO of PSII/PSI components leads to the loss of chlorophyll and carotenoid pigments. The minimal loss of chlorophylls and carotenoid pigments on exposure to GSNO reaffirms that GSNO be preferred as NO-donor

PSII and PSI components were both NO-sensitive

Both PSII and PSI photosystems were targets of NO. Damage to PSII/PSI from SNP or GSNO under normal light or HL was more severe than when kept in complete darkness (Figs. 5.19 and 5.20). To ensure NO-mediated damage to PSII in *V. faba*, light was necessary (Ördög et al., 2013). The damage to PSII depended on the intensity of light and the rate of recovery was influenced by the chloroplast's energetic state (Allakhverdiev and Murata, 2004; Murata et al., 2012; Tikkanen et al., 2014). However, Chl fluorescence characteristics demonstrated that PSII was more susceptible to NO than PSI. Several authors observed the significant sensitivity of PSII components to NO (Ördög et al., 2013; Misra et al., 2014; Sunil et al., 2020b). In the presence of NO, either PSII-related parameters were downregulated (Wodala et al., 2005, 2008; Chang et al., 2013) or PSI-related reactions were upregulated (Wodala and Horvath 2008). In our studies, SNP inhibited PSII components more effectively than GSNO. Exogenous NO at high concentrations greatly hindered the transfer of PSII electrons between Q_A and Q_B (Diner and Petrouleas, 1990; Wodala et al., 2008, 2010; Vladkova et al., 2011).

Fv/Fm status of Chl a fluorescence can be used as a stress marker in leaves. SNP decreased the Fv/Fm in potato leaves, but it had no effect on the NPQ (Yang et al., 2004). On the other hand, another NO donor (GSNO) downregulated both Fv/Fm and NPQ in pea leaves (Wodala et al., 2008). All of the measured PSII parameters showed that, following treatment with NO, the majority of reaction centres switched from photochemistry to heat dissipation, possibly as a result of a structural reorganisation in PSII and its

antenna complexes (Finazzi et al., 2004; Wodala et al., 2008; Ördög et al., 2013).

Wodala and Horvath (2008) also noted that GSNO-induced NO had higher PSI quantum efficiency in pea leaves. In our investigations, the considerable shift in PSI photochemistry was reflected in the marked change in PSI components with NO. In contrast to Y(ND) in the presence of SNP under HL, the levels of Y(NA) were substantially greater, causing a buildup of electrons on the PSI donor side. In order to disperse the electrons and prevent an excessive reduction of PSI P700, excess Y(NA) may stimulate cyclic electron transport (Takagi et al., 2016). We suggest that inhibition of PSII components, restriction of the donor side of PSI Y(ND), and stimulation on the acceptor side Y(NA) constitute a measurement of PSI-based cyclic electron transport. As a result, the drastic reduction of PSII and PSI by SNP appears to be somewhat offset by the increase of cyclic electron transport. On the other hand, GSNO only slightly restricted PSII and PSI. Our findings support the notion that PSI is more robust than PSII and that PSII has suffered serious damage at HL (Sonoike, 2011; Tikkanen et al., 2014; Gururani et al., 2015).

Conclusions from this chapter

1. Exposure to NO-donors elevated the levels of NO and ROS, particularly under high light. The decrease in NO and ROS by NO scavenger confirmed accumulation of NO.
2. Modulation of photorespiratory enzymatic contents was monitored by activity, protein content, and transcripts on exposure to NO-donors.

SNP upregulated the photorespiratory enzyme components, while GSNO was not as powerful as HL in upregulating photorespiration.

3. NO-donors enhanced the levels of nitrosothiols and tyrosine nitrated proteins, while the decrease in aconitase confirming the nitro-oxidative stress occurred in the presence of SNP and GSNO.
4. NO-donors decreased the photosynthesis that may be due to the interference by NO to PSII. They decreased respiration, possibly due to the inhibition of the cytochrome pathway.
5. NO reduced the total chlorophyll and carotenoid contents which might be due to the interference by NO of PSII/PSI.
6. Chlorophyll fluorescence patterns revealed the marked sensitivity of PSII, followed by PSI to NO. The damage to PSII and PSI were aggravated by NO in presence of HL.

Chapter 6

Responses of Photorespiratory Enzymes Components in Leaves of *Arabidopsis thaliana* by Low O₂ and Aminooxyacetic Acid (AOA)

Chapter 6

RSEPNSES OF PHOTORESPIRATORY ENZYMES COMPONENTS IN LEAVES OF *ARABIDOPSIS THALIANA* BY LOW O₂ AND AMINOXYACETIC ACID (AOA)

Introduction

Modulating photorespiration affected the redox status and metabolic components of leaves. There were many approaches through which restricted photorespiration. These include high CO₂, low O₂, photorespiratory inhibitors, and photorespiratory mutants. There are advantages as well as limitations to each approach. High CO₂-mediated restriction of photorespiration needs a specially built sealed growth chamber where CO₂ levels of 3000 µL/L or more were required (Queval et al., 2007). Low O₂ was one of the simple and easy approaches to restrict photorespiration. Numerous techniques, such as nitrogen purging, boiling at low pressure, or sonicating at low pressure, were used to minimize the amount of oxygen in the solution. The quick and effective way to minimize O₂ concentration in the solution was purging with N₂ gas for 20 to 30 minutes (Butler et al., 1994; Riazunnisa et al., 2006).

Photorespiratory inhibitors affected the enzymes or transporters located in specific organelles, thus restricting photorespiration. Compounds such as aminoacetonitrile (AAN), aminooxyacetic acid (AOA), glycine hydroxamate (GHA), phosphinothricin, α-hydroxy-2-pyridinemethansulfonic acid (HPMS), and isonicotinyl hydrazide (INH) and sodium fluoride (NaF), etc. were used to restrict the photorespiration. AAN, GHA, and AOA inhibited the glycine decarboxylase, blocking glycine decarboxylation to serine or

ammonia, inhibiting the photorespiratory pathway, and decreased the photosynthetic efficiency (Riazunnisa et al., 2006; Kleczkowski et al., 1987; Han et al., 2018). Phosphinothricin irreversibly inhibited the glutamine synthetase (GS), preventing ammonium reassimilation and, thus, restricting the photorespiratory cycle (Wendler et al., 1990; Takano and Dayan, 2020). HPMS blocked the glycolate to glyoxylate conversion, and INH blocked the glycine to serine or ammonia conversion, while sodium fluoride inhibited phosphoglycolate phosphatase (PGLP) activity, thus restricting photorespiration (Martin et al., 1983; Hewitt et al., 1990; Kang et al., 2018).

The other approach to study photorespiration was to use photorespiratory mutants deficient in key genes such as *pglp1* (phosphoglycolate phosphatase), *gox1/gox2* (glycolate oxidase), *cat2* (catalase), *hpr1/hpr2* (hydroxypyruvate reductase), *glyk* (glycerate kinase), etc. Most of these photorespiratory mutants did not grow in normal air, but *hpr1* could survive (active photorespiration). The photorespiratory mutants were grown in high CO₂-enriched air (suppressed photorespiration). For that, we need a plant growth chamber which was having CO₂-enriched system. These plants, when transferred from high CO₂ to normal air, showed chlorotic and bleaching leaves like symptoms (Somerville, 2001; Timm et al., 2012, 2013; Eisenhut et al., 2019; Wang et al., 2022).

In this chapter, we attempted to study the effects of low O₂ and AOA on the redox status and photorespiratory enzyme components in leaves. We have used AOA and low O₂ to restrict photorespiratory metabolism. We observed that restricting photorespiration elevated the ROS levels and

downregulated the photorespiratory enzyme components, showing the importance of photorespiration under abiotic stress conditions.

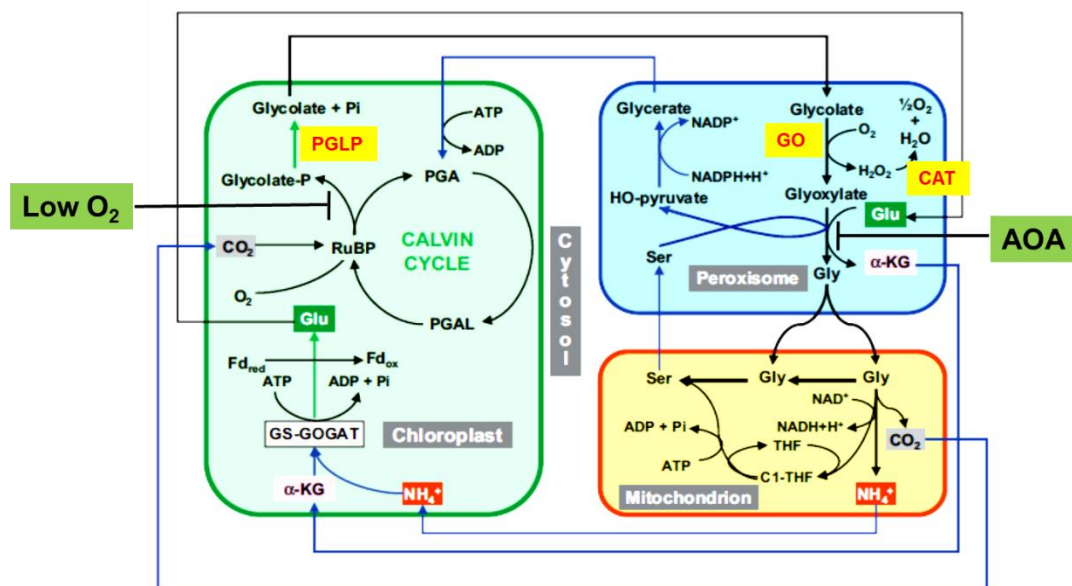


Figure 6.1. Integrated photorespiratory carbon nitrogen cycle scheme. Photorespiratory metabolism was restricted by either low oxygen (O₂) or AOA in this study (modified from Mishra, 2014).

Results

Accumulation of ROS (H₂O₂ and superoxide)

The pattern of ROS accumulation on exposure to low O₂ and AOA under dark, ML, and HL was monitored by NBT or DAB staining. The ROS levels (both superoxide and H₂O₂) increased in ML and further increased under HL. When NBT was present, blue color formazan appeared (Fig. 6.2A). Superoxide content increased on exposure to low O₂ and AOA over respective control. When stained leaves were used to quantify the superoxide levels, it increased in a range up to 2 to 3-fold by AOA and by low O₂ in the dark or ML or HL (Fig. 6.3A). Similarly, the H₂O₂ content in *Arabidopsis* leaves was monitored by DAB staining, as shown by the precipitation of brown color in the leaves (Fig. 6.2B). When stained leaves were used to quantify the H₂O₂

levels, the H_2O_2 content too increased by up to 2 to 3-fold under low O_2 or in the presence of AOA (Fig. 6.3B).

Changes in the photorespiratory enzymes: Activities and protein levels

We checked the consequences of restricted photorespiratory metabolism on some of the enzymes by low O_2 or AOA. Among the three enzymes studied, GO and CAT is in peroxisomes, while PGLP is a chloroplastic one. The activity of CAT and PGLP decreased with low O_2 or AOA treatment compared to that of the control. The activity of GO too increased on exposure to low O_2 , while on exposure to AOA, the activity of GO increased in the dark but decreased particularly under ML and HL conditions (Fig. 6.4).

Subsequently, we checked the protein levels of these two enzymes by western blotting using specific antibodies. Ponceau staining was done to ensure equal loading of Rubisco. In the case of light treated with photorespiratory inhibitors, the protein levels of GO and CAT decreased in ML and HL (Figs. 6.5 and 6.6). However, AOA appeared more effective in decreasing protein levels. Due to the lack of an antibody, the protein levels of PGLP could not be determined.

Transcripts of photorespiratory enzymes

When photorespiration was restricted, the activities and the protein levels of GO, CAT, and PGLP were all modulated. We attempted to use semi-quantitative RT-PCR to examine the transcripts of genes related to photorespiratory metabolism. Transcripts of the following enzymes were looked at: different isoforms of catalase (CAT1 and CAT2), glycolate oxidase (GOX1 and GOX2), and PGLP1. Actin 8 was used as an internal control.

There was not much change in the transcripts of CAT1 in the dark, while it decreased in ML and HL-treated conditions. The transcripts of CAT2 were reduced when the leaves were treated with AOA. Similarly, the transcripts of GOX1 and GOX2 were also decreased in the presence of AOA. The transcripts of PGLP1 were not much affected in the dark or ML but decreased on exposure to HL in the presence of AOA (Fig. 6.7). The transcripts of CAT1 and CAT2 were slightly decreased in dark or ML when leaves were under low O₂. But the transcripts of GOX1 and GOX2 were higher in low O₂. The transcripts levels of PGLP1 were also not much changed in dark conditions, while downregulated in ML and HL treated with low O₂. (Fig. 6.8).

Discussion starts on page no. 117.

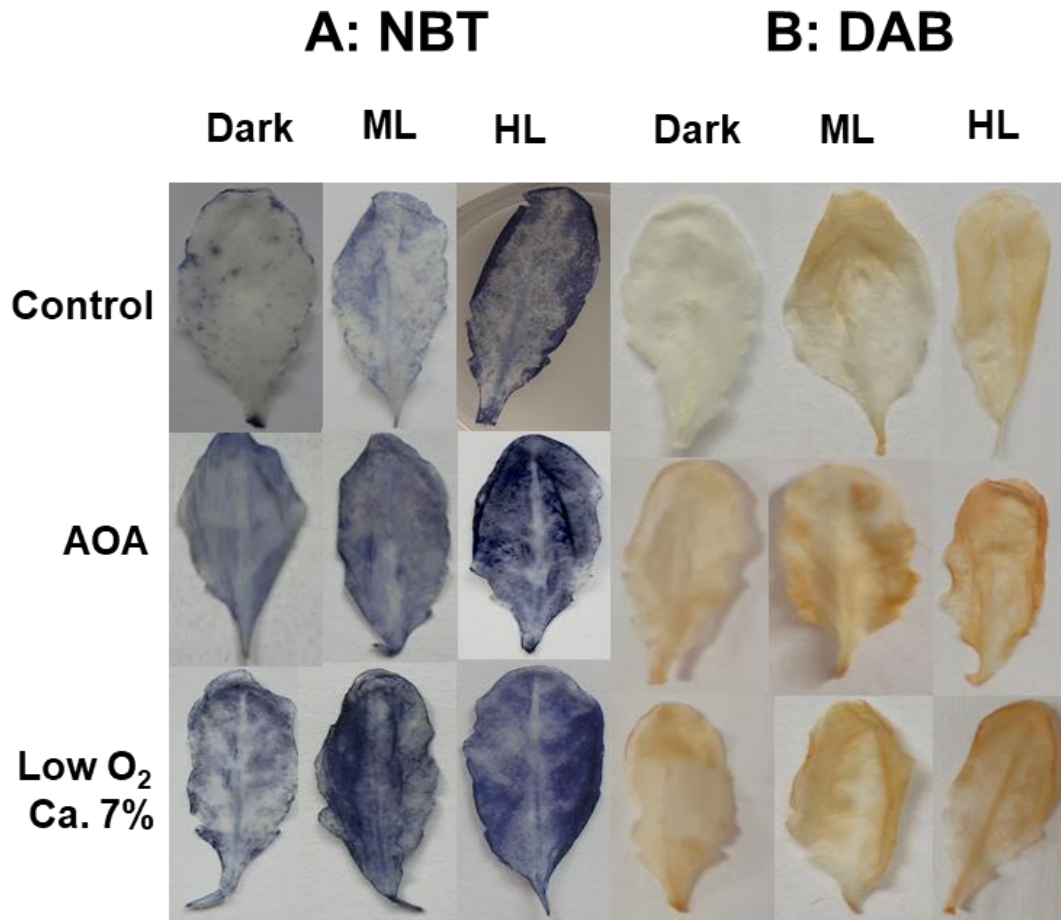


Figure 6.2. Superoxide and H₂O₂ levels, visualized by DAB and NBT staining in leaves of *Arabidopsis thaliana* upon treatment with photorespiratory inhibitors. The blue color represented the superoxide levels (A), while the brown color showed up the accumulation of H₂O₂ (B). The leaves were exposed to inhibitors for 3 h and left in darkness or moderate (ML, 150 $\mu\text{E m}^{-2} \text{s}^{-1}$), or high light (HL, 600 $\mu\text{E m}^{-2} \text{s}^{-1}$). The brown and blue coloration leaves were used for quantification (see Fig. 6.3).

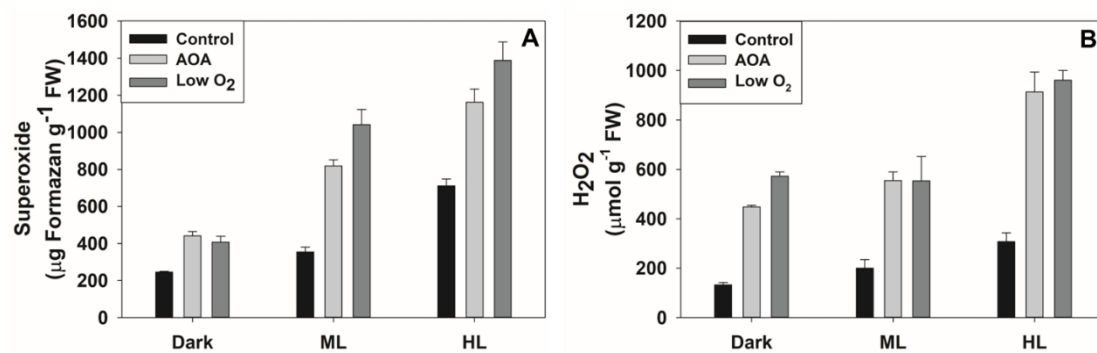


Figure 6.3. Superoxide and H_2O_2 quantified from NBT and DAB-stained leaves of *Arabidopsis thaliana*. The treatments and averages were all as in Fig. 6.2.

