DOES ALTERNATIVE OXIDASE PLAY A ROLE IN PLANT ADAPTATION TO ANOXIA AND POST-ANOXIA?

By

Jayamini Jayawardhane

A thesis submitted to the School of Graduate Studies in conformity with the requirements for the degree of Master of Science

Department of Biology

Memorial University of Newfoundland, St. John's, Canada

December 2019

This work is dedicated to my loving parents, husband and the teachers

Abstract

Low oxygen stress and reaeration have detrimental effects on plants. Transgenic tobacco plants differentially expressing alternative oxidase (AOX) were used to investigate its role during anoxia and post-anoxia. Superoxide, hydrogen peroxide and malondialdehyde (MDA) levels increased under anoxia and post-anoxia exhibiting lower level in AOX-overexpressing lines. Nitric oxide (NO) emission increased during anoxia stronger in overexpressing lines while nitrosylation of proteins followed a reverse pattern. ATP/ADP ratio decreased during anoxia and increased with reaeration being higher in overexpressing lines. Alcohol dehydrogenase and aconitase increased during anoxia and decreased during post-anoxia in overexpressing lines. AOX-knockdown lines had higher SOD and lower catalase activity than overexpressors. The activities of enzymes of ascorbate-glutathione cycle decreased during both conditions being higher in overexpressing lines. We conclude that AOX is involved in NO turnover and plays a protective role by reducing the level of reactive oxygen species and sustaining energy requirements during anoxia and post-anoxia.

Acknowledgement

This thesis is dedicated to my parents, Mr. Sunil Jayawardhane and Mrs. KM Florida Kanthie, my husband Dr. L. Mihiran Muditha Upeksha and all the siblings who have been the great pillars of my scientific career and the success.

The study presented here would not have been a success without the collaboration and utmost support of many people and hereby I wish to extend my heartiest gratitude for them. First and foremost, my deepest appreciation and gratitude are offered to my supervisor Dr. Abir U. Igamberdiev and the co-supervisor Dr. Natalia Bykova and supervisory committee members, Dr. Julissa Roncal, Department of Biology, Memorial University of Newfoundland, for devoting their valuable time for excellent supervision, motivation, encouragement, untiring guidance and useful suggestions throughout the whole work. Further, I extend my heartiest thanks to Dr. Natalia Bykova for devoting her valuable support and guidance during my training period on proteomics at Agriculture and Agri-Food Canada, Morden, Winnipeg. I gratefully acknowledge Dr. Paul Marino, Dr. Thomas Chapman, Dr. Brian Staveley, Ms. Cherie Hussey, Ms. Andrea Darby-King, Ms. Tracey Bishop, Mr. Alvin Kenny, Ms. Sylvia Barlett, Ms. Kate Carson, Ms. Stephanie Legar, Ms. Colleeen Furlong, Ms. Jody-Lynn Burke at Memorial University and all research staff at Agriculture and Agri-Food Canada, Morden, Winnipeg, especially Ms. Natasa Radovanovic and Ms. Slavica Djurie for their valuable support and facilities provided with relevant guidance.

I would like to convey my gratitude to the NSERC grant holders for financial assistance during the project, Dr. Natalia Bykova for financial assistance during the research training on proteomics at Morden Agriculture and Agri-Food Centre and Dr. Greg C. Vanlerberghe for providing research materials during the project.

It is a great pleasure to convey my heartiest gratitude to my colleagues, Ms. MKPS Wijesinghe, Mr. Devin Cochrane, Ms. Amrita Ghosh, Mr. Juran C.Goyali, Ms. Somaieh Zafari and all others for making the Newfoundland life a success filled with unforgettable memories.

Table of contents:

List of tables

List of figures

List of abbreviations

AA	Ascorbic acid
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AOX	Alternative oxidase
AP	Alternative pathway
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
B7, B8	*Aox1a* overexpressing tobacco transgenic lines
$CaCl_2$	Calcium chloride
CAT	Catalase
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CP	Cytochrome pathway
CO_2	Carbon dioxide
COX	Cytochrome oxidase
Cys	Cysteine
Cyt c	Cytochrome c
DHA	Dehydroascorbic acid
DHAR	Dehydroascorbate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5’-dithiol-bis 2-nitrobenzooic acid
EDTA	2,2',2",2"'-(Ethane-1,2-diyldinitrilo) tetraacetic acid
$FADH_2$	Flavin adenine dinucleotide
FW	Fresh weight
GPX	Guaiacolperoxidase
GR	Glutathione reductase

GSNO	*S*-nitrosoglutathione
GSNOR	GSNO reductase
GSH	Glutathione
GSSG	Glutathione disulfide
HA	Hydroxylamine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
KCl	Potassium chloride
KCN	Potassium cyanide
KI	Potassium iodide
KOH	Potassium hydroxide
LP	Lipid peroxidation
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate
mETC	Mitochondrial electron transport chain
MES-KOH	2-[N-Morpholino] ethanesulfonic acid- potassium hydrochloride
$MgCl_2$	Magnesium chloride
Myxo	Myxothiazol
NAD^+	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NBT	Nitro-blue tetrazolium

NR	Nitrate reductase
NO	Nitric oxide
$NO2^{\cdot}$	Nitrogen dioxide
NO_2^-	Nitrite
N_2O_3	Dinitrogen trioxide
$O_{2.}^-$	Superoxide
$_1O_2$	Singlet oxygen
O_3	Ozone
$OH^{\cdot}$	Hydroxyl radical
$OH^{\cdot}{}_2$	Hydroperoxyl radical
$ONOO^-$	Peroxynitrite
PA	Polyamine
PCD	Programmed cell death
PDC	Pyruvate dehydrogenase complex
Pi	Inorganic phosphate
Pgb	Phytoglobin
PG	Propyl gallate
PM NiNOR	Plasma membrane bound nitrite: NO reductase
PPFR	Photosynthetic photon fluence rate
PUFA	Poly-unsaturated fatty acids
PVP	Polyvinylpyrrolidone
Qi	Quinone reduction site
RI9, RI29	*Aox1a* RNA interference tobacco transgenic lines (knockdown/ downregulating)
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SHAM	Salicylhydroxamic acid
SNO	Nitrosothiol
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Tricarboxylic acid
Tris-HCL	Hydrochloride acid
W	Wild type
XOR	Xanthine oxidoreductase
YADH	Yeast Alcohol Dehydrogenase

Chapter 1 Background and literature review

1 Introduction

1.1 Plant cellular respiration

Cellular respiration involves metabolic reactions that oxidize a diverse group of molecules such as carbohydrates, fatty acids, proteins etc. and use the released energy to produce adenosine triphosphate (ATP). This process drives through in three distinct phases which includes glycolysis, the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (Figure 1.1) (Taiz *et al.*, 2014). Glycolysis occurs in the cytosol by oxidizing glucose into pyruvate and producing both ATP and NADH. Pyruvate is subsequently transported into the mitochondrial matrix for further oxidation and converted to acetyl CoA, which is completely oxidized to CO_2, generating ATP, NADH and $FADH_2$ (Taiz *et al.*, 2014). Further, ATP is generated additionally by the oxidation of NADH and $FADH_2$ via a respiratory electron transport chain in the mitochondria (Figure 1.2) (Taiz *et al.*, 2014). This pathway is denoted as the cytochrome pathway where some of organisms possess a second, alternative pathway of mitochondrial respiration when the cytochrome pathway is impaired or restricted (Taiz *et al*., 2014).