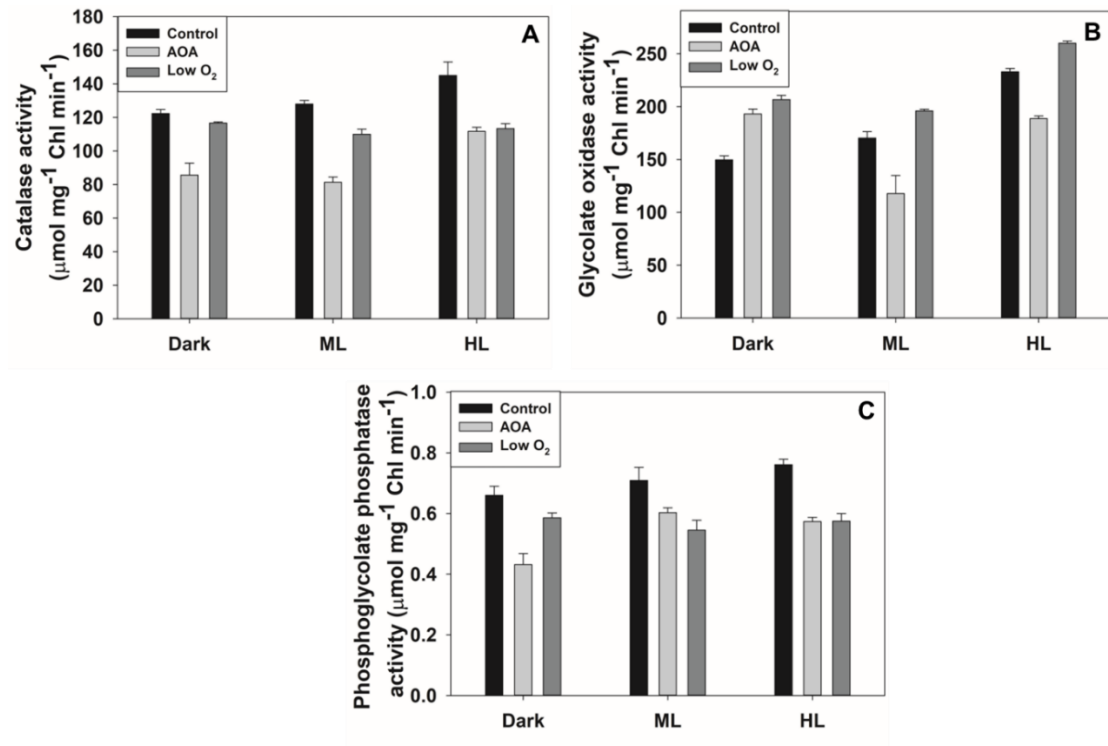


Figure 6.4. Photorespiratory enzyme activities, CAT (A), GO (B), and PGLP (C), were measured in leaves of *Arabidopsis thaliana*. The treatments and averages were all as in Fig. 6.2.

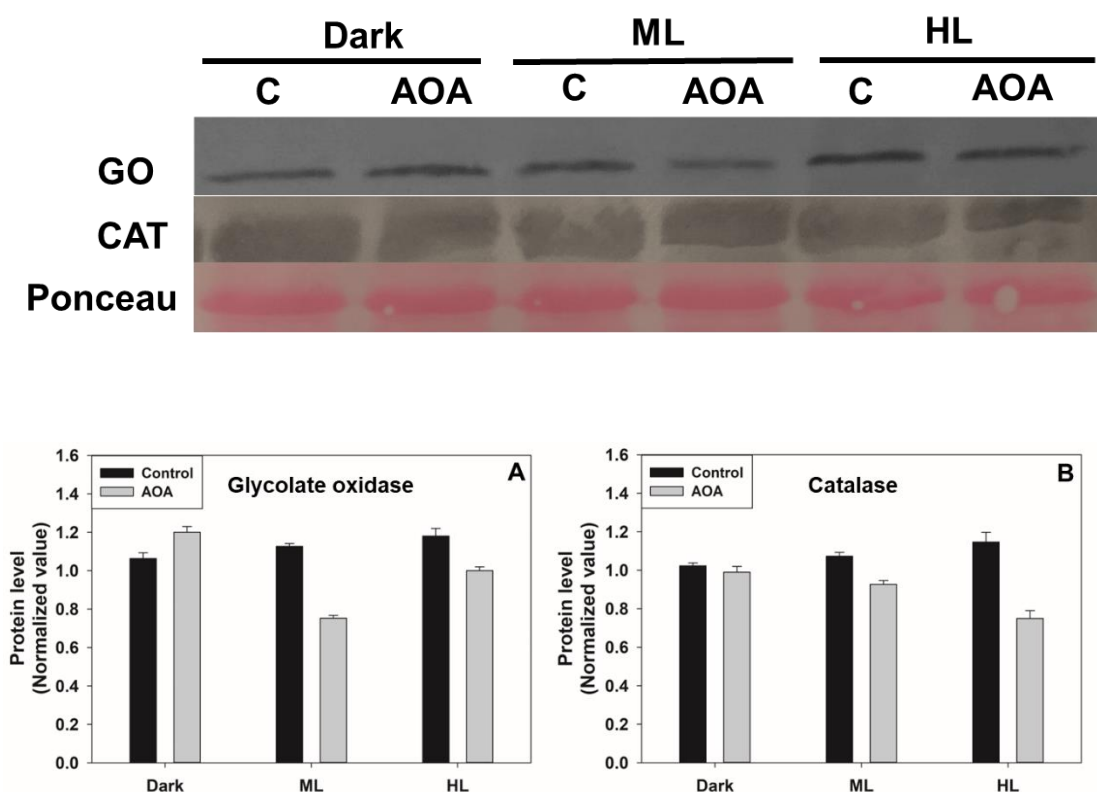


Figure 6.5. The protein levels of GO and CAT were measured in leaves of *Arabidopsis thaliana* upon exposure to AOA, a photorespiratory inhibitor (Top panel). Based on image J, the ratios of GO and CAT protein to Rubisco large subunit were computed (Bottom panel, A and B). The treatments and averages were all as in Fig. 6.2.

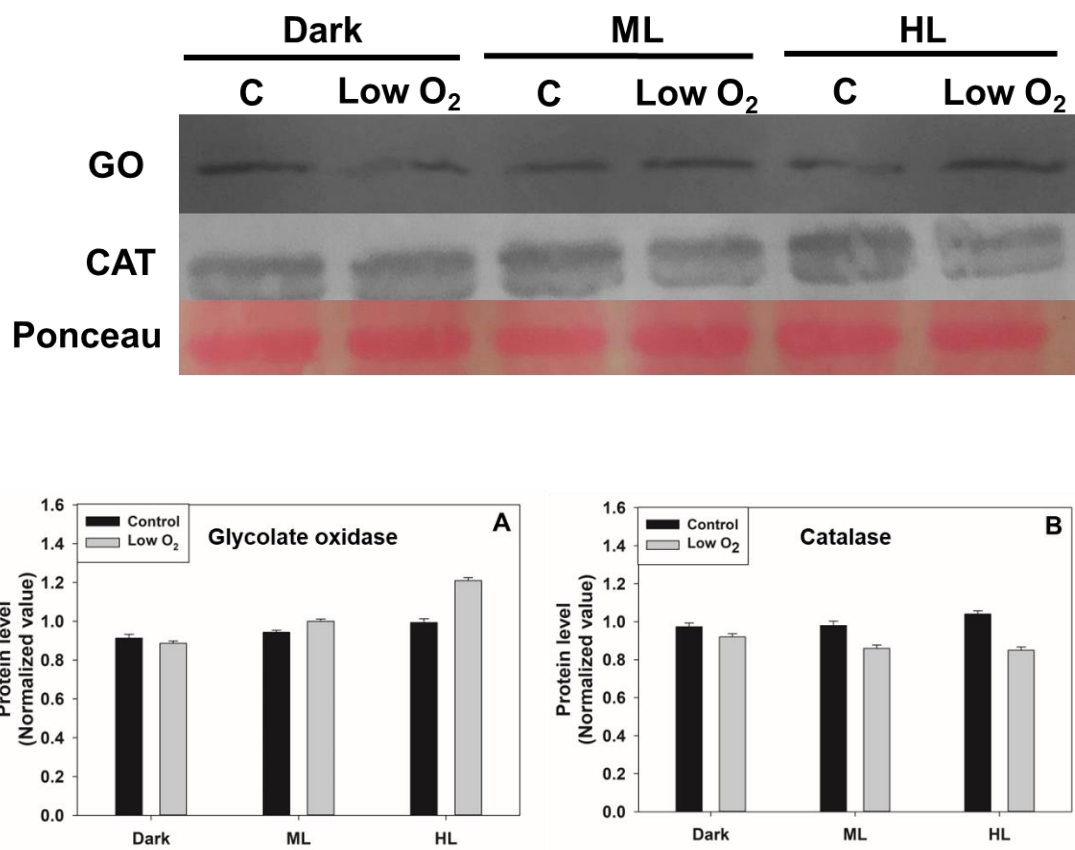


Figure 6.6. The protein levels of GO and CAT were measured in leaves of *Arabidopsis thaliana* upon exposure to low O₂ (Top panel). Based on image J, the ratios of GO and CAT protein to Rubisco large subunit were computed (Bottom panel, A and B). The treatments and averages were all as in Fig. 6.2.

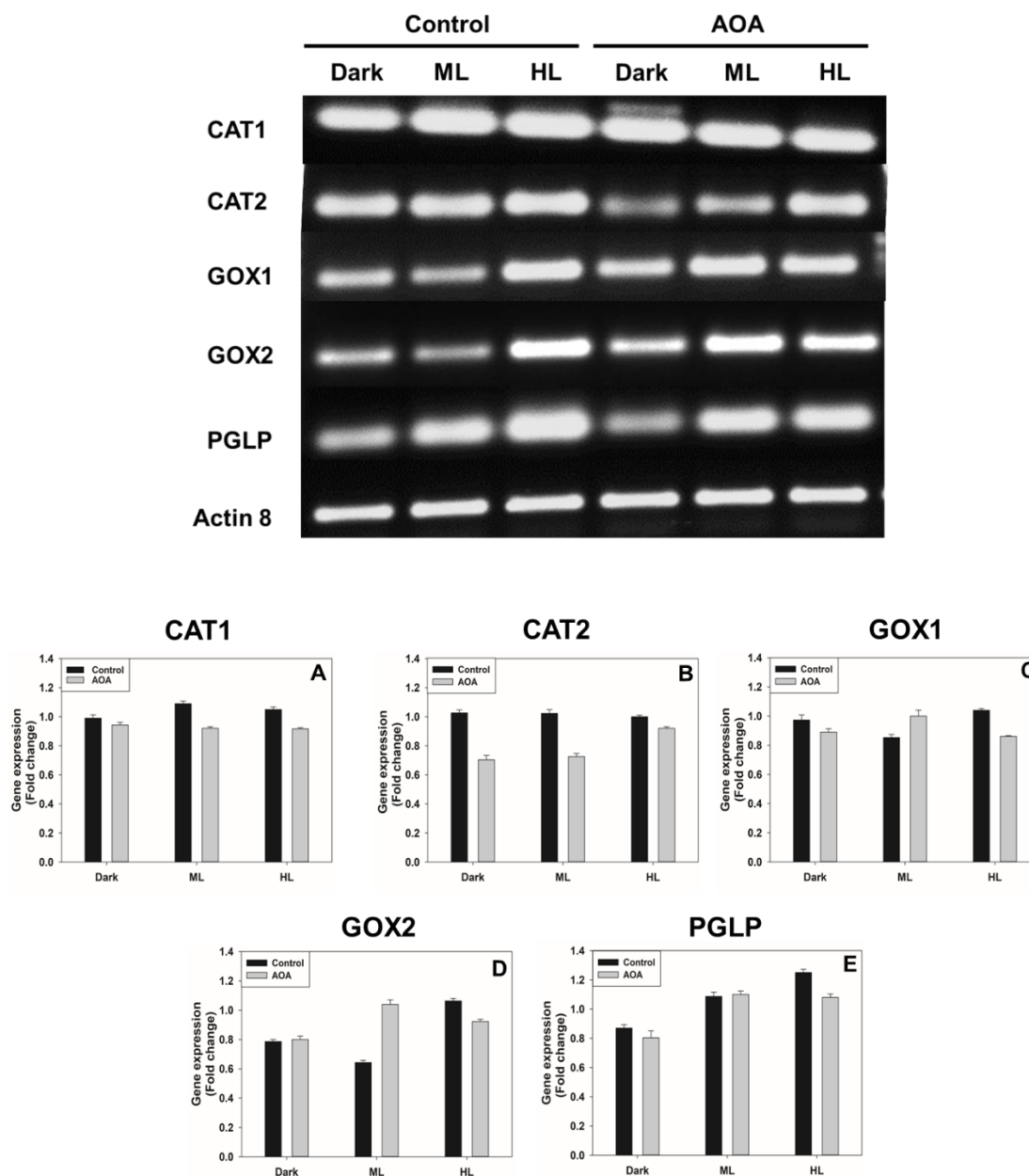


Figure 6.7. The transcripts of selected photorespiratory metabolism by semi-quantitative RT-PCR in *Arabidopsis thaliana* leaves after AOA treatment. The scans are shown in Top panel. Actin 8 was the internal control. The quantified values were given in Bottom panel (A to E); CAT1 (A), CAT2 (B), GOX1 (C), GOX2 (D), and PGLP (E). The treatments and averages were all as in Fig. 6.2.

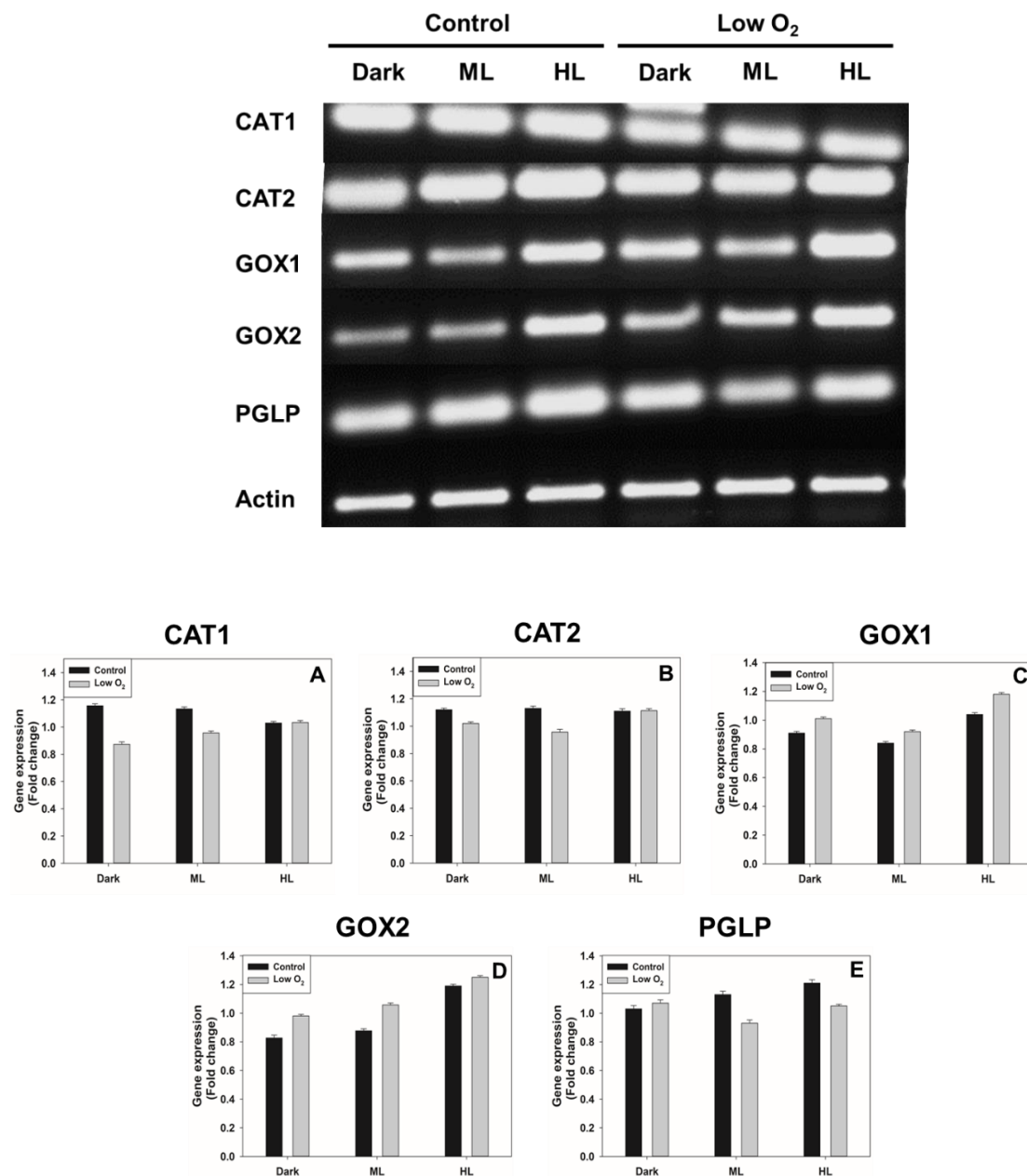


Figure 6.8. The transcripts of selected photorespiratory metabolism by semi-quantitative RT-PCR in *Arabidopsis thaliana* leaves after low O₂ treatment. The scans are shown in Top panel. Actin 8 was the internal control. The quantified values were given in Bottom panel (A to E); CAT1 (A), CAT2 (B), GOX1 (C), GOX2 (D), and PGLP (E). The treatments and averages were all as in Fig. 6.2.

Discussion

We tried to assess the consequences of low O₂ and treatment with AOA (an inhibitor of glycine formation) on photorespiratory metabolism in *Arabidopsis*. When photorespiration was restricted by either low O₂ or AOA, there was a marked elevation in ROS levels (both H₂O₂ and superoxide) and downregulation of photorespiratory enzyme components.

High accumulation of ROS

The ROS levels (both H₂O₂ and superoxide) were elevated under restricted photorespiratory conditions irrespective of dark or light incubation (Figs. 6.2 and 6.3). Such increases in the H₂O₂ and superoxide content were observed in rice under O₂-deficient conditions (Yamaguchi et al., 2017). An increase in H₂O₂ and superoxide levels were also observed in tobacco on exposure to hypoxia (Jayawardhane et al., 2020), while H₂O₂ levels increased in *Arabidopsis thaliana* under low oxygen treatment (Liu et al., 2017). In another study, exposure to AOA increased the ROS in *Dunaliella tertiolecta* (Kamalanathan et al., 2022). In *hpr1* mutants, ROS and harmful metabolic intermediates accumulated under high light intensity, resulting in growth retardation and damage to PSII (Wang et al., 2022).

From our results, we emphasize that ROS production gets enhanced under restricted photorespiratory conditions. Low concentrations of ROS function as a signaling molecule, but at high concentrations behaves as a toxic molecule. The elevated ROS can be harmful to physiological processes such as photosynthesis, respiration, and could even lead to cell death in

plants (Mittler et al., 2002, 2017). For e.g., a high concentration of AOA could induced cell death in *Arabidopsis* (Errakhi et al., 2008).

Downregulation of selected photorespiratory enzyme components

Our results revealed that restricting photorespiration downregulated the photorespiratory enzyme components located in chloroplast and peroxisome (Fig. 6.4). Photorespiratory metabolism was extremely sensitive to inhibitors such as AAN, AOA, or GHA. Among these, AAN and GHA inhibited the glycine decarboxylase, which blocks the glycine degradation to serine or ammonia, while AOA inhibited photorespiration by preventing the conversion of glyoxylate to glycine. As a consequence, photosynthesis was suppressed (Kleczkowski et al., 1987; Riazunnisa et al., 2006). In our study, we used low O₂ and AOA to restrict photorespiration. Purging with N₂ gas resulted in low O₂, slowing down the RuBP oxygenase activity. In contrast to that, AOA inhibited photorespiration by preventing glyoxylate conversion to glycine.

The effects of low O₂ on GO activity under restricted photorespiratory conditions have been ambiguous. There was a decrease in the CAT, GO, and PGLP activity in barley plants exposed to low O₂ (Hodges and Forney, 2000; Yordanova et al., 2003). On exposure to phosphinothricin, an inhibitor of photorespiration, GO, and catalase activity decreased in *Zea mays* (Gonzalez-Moro et al., 1993). Similarly, AOA inhibited the glycolate dehydrogenase activity in *Euglena gracilis* Z (Yokota and Kitaoka, 1987). On the other hand, the GO activity increased under hypoxia conditions (Nichugovskaya and Shevchenko, 1990).

Photorespiratory metabolites were often modulated when photorespiration was restricted. The glycine/serine ratio increased in the presence of GDC inhibitors or a glutamine synthetase deficiency, showing glycine accumulation (Bauwe and Kolukisaoglu, 2003). Exposure to phosphinothricin interrupted the photorespiratory cycle, inhibited NH_3 accumulation, and resulted in a reduction in amino acids such as glutamine, glutamate, glycine, serine, aspartate, and alanine (Wendler et al., 1992; Lebedev et al., 2019). Treatment with phosphinothricin and AOA also caused an accumulation of glycolate and, to a lesser extent, glyoxylate and decreased photosynthesis (Gonzalez-Moro et al., 1993, 1997).

Relation between the status of ROS levels in the leaf with photorespiration

The operation of photorespiration appeared to be strongly related to the redox state of leaves. Several reports showed that the photorespiratory enzymes were upregulated in plants on exposure to abiotic stress. There was an increase in the activities, protein, and transcripts levels of key photorespiratory enzymes such as GO, CAT, or HPR under drought, high temperature, or photo-oxidative stress was noticed (Yuan et al., 2016; Cui et al., 2016; Bapatla et al., 2021). Such upregulation of photorespiratory enzymes demonstrated the importance of photorespiration to cope up with abiotic stress. Sunil et al. (2019) suggested photorespiration could protect photosynthesis against oxidative damage under abiotic stress conditions such as HL and drought.

In the present work, restriction of photorespiration raised the ROS levels while downregulating the photorespiratory enzymes. These results imply that unrestricted photorespiration can help keep the ROS levels low.

Conclusions from this chapter

1. The levels of both superoxide and H_2O_2 increased on exposure to low O_2 or the presence of AOA, irrespective of darkness or ML/HL. Thus, restriction of photorespiration elevated the levels of ROS/oxidative stress in plant leaves.
2. This observation implies that the normal operation of photorespiration would be minimizing the ROS levels in leaves.
3. The activities, protein content, and transcripts of photorespiratory enzymes decreased under restricted photorespiratory conditions. We, therefore, believe that the downregulation of photorespiratory metabolism at low O_2 or in the presence of AOA, was the reason for enhanced oxidative stress in leaves.
4. We propose that photorespiration is essential under normal oxygen and can keep optimal levels of ROS, and maintain the activities of photorespiratory enzymes.

Chapter 7

**Photorespiratory Enzymes components in
Arabidopsis mutants lacking genes
involved in redox balancing components**

Chapter 7

PHOTORESPIRATORY ENZYMES COMPONENTS IN *ARABIDOPSIS* MUTANTS LACKING GENES INVOLVED IN REDOX BALANCING COMPONENTS

Introduction

Photorespiration plays a crucial role in optimizing photosynthetic CO₂ fixation and to protect against photoinhibition. Work on photorespiratory mutants suggested that photorespiration may act as a defense mechanism against photoinhibition (Wingler et al., 2000; Voss et al., 2013; Saji et al., 2017). Photorespiratory metabolism is distributed among different compartments, such as peroxisome, mitochondria, and chloroplast (Bauwe et al., 2010; Deller et al., 2016). As a result, it might help maintain the redox balance among these compartments (Eisenhut et al., 2019). *Arabidopsis thaliana* is chosen as a model flowering plant for a variety of reasons, including its small/well-characterized genome, short life cycle, ease of genetic manipulation, and the decoding of its entire genome (Meinke et al., 1998; Garcia-Hernandez et al., 2002). Numerous mutants, transgenics, and established methods are readily available, providing enormous potential for physiological and biochemical research using *Arabidopsis* (Hayashi and Nishimura, 2006).

In Chapter 4, we showed that photorespiratory enzyme components were upregulated in leaf discs of pea under photooxidative and oxidative stress conditions. These conditions were imposed using menadione. It would be interesting to know photooxidative/oxidative stress induced by MD

regulated the photorespiratory metabolism in *Arabidopsis thaliana* mutants lacking key redox components. We chose three mutants—*nadp-mdh*, *aox1a*, and *vtc* that were deficient in redox-balancing components to examine the modulation of photorespiratory metabolism. Among these three, the *nadp-mdh* and *aox1a* mutants lacked the major redox-regulating elements located in the mitochondria and chloroplasts. Likewise, *vtc* mutant had the least amount of ascorbate, one of the main antioxidants in plant tissues.

Mutants used:

- ***nadp-mdh***: lacks NADP-dependent malate dehydrogenase (Hebbelmann et al., 2012).
- ***aox1a***: deficient in mitochondrial alternate oxidase (Strodtkotter et al., 2009).
- ***vtc***: ascorbate-deficient, ca. 30 % of wild-type (Pastori et al., 2003).

We attempted to study photorespiratory metabolism utilizing *Arabidopsis* leaves of both WT (Col-0) and mutants. We created ROS in mitochondria using MD and assessed the effects under dark, ML or HL. Except for the *vtc* mutants, which had sluggish growth, all the mutants had similar phenotype as the WT (Fig. 3.2, in **Chapter 3**, Materials and Methods).

Results

Changes in levels of ROS

The pattern of ROS accumulation on exposure to MD under dark, ML, and HL was monitored by NBT or DAB staining in *Arabidopsis thaliana* WT and mutants (*nadp-mdh*, *aox1a*, and *vtc*). ROS levels (both superoxide and H₂O₂) increased markedly under HL. Superoxide levels increased on

treatment with MD, particularly under HL, as indicated by NBT-staining (Fig. 7.1). Similar to WT, superoxide accumulated in *nadp-mdh* and *aox1a* mutants. The superoxide levels increased by 4.5 to 5.5-fold in WT and mutants (*nadp-mdh* and *aox1a*) plants when exposed to MD in ML or HL. The changes were marginal in the dark treated with MD. In contrast, to that, the superoxide levels were increased by 5 to 7.5-fold in *vtc* mutants when treated with MD (Fig. 7.2).

The H₂O₂ levels in WT and mutants were monitored by DAB staining, as shown by the appearance of brown color (Fig. 7.3). The extent of H₂O₂ accumulation in *nadp-mdh* and *aox1a* was similar to that of WT. When stained leaves were used to quantify the H₂O₂ levels, the H₂O₂ content too increased by up to 1.5 to 2.3-fold in ML and HL in WT and mutants (*nadp-mdh* and *aox1a*). Accumulation of H₂O₂ was more in *vtc* mutant than that of WT, *nadp-mdh*, and *aox1a*, even without MD treatment. H₂O₂ content increased in the *vtc* mutant up to 1.8 to 3.3-fold when exposed to MD (Fig. 7.4).

Changes in activities of photorespiratory enzymes

We checked the consequences of oxidative/photooxidative stress on the photorespiratory enzymes by HL and MD. Among the enzymes studied, GO, CAT, and HPR are peroxisomal, while GK and PGLP are chloroplastic. The GO activity increased in the WT and mutants, particularly under HL treated with MD, while not much changes were noticed under dark conditions. The rise in GO activity was maximal in WT. Surprisingly, the lowest GO activity was noticed in the *vtc* mutant (Fig. 7.5). Similarly, the CAT activity was increased on exposure to MD in HL. The extent of increase was similar in WT and mutants (*nadp-mdh* and *aox1a*), while the maximum increase in CAT

activity was noticed in *vtc* mutant (Fig. 7.6). There were not much change in HPR activity in WT or in mutants when treated with MD (Fig. 7.7). The PGLP activity was increased in WT and mutants on exposure to MD, particularly under HL condition (Fig. 7.8).

Aconitase activity, a mitochondrial enzyme considered a marker of oxidative stress, was decreased in MD-treated samples in WT and in mutants (*nadp-mdh* and *aox1a*). The maximum inhibition of aconitase activity was noticed in *vtc* mutant (Fig. 7.9).

Discussion starts on page 134.

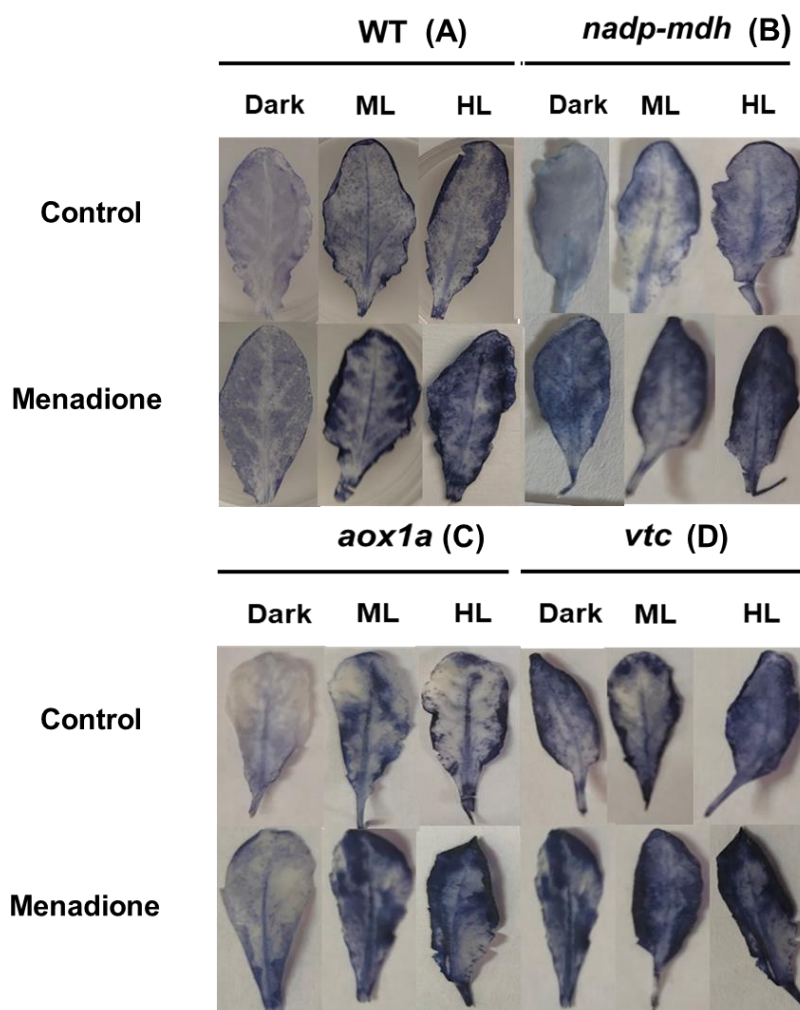


Figure 7.1. Superoxide levels, visualized by NBT staining in *Arabidopsis thaliana* wild type (A) and mutants (*nadp-mdh*, B; *aox1a*, C; *vtc*, D) leaves upon exposure to MD for 3 h in dark, moderate light (ML, $150 \mu\text{E m}^{-2} \text{s}^{-1}$) and high light (HL, $600 \mu\text{E m}^{-2} \text{s}^{-1}$). The blue color represents the superoxide, and the NBT-stained leaves were used for quantification (see Fig. 7.2).

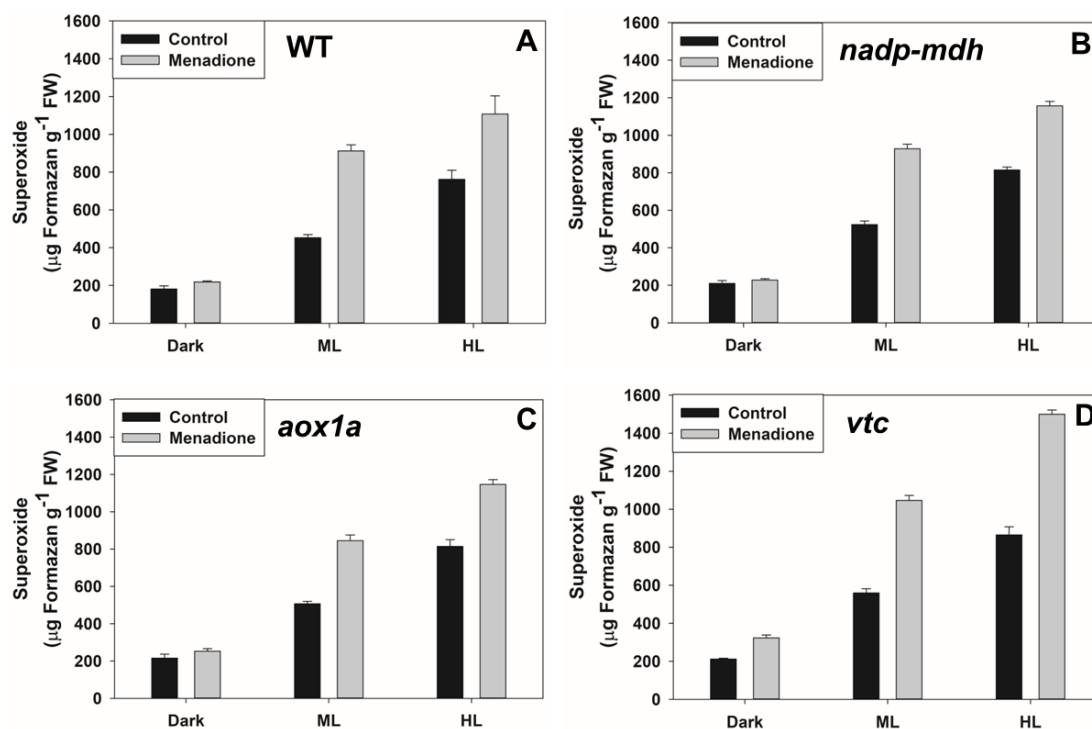


Figure 7.2. Superoxide levels, as indicated by NBT stained leaves of *Arabidopsis thaliana* wild type (A) or mutants: *nadp-mdh*, (B); *aox1a*, (C); *vtc*, (D). Further details of experimental conditions were as in Fig. 7.1.

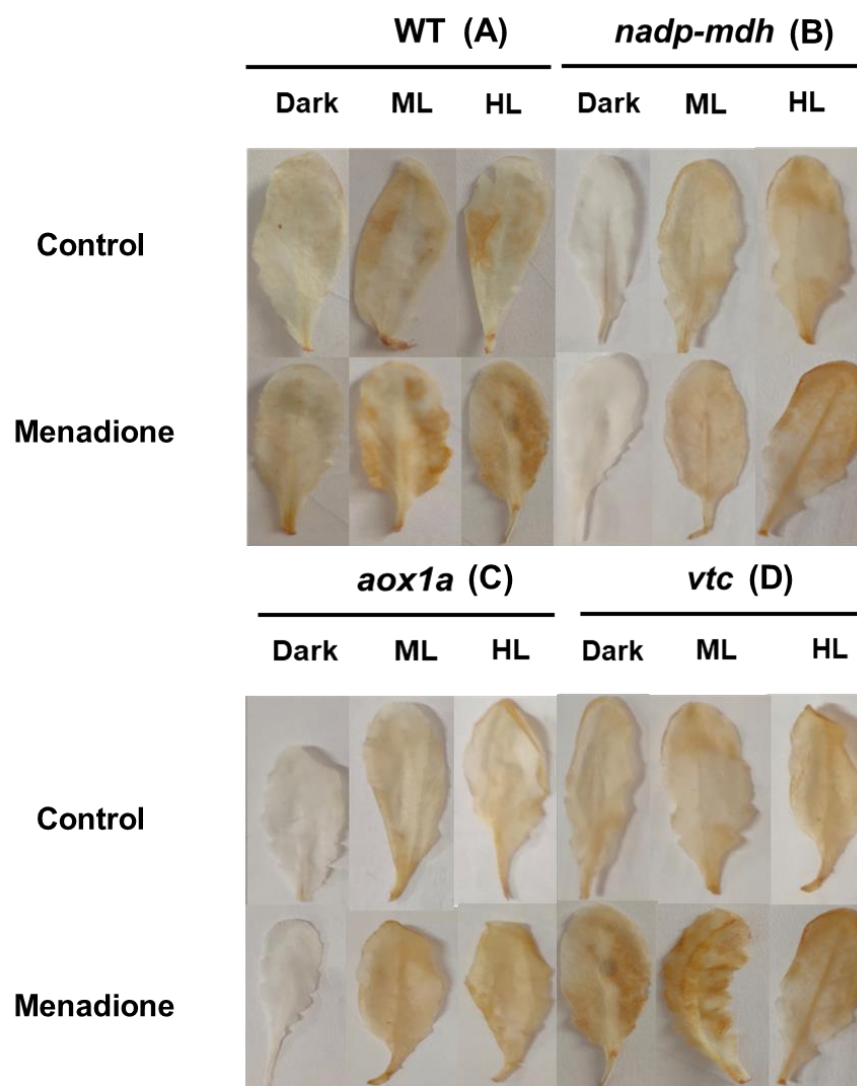


Figure 7.3. H_2O_2 levels, visualized by DAB staining in leaves of *Arabidopsis thaliana* wild type (A) or mutants: *nadp-mdh*, (B); *aox1a*, (C); *vtc*, (D). The brown color showed the pattern of H_2O_2 accumulated, and the DAB-stained leaves were used for quantification (see Fig. 7.4). Further details of experimental conditions were as in Fig. 7.1.

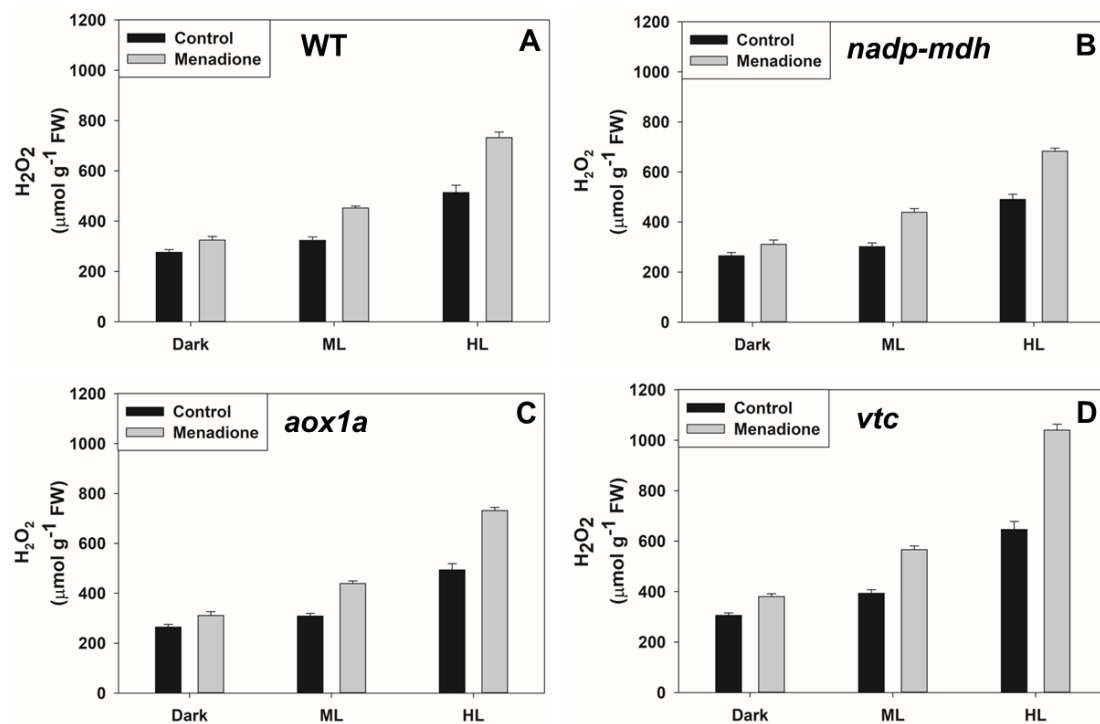


Figure 7.4. H_2O_2 levels in leaves of *Arabidopsis thaliana*, as indicated by DAB-staining. Wild type (A) or mutants: *nadp-mdh*, (B); *aox1a*, (C); *vtc*, (D). Further details of experimental conditions were as in Fig. 7.1.

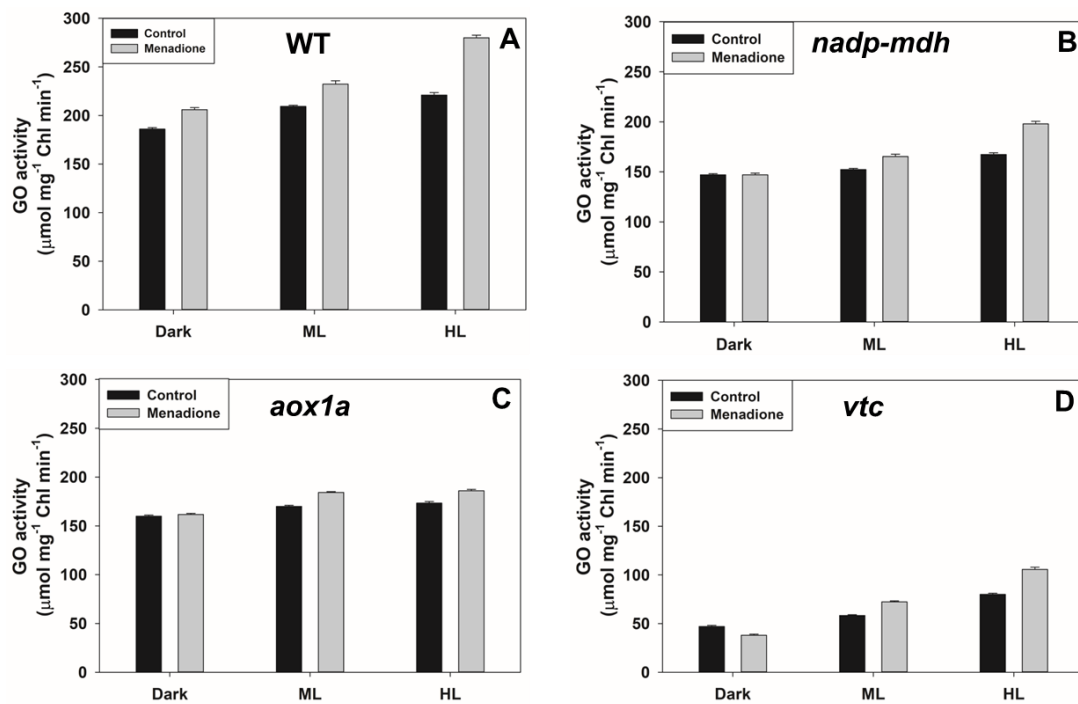


Figure 7.5. The activity of GO in wild type (A) or the three mutants: *nadp-mdh*, (B); *aox1a*, (C); *vtc*, (D). of *Arabidopsis thaliana*, upon exposure to MD. Further details of experimental conditions were as in Fig. 7.1.

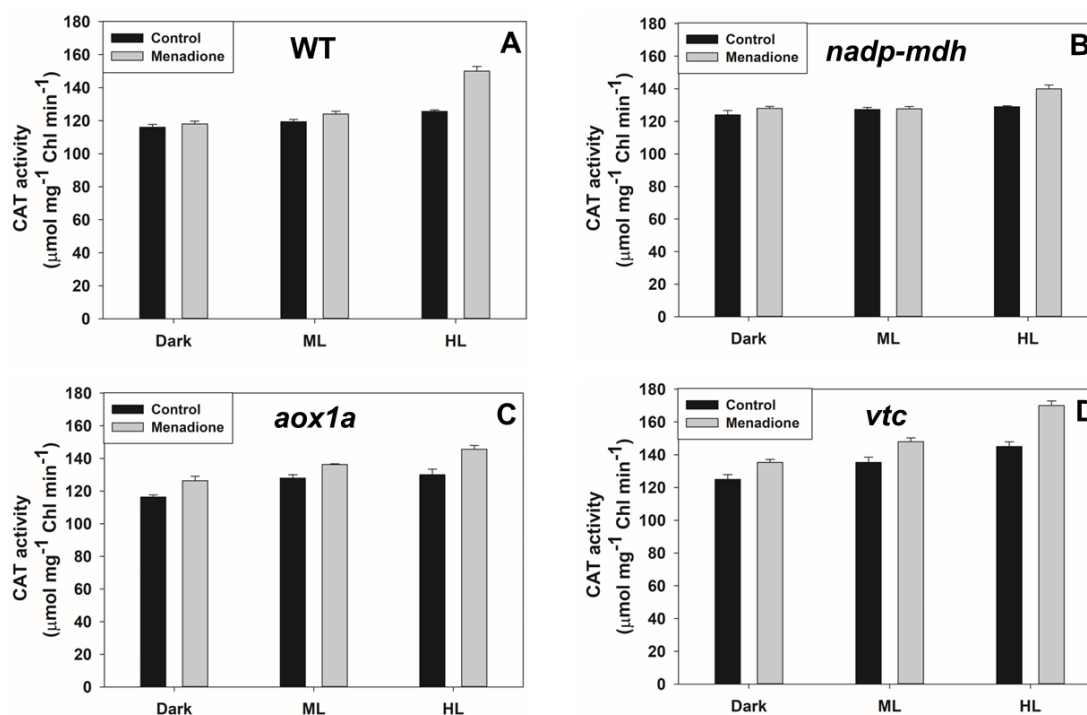


Figure 7.6. The activity of CAT in wild type (A) or the three mutants: *nadp-mdh*, (B); *aox1a*, (C); *vtc*, (D). of *Arabidopsis thaliana*, upon exposure to MD. Further details of experimental conditions were as in Fig. 7.1.

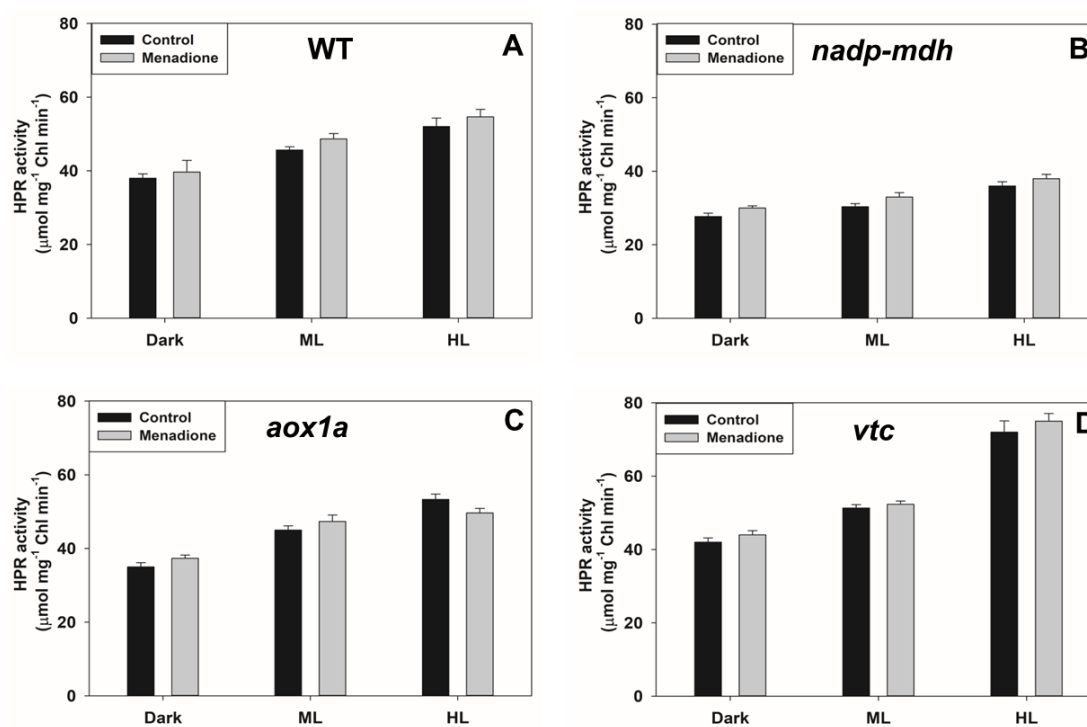


Figure 7.7. The activity of HPR in wild type (A) or the three mutants: *nadp-mdh*, (B); *aox1a*, (C); *vtc*, (D). of *Arabidopsis thaliana*, upon exposure to MD. Further details of experimental conditions were as in Fig. 7.1.

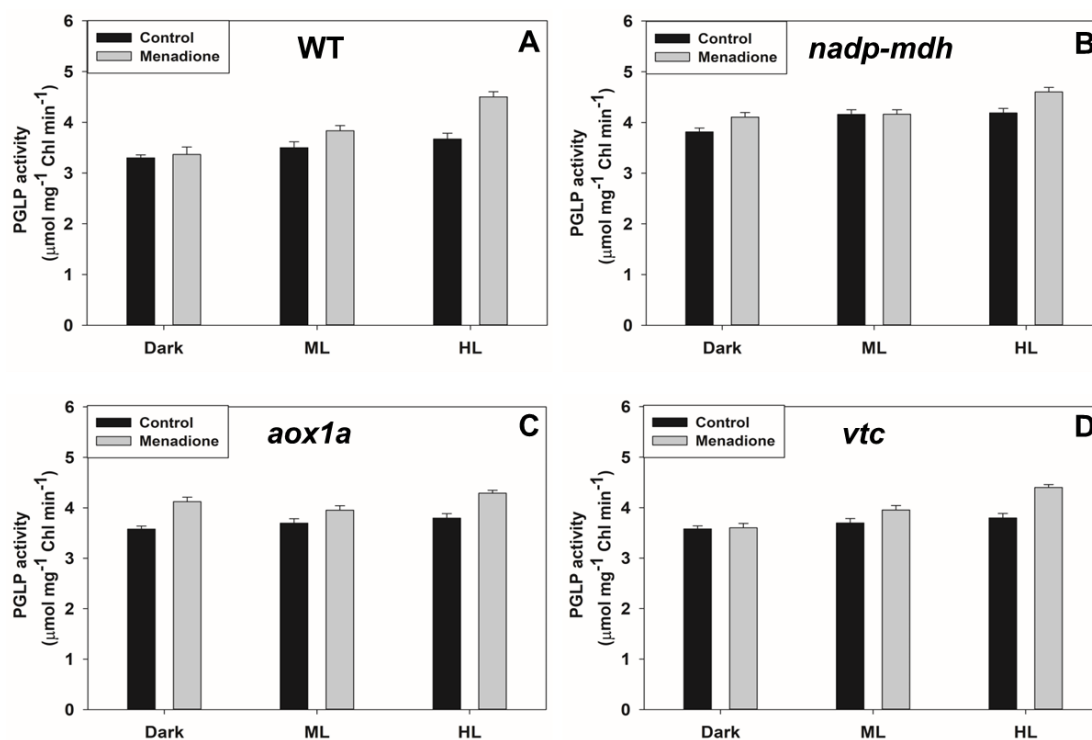


Figure 7.8. The activity of PGLP in wild type (A) or the three mutants: *nadp-mdh*, (B); *aox1a*, (C); *vtc*, (D). of *Arabidopsis thaliana*, upon exposure to MD. Further details of experimental conditions were as in Fig. 7.1.

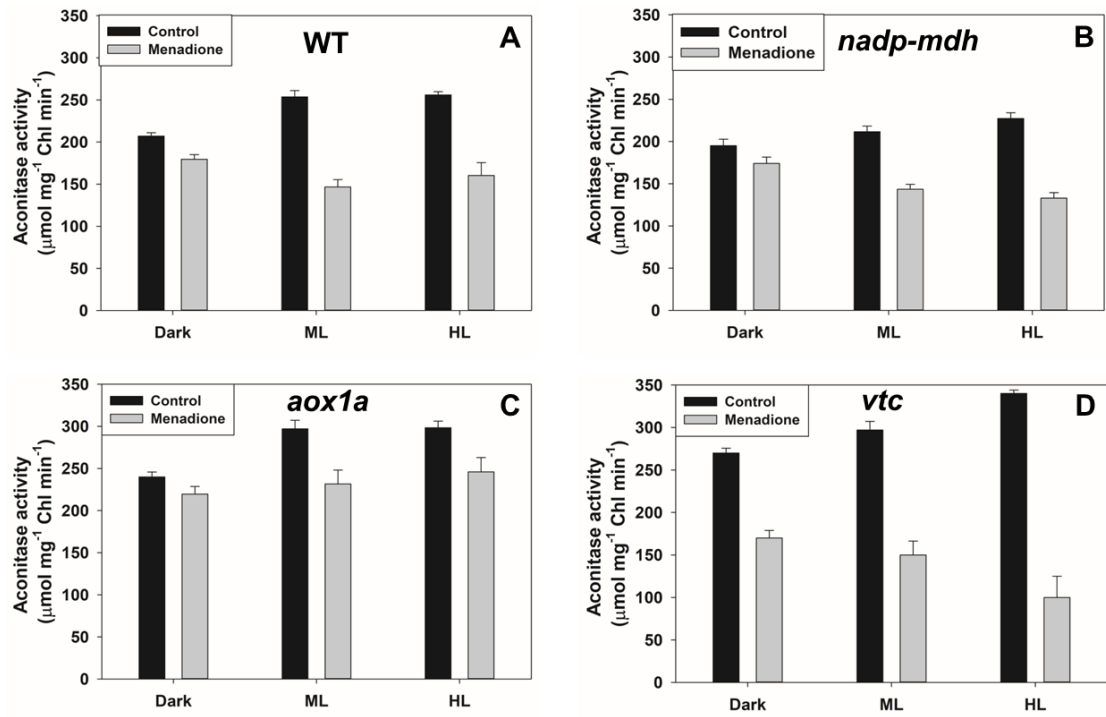


Figure 7.9. The activity of aconitase in wild type (A) or the three mutants: *nadp-mdh*, (B); *aox1a*, (C); *vtc*, (D). of *Arabidopsis thaliana*, upon exposure to MD. Further details of experimental conditions were as in Fig. 7.1.

Discussion

Under stressful conditions, elevated ROS levels can have detrimental impacts on plant metabolism (Szymanska et al., 2017). There are reports which indicated the importance of photorespiratory metabolism under abiotic stress. However, there were no reports on the response of photorespiratory components to oxidative stress by MD in mutants. We tried to assess the consequences of oxidative stress induced by MD on the ROS and photorespiratory metabolism in *Arabidopsis thaliana* WT and in mutants (*nadp-mdh*, *aox1a*, and *vtc*). Our findings highlighted the fact that MD-induced oxidative stress was considerable and highly prominent under HL. The current chapter shows that redox disruptions caused by MD in mitochondria enhanced ROS, and these effects were pronounced in ascorbate deficient *vtc* mutants with compromised redox states.

High accumulation of ROS (superoxide and H₂O₂) in WT and mutants

Zhang et al. (2010) reported an increase in the ROS levels was noticed in WT and *aox1a* mutant of *Arabidopsis* under HL. Similarly, a marked increase in H₂O₂ levels was noticed in *nadp-mdh* mutant of *Arabidopsis thaliana* on exposure to HL (Heyno et al., 2014). In the present work too, the ROS levels were markedly higher in *vtc* mutant than that of WT and *nadp-mdh*, *aox1a* (Figs. 7.1 to 7.4). Thus, the oxidative stress was severe in *vtc* mutant.

A marked increase in the H₂O₂ was noticed in the *vtc* mutant of *Arabidopsis* on exposure to HL (Heyneke et al., 2013; Yao et al., 2015). Under oxidative stress, particularly under HL, the levels of superoxide and

H₂O₂ were raised in pea leaf discs and *Arabidopsis thaliana* (Baxter et al., 2007; Obata et al., 2011; Aswani et al., 2019; Bapatla et al., 2021). Similarly, the levels of ROS were increased in *vtc* mutant than in *Arabidopsis* WT when grown under long-day conditions or infiltrated with a bacterial suspension (Kotchoni et al., 2009; Mukherjee et al., 2010).

Despite the deficiency, the *aox1a* and *nadp-mdh* mutants exhibited similar increases in superoxide or H₂O₂ levels as in the wild type. On the other hand, deficiency of ascorbate made *vtc* mutants raise the superoxide or H₂O₂ much more than that in the wild type. This point suggests that the modulation/response of ROS in plants subjected to oxidative/photooxidative stress creates much stronger stress, if the antioxidant system is weak, as in *vtc* plants. On the other hand, the *aox1a* and *nadp-mdh* plants are known to have primed responses (Clifton et al., 2006; Vishwakarma et al., 2015; Kandoi et al., 2018). We emphasize the significance of ROS in signaling, which helps to coordinate actions in plant cells. Obviously, under the confluence of stresses, plant adaptation depends on ROS modulation (Cerny et al., 2018).

Modulation of photorespiratory enzymes by MD in peroxisomes and chloroplasts, particularly under high light

Our earlier results suggested that photorespiratory enzymes located in different subcellular organelles of photorespiratory metabolism were upregulated under photooxidative and oxidative stress induced by HL and MD (Bapatla et al., 2021). GO, CAT and HPR are located in the peroxisomes, while the PGLP and GK are the chloroplastic ones. Similarly, the increase in photorespiratory enzymes (GO, CAT, HPR, or GLYK) was noticed in tomato

and *Jatropha curcas* under drought stress (Yuan et al., 2016; Silva et al., 2015). An increase in GO and CAT activities was observed in *Ricinus communis* (Neto et al., 2016). These results suggested that photorespiration could help in redox adjustment in different compartments of plant cells.

Among the photorespiratory enzymes, the stimulation of GO during stress was quite subdued in *vtc* mutants. The reason for this needs to be studied further. It is possible that the lack of ascorbate makes the leaves unable to respond and adapt to stress. The moderate response of photorespiratory enzymes in *aox1a* and *nadp-mdh* plants could be due to the robust ability of these mutants to face stress (Scheibe, 2004; Yoshida et al., 2007; Hebbelmann et al., 2012; Vanlerberghe, 2013). An intriguing comparison was made using the enzyme aconitase, which is found in mitochondria. When exposed to MD and HL, aconitase activity reduced (Fig. 7.9). Aconitase is known to be vulnerable to oxidative stress (Lehmann et al., 2009). Exposure to MD reduced the aconitase activity in heterotrophically grown *Arabidopsis* (Baxter et al., 2007; Lehmann et al., 2012) and in pea (Bapatla et al., 2021). Pretreatment with MD led to oxidative stress that caused mitochondrial proteins to degrade, TCA cycle enzyme metabolism to be inhibited, and alterations in NADPH pools (Sweetlove et al., 2002).

Conclusions from this chapter

1. Deficiency of ascorbate in *vtc* mutants lead to much high ROS levels than that in WT in leaves after treatment with MD in light.
2. Despite the deficiency of chloroplastic or mitochondrial redox balance components in the mutants, oxidative damage was not pronounced in *nadp-mdh* and *aox1a* mutants.

3. In parallel to ROS levels, the photorespiratory enzyme components were upregulated in the mutants under HL. However, the presence of MD did not cause much additional effect.
4. Both WT and mutants decreased aconitase activity, a sign of oxidative stress. The aconitase activity was mostly affected in the vtc mutant, which may be due to low ascorbate content than that of WT and other mutants.

Chapter 8

Patterns of Primary Metabolites Under Oxidative and Photo-oxidative Stress in Leaves of *Arabidopsis thaliana*

Chapter 8

PATTERNS OF PRIMARY METABOLITES UNDER OXIDATIVE AND PHOTO- OXIDATIVE STRESS IN LEAVES OF *ARABIDOPSIS THALIANA*

Introduction

Since oxidative stress is an inherent component of abiotic stress conditions, there is a significant overlap in the molecular and physiological responses of plants to such challenges (Apel and Hirt, 2004; Obata et al., 2011). Metabolic alterations often occur in response to different abiotic stresses, and these metabolite changes can also be regarded as signs of oxidative stress (Baxter et al., 2007; Obata et al., 2011; Batista-Silva et al., 2019). Specific alterations in amino acids, carbohydrates, and their derivatives occur under oxidative stress conditions. Thus, stress-induced changes in metabolites are dynamic and challenging to understand. It is essential to identify the distinctive metabolic indicators of oxidative stress in plants (Obata and Fernie, 2012; Lehmann et al., 2012; Ho et al., 2013; Liu et al., 2020).

An additional indicator of oxidative stress, besides the ROS levels, changes in metabolites (Noctor et al., 2015). The metabolite changes can be measured using the “metabolomics” approach (Fernie et al., 2004; Obata and Fernie, 2012). One can assess the degree of oxidative stress in plants by metabolite fingerprints. For instance, exposure to MD increased the levels of isoleucine and valine in heterotrophic cells (Lehmann et al., 2009). Proline and GABA levels increased to help sustain redox balance under stress (Liu et al., 2020). A decrease in the TCA cycle intermediates such as succinate, fumarate, and malate were observed in *Arabidopsis* on exposure to MV

(Sipari et al., 2020). Similarly, sugars such as sucrose, galactose, and raffinose accumulated under chilling stress to provide additional protection (Morsy et al., 2007).

One of the typical responses to oxidative stress is the accumulation of compatible solutes or osmolytes. Compatible solutes produced in larger quantities helped to sustain cellular metabolism (Hare et al., 1998; Yancey, 2005). Amino acids, raffinose family oligosaccharides (RFOs), soluble sugars, and polyamines are common osmolytes. These osmolytes stabilized proteins and enzymes, preserved osmotic balance, and maintained the plant cell turgor (Jorge et al., 2016).

In this chapter, we attempted to determine the levels of 34 metabolites in *Arabidopsis* leaves on exposure to MD. These metabolites belonged to different groups, such as photorespiratory metabolites, TCA cycle metabolites, compatible solutes, etc. The scheme showing the central pathways associated with amino acid biosynthesis and primary metabolism is represented in Fig. 8.1.

Results

Photorespiratory metabolites

Levels of key photorespiratory metabolites (glycine, hydroxypyruvate, 3-phosphoglycerate, serine, and glycerate) were measured. A decrease in the glycine levels, along with the accumulation of hydroxypyruvate, 3-PGA, and serine was observed. The glycerate levels remained unchanged (Fig. 8.2).

Amino acids and osmolytes

Following oxidative treatment, especially in the light, a majority of amino acids accumulated in *Arabidopsis thaliana* leaves.

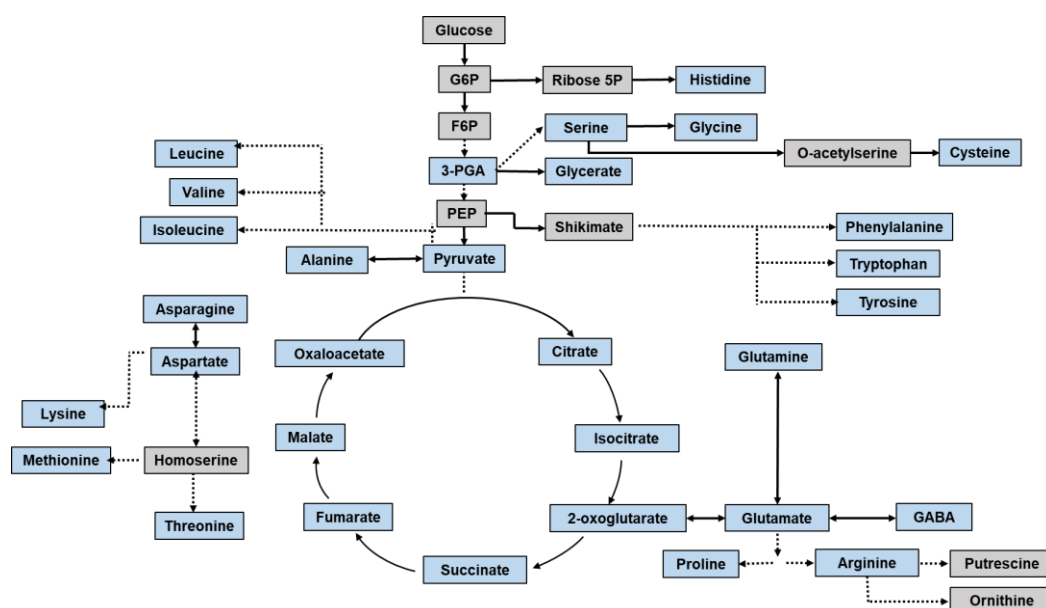


Figure 8.1. Primary metabolism and the related production of amino acids are shown schematically. Measured metabolites were shown in blue box, while those not determined were indicated in grey box (Basic scheme modified from Lehmann et al., 2009).

The levels of alanine, valine, leucine, and isoleucine were reduced on exposure to MD, particularly under HL (Fig. 8.3A-D). The hydroxyl group-containing amino acids such as serine and threonine increased under MD treatment (Fig. 8.3E and F). There was a decreased accumulation of aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Fig. 8.4A-C). Basic amino acids such as lysine, arginine, and histidine were modulated on exposure to MD and HL. Arginine increased while lysine and histidine decreased (Fig. 8.4D-F). Aspartic acid, glutamic acid, and pyruvate accumulated. Marked accumulation of proline and GABA occurred under oxidative/photooxidative stress by MD (Fig. 8.5).

TCA cycle intermediates

Several intermediates of the TCA cycle were measured. Levels of succinate, malate, and citrate were increased, while levels of fumarate, isocitrate, and 2-oxoglutarate were decreased on exposure to MD in light as compared to the respective untreated control (Fig. 8.6).

Other metabolites

The other metabolites, such as glutamine which served as an important nitrogen source of plant growth and development, were decreased under MD treated with light. Asparagine and methionine remained unaffected under photooxidative and oxidative conditions. AMP levels were decreased, particularly under MD treated with light, which now acts as a signaling molecule and plays an important role in sensing and responding to abiotic stresses. The other metabolites, such as cystine and cysteine, were increased particularly under light treated with oxidant (Fig. 8.7).

Discussion starts on page no. 148.

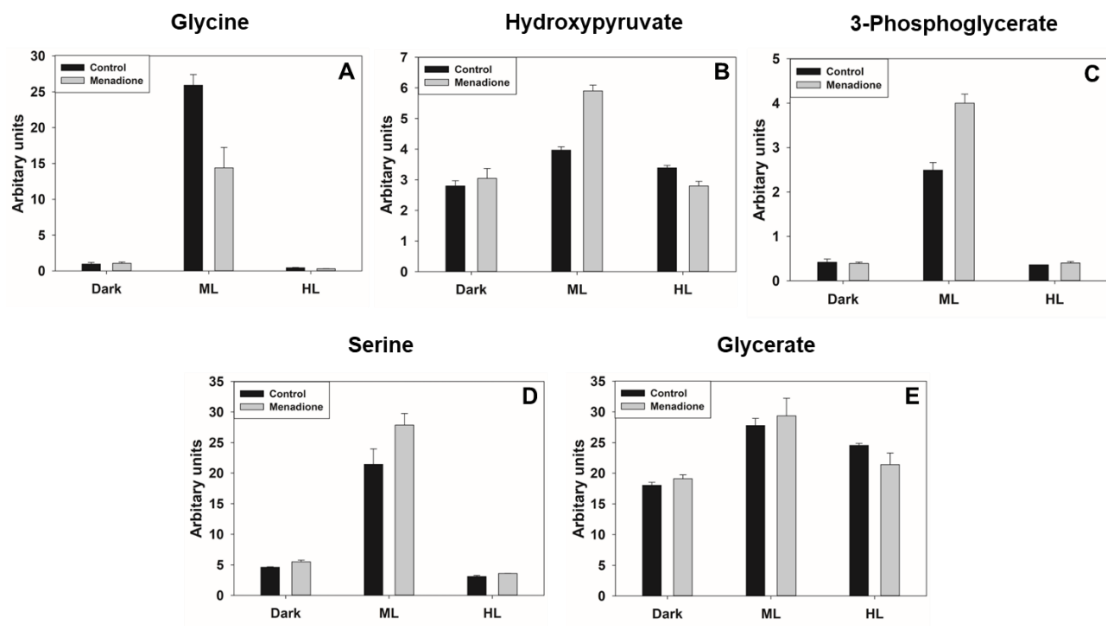


Figure 8.2. Photorespiratory metabolite (glycine, hydroxypyruvate, 3-phosphoglycerate, serine and glycerate) levels in leaves of *Arabidopsis thaliana*. The leaves were exposed to MD for 3 h in either darkness, moderate (ML, $150 \mu\text{E m}^{-2} \text{s}^{-1}$) or high light (HL, $600 \mu\text{E m}^{-2} \text{s}^{-1}$). The values represent averages ($\pm$ SE) from three experiments done on different days.

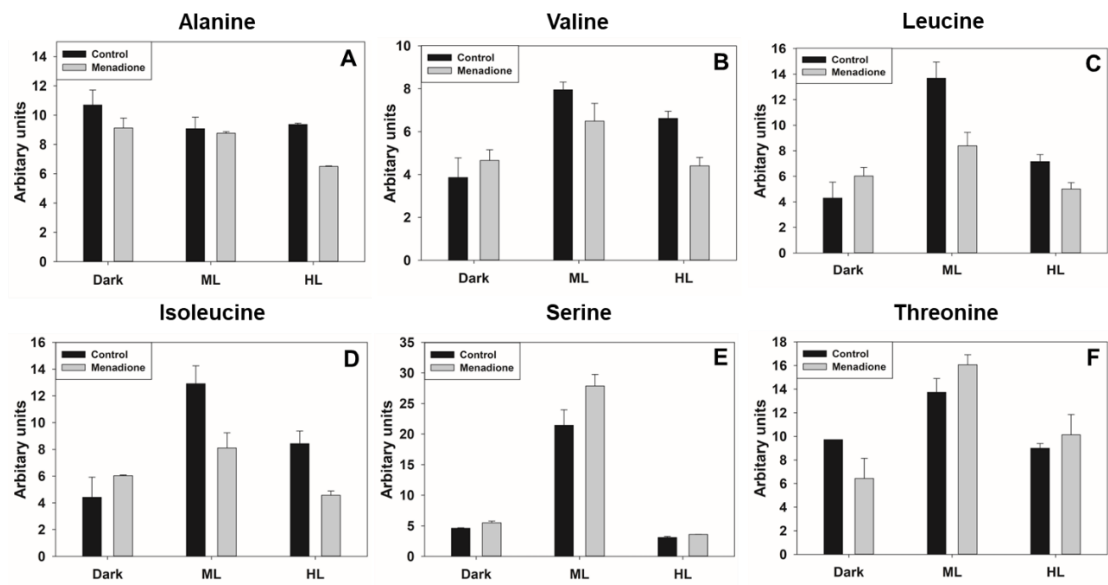


Figure 8.3. The levels of aliphatic amino acids in leaves of *Arabidopsis thaliana*. The treatments and averages were all as in Fig. 8.2.

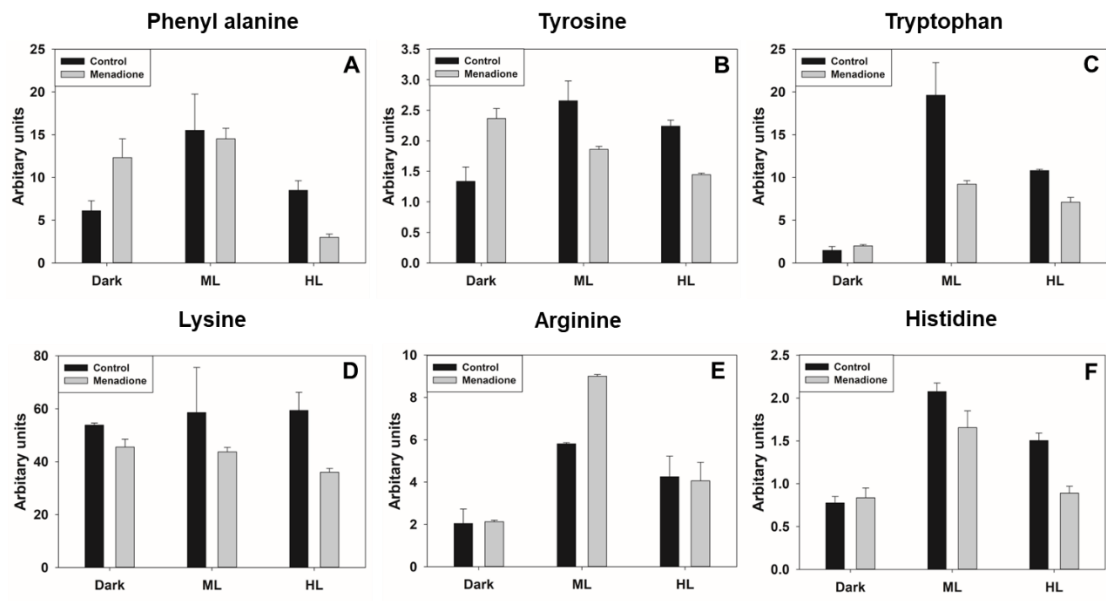


Figure 8.4. Levels of aromatic (phenyl alanine, tyrosine and tryptophan) and basic amino acids (lysine, arginine and histidine) in leaves of *Arabidopsis thaliana*. The treatments and averages were all as in Fig. 8.2.

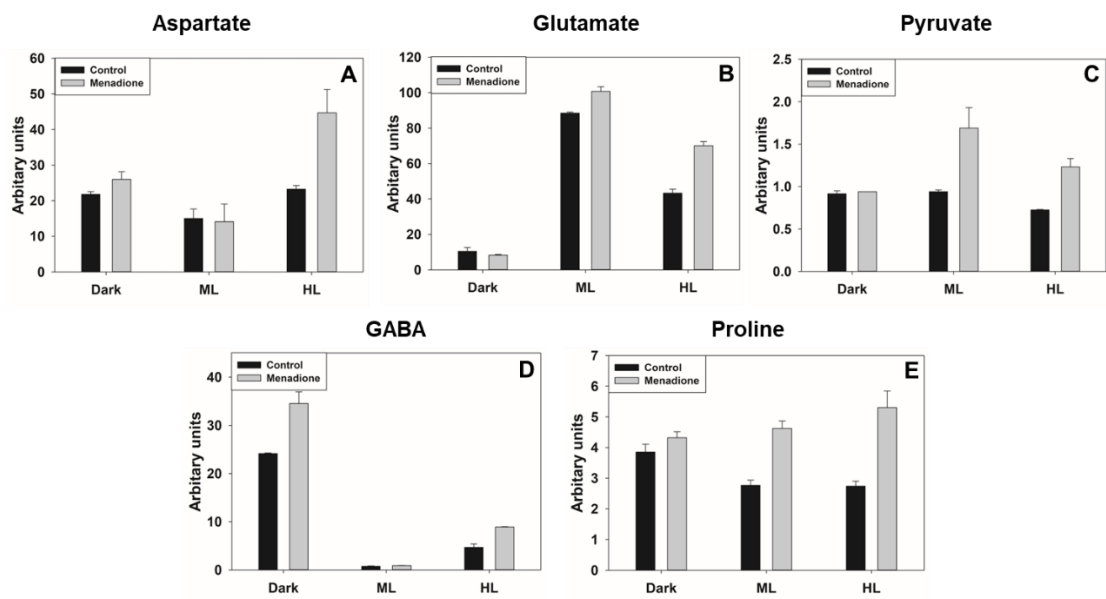


Figure 8.5. Metabolites levels of acidic amino acid (aspartate and glutamate) and compatible solutes (pyruvate, proline and GABA) in leaves of *Arabidopsis thaliana*. The treatments and averages were all as in Fig. 8.2.

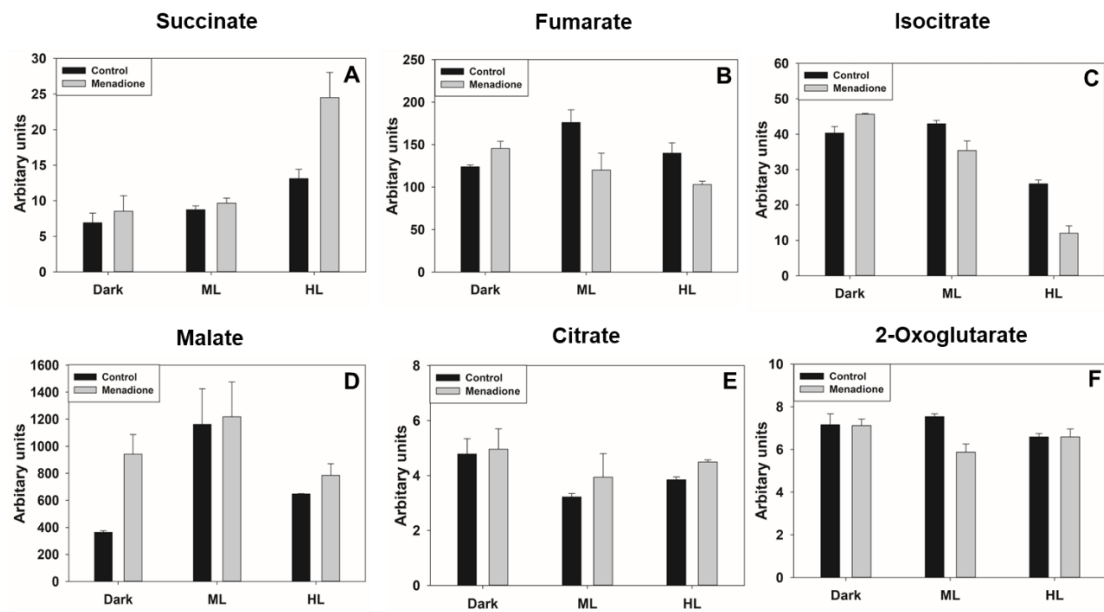


Figure 8.6. Abundance of TCA cycle metabolites (succinate, fumarate, isocitrate, malate, citrate and 2-oxoglutarate) in leaves of *Arabidopsis thaliana*. The treatments and averages were all as in Fig. 8.2.

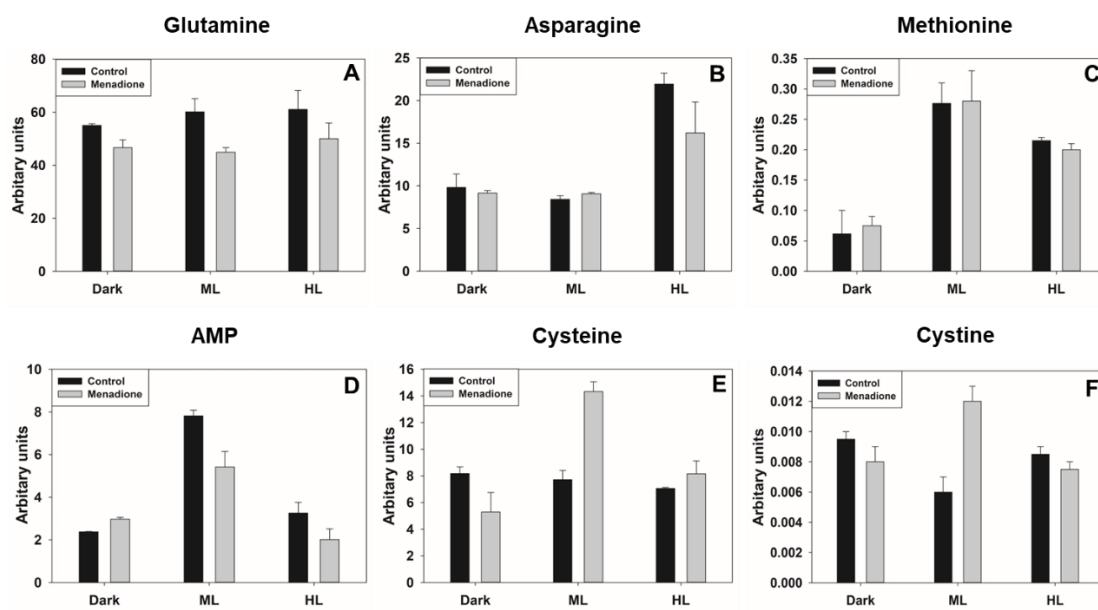


Figure 8.7. Levels of aliphatic amino acids (glutamine, asparagine, methionine, cysteine) and AMP in leaves of *Arabidopsis thaliana*. The treatments and averages were all as in Fig. 8.2.

Discussion

This chapter presented the modulation of 34 primary metabolites, like aliphatic amino acids, aromatic amino acids, photorespiratory metabolites, TCA cycle intermediates, and osmolytes (proline and GABA) under oxidative/photooxidative stress conditions. Oxidative stress was often assessed and related to metabolite changes in stress responses (Noctor et al., 2015). Their levels reflected the redox status of the cell, and whether the plant was under stress or not. A few such metabolites were amino acids, compatible solutes, TCA cycle intermediates, and photorespiratory metabolites (Baxter et al., 2007). Modulation of primary metabolic pathways, which include those of carbon and nitrogen is necessary for plant survival (Krasensky and Jonak, 2012).

Modulation of photorespiratory metabolites under oxidative and photooxidative stress

Photorespiration is modulated under a variety of stress conditions. The upregulation of photorespiratory metabolism in the form of enzyme activities on exposure to oxidative/photooxidative stress were shown in **Chapters 4 and 7**. Photorespiration plays an important role in adaptation of plants to various stresses. Decrease in the glycine levels, whereas accumulation of hydroxypyruvate, 3-PGA, and serine was noticed on exposure to MD (Fig. 8.2). A decrease in glycine and an increase in PGA and HPR upon MD treatment reflected the activation of the photorespiratory pathway (Baxter et al., 2007; Obata and Fernie, 2012).

Accumulation of photorespiratory metabolites were modulated under different stress conditions. Serine and glycine are important building blocks for protein synthesis in mitochondria and perhaps in other regions of the cell. Photorespiration may provide significant amounts of these amino acids (Fernie et al., 2013). Increase in serine and glycine was observed in *Oryza sativa* suspension cells and heterotrophic *Arabidopsis* cells (Ishikawa et al., 2010; Chen and Hoehenwarter, 2015). In contrast to that, glycine levels were decreased under H₂O₂ treatment in heterotrophic *Arabidopsis* cells (Chen and Hoehenwarter, 2015). Numerous primary and secondary metabolic pathways use photorespiratory metabolites as substrates. Plant survival does not require high rates of photorespiration. However, the primary molecular and metabolic responses in plant cells are orchestrated by metabolite and redox signaling cascades, which are closely related to the photorespiratory pathways (Foyer et al., 2009).

Modulation of amino acids

In general, there was modulation in all amino acids and osmolytes on treatment with MD. The activation or deactivation of biosynthetic processes, as well as enhanced or reduced protein breakdown, may contribute to a rise or reduction in the levels of amino acids during stress. Proline, GABA, arginine, and other abundant amino acids are synthesized under varied abiotic stress circumstances. Some of these amino acids served as compatible solutes as well as precursors of secondary metabolites. The levels of GABA and proline, considered compatible solutes, increased under oxidative stress (Fig. 8.5D and E). Even at very high concentrations, these solute molecules are non-toxic and protect by redox equilibrium (Obata and

Fernie, 2012). An increase in proline and GABA was often noticed on exposure to MD in *Arabidopsis thaliana* (Lehmann et al., 2009, 2012). Similarly, an increase in serine and threonine was observed in *Arabidopsis* cells under H₂O₂ and MD treatment (Lehmann et al., 2009; Chen and Hoehenwarter, 2015).

Aspartate and glutamate, which accumulated under oxidative/photooxidative stress (Fig. 8.5A and B), are the important substrates for several other amino acids. Even when plants recovered from stress, these amino acids did not return to their original levels. Aspartate and glutamate levels increased under oxidative stress of *Scrophularia striata* in cell cultures and *Arabidopsis thaliana* on exposure to MD and oxidative stress raised by UV-B (Sadeghenezhad et al., 2016; Gao and Zhang, 2008). Aromatic amino acids like phenylalanine, tyrosine, and tryptophan are also precursors for the synthesis of several secondary compounds, such as pigments. The decrease in aromatic amino acids under MD treatment in *Arabidopsis* suspension cultures was proposed to cause downregulation of the shikimate pathway (Baxter et al., 2007).

Modulation of TCA cycle intermediates

Many biosynthetic pathways depend on TCA cycle intermediates as substrates. Several TCA cycle intermediates were modulated under MD treatment. Levels of succinate, malate, and citrate were increased, while levels of fumarate, isocitrate, and 2-oxoglutarate were decreased (Fig. 8.6), indicating a disturbance in TCA cycle metabolism under oxidative stress. One of the reasons could be due to the sensitivity to oxidative stress of TCA cycle enzymes such as aconitase and pyruvate dehydrogenase (Lehmann et al.,

2009; Sweetlove et al., 2002). The reports on changes in TCA cycle intermediates are ambiguous. The levels of these TCA cycle intermediates were found to increase as in *Arabidopsis thaliana* and *Triticum aestivum* on exposure to H₂O₂, MV, or chlorinated organophosphate esters (Chen and Hoehenwarter, 2015; Sipari et al., 2020; Liu et al., 2020). In contrast, a decrease in fumarate, isocitrate, and 2-oxoglutarate levels were observed in *Arabidopsis thaliana* and *Oryza sativa* suspension cells on exposure to MD and MV (Ishikawa et al., 2010; Lehmann et al., 2012; Sipari et al., 2020).

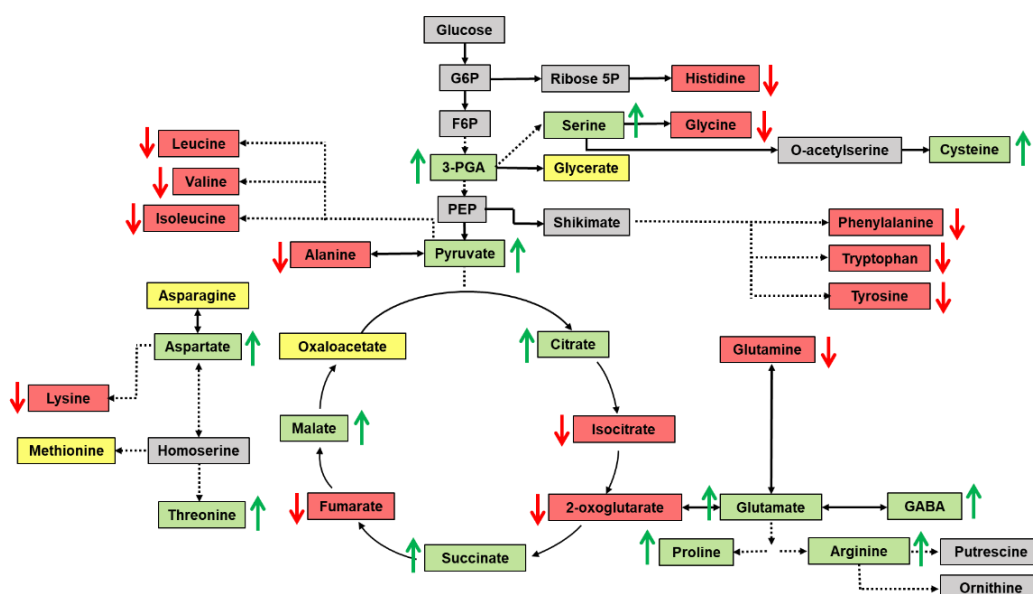


Figure 8.8. Changes in abundance of select primary metabolites upon oxidative/photooxidative stress in leaves of *Arabidopsis thaliana*. Green box/upward arrow denotes an increase, while red box/downward arrow denotes reduction. In contrast to grey ones, which are not analysed, yellow ones suggest no discernible change. The broken arrows denote multiple-step conversion, while a solid arrow represents the single step conversion of the metabolites. The basic scheme was adapted from Lehmann et al. (2009).

Conclusions from this chapter

1. Several metabolites involved in primary metabolism were modulated by MD, even in darkness, obviously to compensate the redox changes or oxidative damage.
2. Metabolite analyses revealed a decrease in glycine and an increase in hydroxypyruvate and phosphoglycerate, reflecting the activation of the photorespiratory pathway.
3. Increase in succinate, malate, and citrate, whereas decrease in fumarate, isocitrate, and 2-oxoglutarate indicated the disturbance in the TCA cycle.
4. The accumulation of compatible solutes in the form of proline and GABA confirmed the oxidative stress by MD and high light.
5. The levels of aliphatic and aromatic amino acid contents, particularly tyrosine and tryptophan, decreased.

Chapter 9

Summary and Conclusions

Chapter 9

SUMMARY AND CONCLUSIONS

Under biotic and abiotic stress, the levels of ROS and RNS invariably increased in plants. These two entities: ROS and RNS, had a strong impact on fundamental metabolic processes like photosynthesis, respiration, and even resulted in cell death (Mittler, 2017; Gong et al., 2020; Kaya et al., 2020; Sychta et al., 2021). ROS are viewed as detrimental byproducts of aerobic metabolism that cannot be avoided but needed for plant cell signalling (Apel and Hirt, 2004). The levels of ROS need to be at optimal levels. Oxidative stress or damage is the result of an imbalance in the production/scavenging of ROS (Mullineaux and Baker, 2010). Photorespiration is now considered to be one of the plant adaptations to oxidative stress.

We examined the influence of an oxidant (MD) and two NO donors (SNP and GSNO) on the photorespiratory enzyme components in pea. Studies were extended to photosynthetic characteristics and related pigments. We also examined the photorespiratory metabolism under restricted photorespiration in *Arabidopsis thaliana*. To verify the findings made on pea under oxidative and photooxidative stress, *Arabidopsis* mutants lacking redox components were employed. At the end of the study, we assessed the response of primary metabolites to oxidative stress, using *Arabidopsis thaliana*. The range of experiments were first with pea (*Pisum sativum*) and later with *Arabidopsis thaliana*. The salient points and their significance are described below.

Changes in ROS, antioxidant systems, and photorespiratory enzyme components under oxidative/photooxidative stress

Our observations on the impact of MD (an oxidant) on ROS formation and changes in antioxidant systems or photorespiratory enzyme components were presented in **Chapter 4**. ROS are crucial cues that help plants adapt to various stresses (Baxter et al., 2007; Lehmann et al., 2009). When pea leaf samples were treated with MD, particularly under HL, there was a marked buildup of ROS (both H_2O_2 and superoxide) (Fig. 4.3). The activities of key photorespiratory enzymes, proteins, and transcript levels were upregulated (Figs. 4.4 to 4.7). The oxidant used in this study, MD acted primarily on mitochondria, but could modulate the enzymes of chloroplasts and peroxisomes. GO, CAT and HPR are peroxisomal enzymes, whereas GLYK and PGLP are chloroplastic. A few authors showed the increase in photorespiratory enzymes under abiotic stress (Silva et al., 2015; Yuan et al., 2016; Neto et al., 2017). But here, we showed the modulation by the stress signal originating in one compartment and its effect on other cellular compartment.

We validated the role of ROS (superoxide and H_2O_2) in upregulating the photorespiratory enzyme components by using Tiron (scavenges superoxide) and catalase (scavenges the H_2O_2). When scavengers of superoxide (tiron) and H_2O_2 (catalase) were added in the incubation media, the increase in ROS levels and photorespiratory enzymes by MD were subdued (Figs. 4.11 and 4.12). Thus, ROS (both superoxide and H_2O_2) generated in mitochondria upregulated the photorespiratory enzyme

components located in other compartments, namely peroxisomes and chloroplasts. This is the first study on the responses of photorespiratory enzymes located in different subcellular compartments by ROS signal from mitochondria.

Modulation of photorespiratory enzymes and photosynthetic characteristics by nitric oxide (NO)

There were many reports on the effect of ROS on photorespiratory metabolism, but the reports on the impact of NO on photorespiratory enzyme components were meagre. We, therefore, checked the modulation by NO of photorespiratory enzyme components and photosynthetic characteristics in pea leaves (**Chapter 5**). NO is a multifaceted free radical found in plants that can modulate various physiological processes (Verma et al., 2020; Lopes-Oliveira et al., 2021; Zhou et al., 2022). The levels of NO and ROS were elevated when leaves were treated with SNP and GSNO, particularly under HL (Figs. 5.1 to 5.3). When cPTIO (a NO scavenger) was present, there was a decrease in NO and ROS levels (Figs. 5.2 and 5.4). The rise in NO and ROS on exposure to SNP and GSNO was noticed in tobacco, sunflower, and *Chlamydomonas reinhardtii* (Ederli et al., 2009; Singh and Bhatia, 2017; Kuo et al., 2020).

The photorespiratory enzymes, activities proteins, and transcript levels located in peroxisomes and chloroplasts were upregulated only marginally under SNP and GSNO treatment (Figs. 5.5 to 5.9). Combining the results obtained with MD and HL (**Chapter 4**) and NO (**Chapter 5**), these results suggest that ROS acted as a preferred signal to modulate photorespiration,

compared to the weak modulation by NO. The suppression of photosynthesis and respiration was severe on exposure to SNP, but was moderate in the presence of GSNO (Fig. 5.17). The inhibition of photosynthesis by NO was noticed often (Ganjewala et al., 2008; Batasheva et al., 2010; Sunil et al., 2020a, b). However, the present results emphasised the sensitivity of respiration as well in pea leaves. The sensitivity of photosynthesis was due to the interference by NO of PSII components followed by PSI. The sensitivity of respiration could be due to the irreversible inhibition of cytochrome c oxidase by NO (Poderoso et al., 2019; Yamasaki et al., 2001).

The minimal loss of chlorophylls and carotenoid pigments on exposure to GSNO (Fig. 5.18), reaffirmed that GSNO be preferred as NO-donor over SNP. Both photosystems (PSII and PSI) appeared to be targets of NO (Figs. 5.19 and 5.20). However, PSII was more sensitive than PSI to NO, as indicated by Chl fluorescence parameters. As per the earlier literature, NO either downregulated PSII-related parameters (Wodala et al., 2005, 2008; Chang et al., 2013) or upregulated the PSI related reactions (Wodala and Horvath 2008). In our studies, SNP exerted stronger inhibition on PSII components than GSNO. Exogenous NO donors strongly inhibited the PSII electron transport between Q_A and Q_B (Diner and Petrouleas, 1990; Wodala et al., 2008; Vladkova et al., 2011).

Modulation of ROS levels and photorespiratory enzyme components in leaves of *Arabidopsis thaliana* when photorespiration was compromised

The available options to restrict photorespiration include high CO₂, low O₂, and the use of photorespiratory inhibitors and mutants. For our study, we

used AOA and low O_2 to restrict photorespiration (**Chapter 6**). AOA decreased the photosynthetic efficiency and inhibited photorespiration by preventing glyoxylate to glycine conversion (Riazunnisa et al., 2006; Kamalanathan et al., 2022). Low O_2 slowed down the activity of RuBP oxygenase and restricted the photorespiratory cycle. When photorespiration was restricted, ROS (both superoxide and H_2O_2) levels were elevated, irrespective of dark or light conditions (Figs. 6.2 and 6.3). Such increases in the H_2O_2 and superoxide content were observed in also rice, tobacco, and *Arabidopsis thaliana* under hypoxic conditions (Liu et al., 2017; Yamaguchi et al., 2017; Jayawardhane et al., 2020). When present, AOA increased ROS levels in *Dunaliella tertiolecta*, too (Kamalanathan et al., 2022). As per our results, ROS production got enhanced under restricted photorespiratory conditions. The elevated ROS can affect the various physiological processes, including photorespiration, and could even lead to cell death in plants (Mittler et al., 2002, 2017).

Restricting photorespiration downregulated the activities, proteins, and transcript levels of three photorespiratory enzymes (Figs. 6.4 to 6.8). The effects of low O_2 on GO activity were ambiguous. For example, there was a decrease in the GO, CAT, and PGLP activity in barley exposed to low O_2 (Hodges and Forney, 2000; Yordanova et al., 2003). On exposure to phosphinothricin and under hypoxic conditions, GO activity increased in *Zea mays* and rice (Gonzalez-Moro et al., 1993; Nichugovskaya and Shevchenko, 1990). The raising of the ROS levels and downregulation of the photorespiratory enzymes by AOA or low O_2 imply that photorespiration, if unrestricted, can help keep the ROS levels low.

Changes in ROS and photorespiratory enzyme components in *Arabidopsis* mutants lacking genes involved in redox related components

The status of photorespiratory metabolism in *Arabidopsis* wild type and three mutants lacking genes involved in redox-stabilizing components was assessed (**Chapter 7**). The mutants were *nadp-mdh* (deficient in malate valve or chloroplastic NADP-malate dehydrogenase), *vtc* (deficient in ascorbate), and *aox1a* (deficient in mitochondrial alternative oxidase). The extent of ROS accumulation on treatment with MD in *nadp-mdh*, *aox1a* mutant was almost similar to WT plants (Figs. 7.1 to 7.4). However, the levels of H₂O₂ and superoxide were much higher in *vtc* mutants than that of WT, obviously due to the deficiency in ascorbate and antioxidant. A marked increase in the H₂O₂ was noticed in *vtc* mutant of *Arabidopsis* on exposure to HL (Heyneke et al., 2013; Yao et al., 2015). Besides HL, exposure to MD also elevated the ROS levels in *Arabidopsis* and pea (Sweetlove et al., 2002; Aswani et al., 2019; Bapatla et al., 2021). We, therefore, emphasise that ROS is crucial in signalling, to coordinate metabolism in plant cells. Under the confluence of stresses, plant adaptation depended on ROS modulation (Cerny et al., 2018).

The increase in activities of GO, CAT, and PGLP, with not much change in HPR under oxidative stress in WT and mutants (*nadp-mdh*, *aox1a*, and *vtc*) (Figs. 7.5 to 7.8). Thus, *vtc* mutant was more vulnerable to oxidative stress, while the *aox1a* and *nadp-mdh* plants were less sensitive, as in earlier reports (Vishwakarma et al., 2015; Kandoi et al., 2018). The moderate response of photorespiratory enzymes in *aox1a* and *nadp-mdh* plants could

be due to the robust ability of these mutants to face stress (Scheibe, 2004; Yoshida et al., 2007; Hebbelmann et al., 2012; Vanlerberghe, 2013).

Modulation of primary metabolites in leaves of *Arabidopsis thaliana*

The changes in 34 chosen metabolites under oxidative/photooxidative stress induced by HL and MD were studied (**Chapter 8**). Their concentrations reflect the cell's redox state and metabolic capability (Noctor et al., 2015). Amino acids, compatible solutes, TCA cycle intermediates, and photorespiratory metabolites were a few of these metabolites (Yancey, 2005; Baxter et al., 2007; Ghassemian et al., 2008; Obata and Fernie, 2012). A decrease in the glycine levels along with the accumulation of hydroxypyruvate, 3-PGA, and serine was noticed on exposure to MD (Fig. 8.2). Such decrease in glycine and an increase in PGA/hydroxypyruvate implied the activation of the photorespiratory pathway (Baxter et al., 2007; Obata and Fernie, 2012).

Marked modulation of amino acids occurred on treatment with MD (Figs. 8.2 to 8.7). The amino acids profile was often altered under oxidative or photooxidative stress (Gao and Zhang, 2008; Chen and Hoehenwarter, 2015; Sadeghenezhad et al., 2016). Proline and GABA served as compatible solutes as well as precursors of secondary metabolites needed under stress conditions. Both these amino acids help in protecting the plants by reducing ROS levels (Obata and Fernie, 2012). Increase in proline and GABA on exposure to MD in *Arabidopsis thaliana* roots was observed (Lehmann et al., 2009, 2012). The decrease in aromatic amino acids like phenylalanine, tyrosine, and tryptophan upon oxidative stress was noticed (Fig. 8.4A-C),

which might be due to the downregulation of the shikimate pathway (Baxter et al., 2007). The modulated levels of several organic acids (Fig. 8.5), indicated a disturbance in TCA cycle metabolism. Such an effect under oxidative stress is in accordance with previous reports (Ishikawa et al., 2010; Lehmann et al., 2012; Sipari et al., 2020; Liu et al., 2020).

In conclusion, there were noticeable alterations in metabolites linked to amino acids, compatible solutes, TCA cycle metabolites, and photorespiratory metabolites under oxidative/photooxidative stress conditions. These modifications might be designed to sustain the energy supply, limit the damage by protective osmolytes, and safeguard the plants from oxidative stress.

The key findings from the current study are as follows:

1. Menadione (MD), an oxidant targeting mitochondria, upregulated the photorespiratory components in chloroplasts or peroxisomes. Obviously, the stress signal generated in mitochondria could communicate with chloroplasts and peroxisomes. Both H_2O_2 and superoxide were involved as the signals in such upregulation of photorespiration.
2. External NO donors (SNP and GSNO) induced NO accumulation but modulated the photorespiratory metabolism only marginally. The reason could be the low ROS levels in the presence of NO.
3. The increase in nitrosothiols and tyrosine nitrated proteins confirmed nitro-oxidative stress, which could be the reason for the inhibition of both photosynthesis and respiration. The components of PSII and PSI were aggravated by NO in the presence of HL. The photosynthetic pigment

levels were also decreased by the interference of NO to PSII.

4. When photorespiration was suppressed by low O₂ or inhibition of glycine production (by AOA), the ROS levels increased, while the photorespiratory enzymes were downregulated. Thus, the operation of photorespiration was essential to keep ROS levels low during abiotic stress.
5. A deficiency in not only *nadp-mdh* but also ASC (*vtc*) or AOX (*aox1a*), as in the selected mutants, elevated the ROS levels (both superoxide and H₂O₂) and upregulated the photorespiratory enzymes located in different subcellular compartments.
6. Despite the disturbance in redox balance elements in the *nadp-mdh* and *aox1a* mutants, there was not much oxidative stress in these mutants. We propose that the deficiency of *NADP-MDH* and *AOX1a* could have primed the plants to endure oxidative/photooxidative stress. In contrast, the *vtc* mutants were quite vulnerable to oxidative stress due to low ascorbate levels.
7. Metabolite analyses revealed the modulation in TCA cycle intermediates, decrease in glycine, and increase in hydroxypyruvate/phosphoglycerate, reflecting the activation of photorespiratory pathway on treatment with MD, a stress inducer. The accumulation of proline and GABA confirmed the oxidative stress by MD, particularly under high light. It is possible that these mutants assist in both energy supply and protection of plants against the oxidative stress.

Concluding remarks and future perspectives

Our studies establish that ROS acted as a major signal over NO to modulate the photorespiratory metabolism. Thus, ROS was a strong modulator of photorespiratory metabolism, while NO is not that much strong as ROS. Photorespiration played a key role in providing tolerance against abiotic stress. In turn, restricted photorespiration makes the plants more vulnerable to stress. Oxidative stress modulated several metabolites possibly located in a different compartment, and this response could protect the plants from oxidative damage induced by MD.

There are several points that need further studies. The present study focussed mostly on photorespiratory carbon metabolism. Since photorespiration is connected to nitrogen uptake and utilization, it would be quite interesting to study the effects of ROS and RNS on the enzyme components related to nitrogen metabolism. A better knowledge of the photorespiratory pathway would result from research on the comparative effects of PTMs induced by ROS and RNS. Against enzymes and proteins relevant to nitrogen metabolism and photorespiration need to be studied. It is important to understand the molecular or biochemical cues that initiate photorespiration, i.e., the mobility of signals like ROS (H_2O_2 and superoxide) or RNS (especially NO) between cellular compartments, and metabolite fluxes. Transporters mediate the dynamic exchange of metabolites across cellular compartments and facilitate proper metabolite flux. Therefore, an interesting but challenging aspect is the role of metabolite transporters and their response to oxidative or nitrosative stress.

Chapter 10

Literature Cited

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LITERATURE CITED

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Appendix

**Research Articles Published and Papers
Presented at Conferences by Deepak Saini**
(First pages of the articles are attached)

LIST OF PUBLICATIONS/ PRESENTATIONS BY DEEPAK SAINI

PUBLICATIONS IN REFEREED JOURNALS

1. **Saini, D.**, Bapatla, R. B., Pandey, J., Aswani, V., Sunil, B., Gahir, S., Bharath, P., Subramanyam, R., & Raghavendra, A. S. (2022). Modulation by S-nitrosoglutathione, a natural nitric oxide donor, of photochemical components of pea (*Pisum sativum*) leaves, as revealed by chlorophyll fluorescence patterns: Aggravation of nitric oxide-effects by light. *Plant Science Today* (Accepted).
2. **Saini, D.**, Rao, D. E., Bapatla, R. B., Aswani, V., & Raghavendra, A. S. (2022). Enzymatic assays of photorespiratory cycle under abiotic stresses. *Methods in Molecular Biology*, Plant Stress Tolerance, 3rd Edition, 2023, Springer Nature Publishers, New York (Accepted).
3. Pandey, J., Devadasu, E. R., **Saini, D.**, Dhokne, K., Marriboina, S., Raghavendra, A. S., & Rajagopal, S. (2022). Changes in photosynthetic apparatus of pea (*Pisum sativum*) leaves under drought stress: Aggregation of LHII and PSI core complexes. *The Plant Journal*, doi.org/10.1111/tpj.16034. In press.
4. Bapatla, R. B., **Saini, D.**, Aswani, V., Rajsheel, P., Sunil, B., Timm. S., & Raghavendra, A. S. (2021). Modulation of photorespiratory enzymes by oxidative and photo-oxidative stress induced by menadione in leaves of pea (*Pisum sativum*). *Plants*, 10, 97.
5. Sunil, B., **Saini, D.**, Bapatla, R. B., Aswani, V., & Raghavendra, A. S. (2019). Photorespiration is complemented by cyclic electron flow and the alternative oxidase pathway to optimize photosynthesis and protect against abiotic stress. *Photosynthesis Research*, 139, 67-79.

PRESENTATIONS AT SCIENTIFIC MEETINGS

1. **Saini, D., & Raghavendra, A. S. (2022).** Modulation of photorespiratory enzymes by oxidative and photo-oxidative stress induced by menadione in leaves of pea, *Pisum sativum*. Oral presentation at '*International Conference on Physiological and Molecular Mechanisms for Abiotic Stress Tolerance in Plants*' held at Department of Botany, University of Calicut, Kerala on 26th October-28th October, 2022.
2. **Saini, D., & Raghavendra, A. S. (2020).** Impact of nitric oxide release by NO donors on photorespiratory metabolism in leaf discs of pea, *Pisum sativum*. Oral presentation at '*National Conference on Frontiers in Plant Biology*' held at Department of Plant Sciences, University of Hyderabad, Hyderabad on 31st January-1st February, 2020.
3. **Saini, D., & Raghavendra, A. S. (2019).** Modulation of photorespiratory metabolism by SNP and GSNO in leaf discs of pea, (*Pisum sativum*). Oral presentation at '*GIAN-2019*' held at University of Hyderabad, Hyderabad, during 26th August-6th September, 2019.
4. **Saini, D., & Raghavendra, A. S. (2018).** Effect of nitric oxide (NO) on photorespiratory metabolism in leaf discs of pea (*Pisum sativum*). Poster presentation at '*6th International Conference on Molecular Signaling*' held at University of Hyderabad, Hyderabad during 8th February-10th February, 2018
5. **Saini, D., & Raghavendra, A. S. (2018).** Upregulation of photorespiratory enzymes by nitric oxide (NO) despite being

antioxidant in leaf discs of pea (*Pisum sativum*). Poster presentation at '4th International Plant Physiology Congress' held at Lucknow, during 2nd December-5th December, 2018

6. **Saini, D., & Raghavendra, A. S. (2017).** Modulation of photosynthesis and photorespiration by reactive oxygen species (ROS) and nitric oxide (NO) in leaves of pea (*Pisum sativum*). Oral presentation at 'SAKURA Exchange Program in Science' held at Okayama University, Okayama, Japan during 4th September-11th September, 2017
7. **Saini, D., & Raghavendra, A. S. (2017).** Effect of sodium nitrite on chlorophyll content and levels of nitric oxide and reactive oxygen species during high light in leaf discs of pea (*Pisum sativum*). Poster presentation at 'BIOQUEST', University of Hyderabad, Hyderabad held during 12th October-13th October, 2017
8. **Saini, D., & Raghavendra, A. S. (2017).** Effect of H₂O₂ and SNP (nitric oxide donor) on antioxidant defense mechanisms in leaf discs of pea (*Pisum sativum*). Poster presentation at '8th International Conference on Photosynthesis and Hydrogen Energy Research for Sustainability' held at University of Hyderabad, Hyderabad during 30th October-4th November, 2017

November 26, 2022

Dear Author(s),

We have reached a decision regarding your submission (PST # 2248) to ***Plant Science Today***, "**Modulation by S-nitrosoglutathione, a natural nitric oxide donor, of photochemical components of pea (*Pisum sativum*) leaves, as revealed by chlorophyll fluorescence patterns: Aggravation of nitric oxide-effects by light**", submitted by

Deepak Saini, Ramesh B. Bapatla, Jayendra Pandey, Vetcha Aswani, Bobba Sunil, Shashibhushan Gahir, Pulimamidi Bharath, Rajagopal Subramanyam and Agepati S. Raghavendra*

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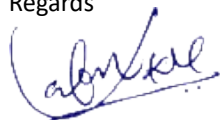
Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

*Corresponding author: asrsls@gmail.com

Our decision is to: **Accept submission**

Date of acceptance: 26 November 2022

Regards



Dr. K.K. Sabu

Editor-in-Chief, Plant Science Today
& Principal Scientist

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Reversible changes in structure and function of photosynthetic apparatus of pea (*Pisum sativum*) leaves under drought stress

Jayendra Pandey , Elsinraju Devadasu[†] , Deepak Saini , Kunal Dhokne , Sureshbabu Marriboina ,
Agepati S. Raghavendra  and Rajagopal Subramanyam^{*} 

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SUMMARY

The effects of drought on photosynthesis have been extensively studied, whereas those on thylakoid organization are limited. We observed a significant decline in gas exchange parameters of pea (*Pisum sativum*) leaves under progressive drought stress. Chl *a* fluorescence kinetics revealed the reduction of photochemical efficiency of photosystem (PS)II and PSI. The non-photochemical quenching (NPQ) and the levels of PSII subunit PSBS increased. Furthermore, the light-harvesting complexes (LHCs) and some of the PSI and PSII core proteins were disassembled in drought conditions, whereas these complexes were reassociated during recovery. By contrast, the abundance of supercomplexes of PSII-LHCII and PSII dimer were reduced, whereas LHCII monomers increased following the change in the macro-organization of thylakoids. The stacks of thylakoids were loosely arranged in drought-affected plants, which could be attributed to changes in the supercomplexes of thylakoids. Severe drought stress caused a reduction of both LHCI and LHCII and a few reaction center proteins of PSI and PSII, indicating significant disorganization of the photosynthetic machinery. After 7 days of rewatering, plants recovered well, with restored chloroplast thylakoid structure and photosynthetic efficiency. The correlation of structural changes with leaf reactive oxygen species levels indicated that these changes were associated with the production of reactive oxygen species.

Keywords: disassembly of LHCII and PSI core, drought stress, non-photochemical quenching, pigment–protein complexes, photosystem, *Pisum sativum*, thylakoid organization.

INTRODUCTION

Drought stress is a significant environmental constraint that hampers crop yield, particularly in arid and semi-arid regions. There is an urgent need to develop varieties that can grow under limited water conditions without compromising yields. In this direction, it is crucial to understand the physiological, molecular, and photosynthetic responses of crop species under drought. Upon exposure to drought stress, plants displayed various morphological symptoms such as wilting, desiccation of leaves, chlorosis, leaf curling, burning of leaf edges, and necrosis (Seleiman et al., 2021). Parallely, the stomatal conductance decreased to reduce the water evaporation through leaves (Li et al., 2017). Low stomatal conductance decreases intercellular CO₂, minimizing the supply for photosynthesis (Kelly et al., 2016). As a result, plants water use efficiency (WUE) decreased. Similarly, the leaf relative water content (RWC), an indicator of stress intensity, was also lowered.

The drought-induced imbalance between light capture and its utilization leads to the accumulation of reactive oxygen species (ROS) in the chloroplast and, subsequently, the disorganization of thylakoid membranes (Das & Roychoudhury, 2014). To combat ROS, plants utilize several antioxidant enzymes, such as ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) during drought stress (Thakur & Anand, 2021). The net photosynthetic rate, chlorophyll (Chl) fluorescence, and antioxidant activities were significantly altered under water deficit conditions (Iqbal et al., 2019). The fast Chl *a* fluorescence is an efficient parameter to monitor the photosystem (PS)II and PSI photochemistry (Sánchez-Reinoso et al., 2019; Sipka et al., 2021). However, drought stress can limit the availability of water molecules for the photolysis of water, affecting the efficiency of the PSII oxygen-evolving complex (OEC), particularly D1 activity of PSII (Sasi et al., 2018).

Article

Modulation of Photorespiratory Enzymes by Oxidative and Photo-Oxidative Stress Induced by Menadione in Leaves of Pea (*Pisum sativum*)

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Citation: Bapatla, R.B.; Saini, D.; Aswani, V.; Rajsheel, P.; Sunil, B.; Timm, S.; Raghavendra, A.S. Modulation of Photorespiratory Enzymes by Oxidative and Photo-Oxidative Stress Induced by Menadione in Leaves of Pea (*Pisum sativum*). *Plants* **2021**, *10*, 987. <https://doi.org/10.3390/plants10050987>

Academic Editors: Magda Pál and Pedro Diaz-Vivancos

Received: 27 March 2021

Accepted: 12 May 2021

Published: 15 May 2021

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Abstract: Photorespiration, an essential component of plant metabolism, is concerted across four subcellular compartments, namely, chloroplast, peroxisome, mitochondrion, and the cytoplasm. It is unclear how the pathway located in different subcellular compartments respond to stress occurring exclusively in one of those. We attempted to assess the inter-organelle interaction during the photorespiratory pathway. For that purpose, we induced oxidative stress by menadione (MD) in mitochondria and photo-oxidative stress (high light) in chloroplasts. Subsequently, we examined the changes in selected photorespiratory enzymes, known to be located in other subcellular compartments. The presence of MD upregulated the transcript and protein levels of five chosen photorespiratory enzymes in both normal and high light. Peroxisomal glycolate oxidase and catalase activities increased by 50% and 25%, respectively, while chloroplastic glycerate kinase and phosphoglycolate phosphatase increased by ~30%. The effect of MD was maximum in high light, indicating photo-oxidative stress was an influential factor to regulate photorespiration. Oxidative stress created in mitochondria caused a coordinative upregulation of photorespiration in other organelles. We provided evidence that reactive oxygen species are important signals for inter-organelle communication during photorespiration. Thus, MD can be a valuable tool to modulate the redox state in plant cells to study the metabolic consequences across membranes.

Keywords: cellular compartments; chloroplasts; high light; menadione; mitochondria; oxidative stress; peroxisomes; photorespiration

1. Background

Photosynthesis in higher plants is affected by abiotic stress such as oxidative conditions and high light (HL) intensities. Prolonged exposure to such stress can damage the photosynthetic apparatus, particularly PSII, resulting in photoinhibition [1–3]. Nevertheless, plants try to protect photosynthesis against photoinhibition by operating different compartments of their cells [4]. Interactions between chloroplasts and mitochondria through peroxisomes and cytosol are essential for optimizing photosynthesis [4–7]. In an earlier report, Saradadevi and Raghavendra [8] demonstrated that mitochondrial oxidative electron transport and phosphorylation could protect photosynthesis against photodamage in pea mesophyll protoplasts. In addition to dark mitochondrial respiration, photorespiration is now acknowledged to be a protective mechanism against photoinhibition, as indicated by the classic work on photorespiratory mutants [9–11]. Readers interested in photorespiratory metabolism can refer to several reviews, which appeared periodically [12–16]. In addition, photorespiration is a classic example of the compartmentation in different cellular



Photorespiration is complemented by cyclic electron flow and the alternative oxidase pathway to optimize photosynthesis and protect against abiotic stress

Bobba Sunil¹ · Deepak Saini¹ · Ramesh B. Bapatla¹ · Vetcha Aswani¹ · Agepati S. Raghavendra¹

Received: 6 June 2018 / Accepted: 24 August 2018 / Published online: 5 September 2018
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Abstract

Optimization of photosynthetic performance and protection against abiotic stress are essential to sustain plant growth. Photorespiratory metabolism can help plants to adapt to abiotic stress. The beneficial role of photorespiration under abiotic stress is further strengthened by cyclic electron flow (CEF) and alternative oxidase (AOX) pathways. We have attempted to critically assess the literature on the responses of these three phenomena—photorespiration, CEF and AOX, to different stress situations. We emphasize that photorespiration is the key player to protect photosynthesis and upregulates CEF as well as AOX. Then these three processes work in coordination to protect the plants against photoinhibition and maintain an optimal redox state in the cell, while providing ATP for metabolism and protein repair. H_2O_2 generated during photorespiratory metabolism seems to be an important signal to upregulate CEF or AOX. Further experiments are necessary to identify the signals originating from CEF or AOX to modulate photorespiration. The mutants deficient in CEF or AOX or both could be useful in this regard. The mutual interactions between CEF and AOX, so as to keep their complementarity, are also to be examined further.

Keywords Alternative oxidase · Chloroplasts · Glycine decarboxylase · Photoinhibition · Reactive oxygen species · Redox homeostasis

Introduction

Plants are sessile and often exposed to stressful environments due to suboptimal or supraoptimal conditions like drought, high or low CO_2 , high or low light, high or low temperature, limiting N/P/K and heavy metal stress. The abiotic stress causes alterations in metabolism, nutrient uptake, growth and development. Severe stress results in damage and cell death (Wang et al. 2008; Verslues et al. 2006). The basal factor during most of the stresses is the excess production of reactive oxygen species (ROS) much more than its removal by scavenging (Miller et al. 2010; Suzuki et al. 2012). The ROS include radicals such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals. Besides the accumulation of ROS, abiotic stress frequently

causes an imbalance in the supply/demand of ATP/NADPH, changes in membrane structure and additional damage due to lipid peroxidation products (Mignolet-Spruyt et al. 2016). The production of ROS and the energy/redox imbalance occurs among different compartments of cell.

Plants have the ability to respond and acclimate to these adverse conditions. In order to protect themselves, plants employ adaptive mechanisms mainly to scavenge the excess ROS using antioxidants (e.g. ascorbate, glutathione, carotenoids, flavonoids) and/or antioxidant enzymes (e.g. catalase, superoxide dismutase) (Gratao et al. 2005; Scandalios 2005; Racchi 2013; Acosta-Motos et al. 2017). Additional protective mechanisms attempt to achieve an energy balance (ATP/NADPH). There are excellent reviews on the adaptive responses of plants to various stresses (to cite a few: Miller et al. 2010; Baxter et al. 2014; Golldack et al. 2014; Mignolet-Spruyt et al. 2016; Acosta-Motos et al. 2017; Szymanska et al. 2017; Zarattini and Forlani 2017).

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Impact of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on photorespiratory enzyme components in leaves of *Pisum sativum* and *Arabidopsis thaliana*

by Deepak Saini


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The above ② were a part of the present thesis of Deepak Saini. Thus, the final similarity index would be 6% and within the acceptable limit.

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Stefan Timm, Nicole Klaas, Janice Niemann, Kathrin Jahnke et al. "Simultaneous adjustments of major mitochondrial pathways through redox regulation of dihydrolipoamide dehydrogenase (mtLPD1)", Cold Spring Harbor Laboratory, 2022

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"Taurine modulates dynamics of oxidative
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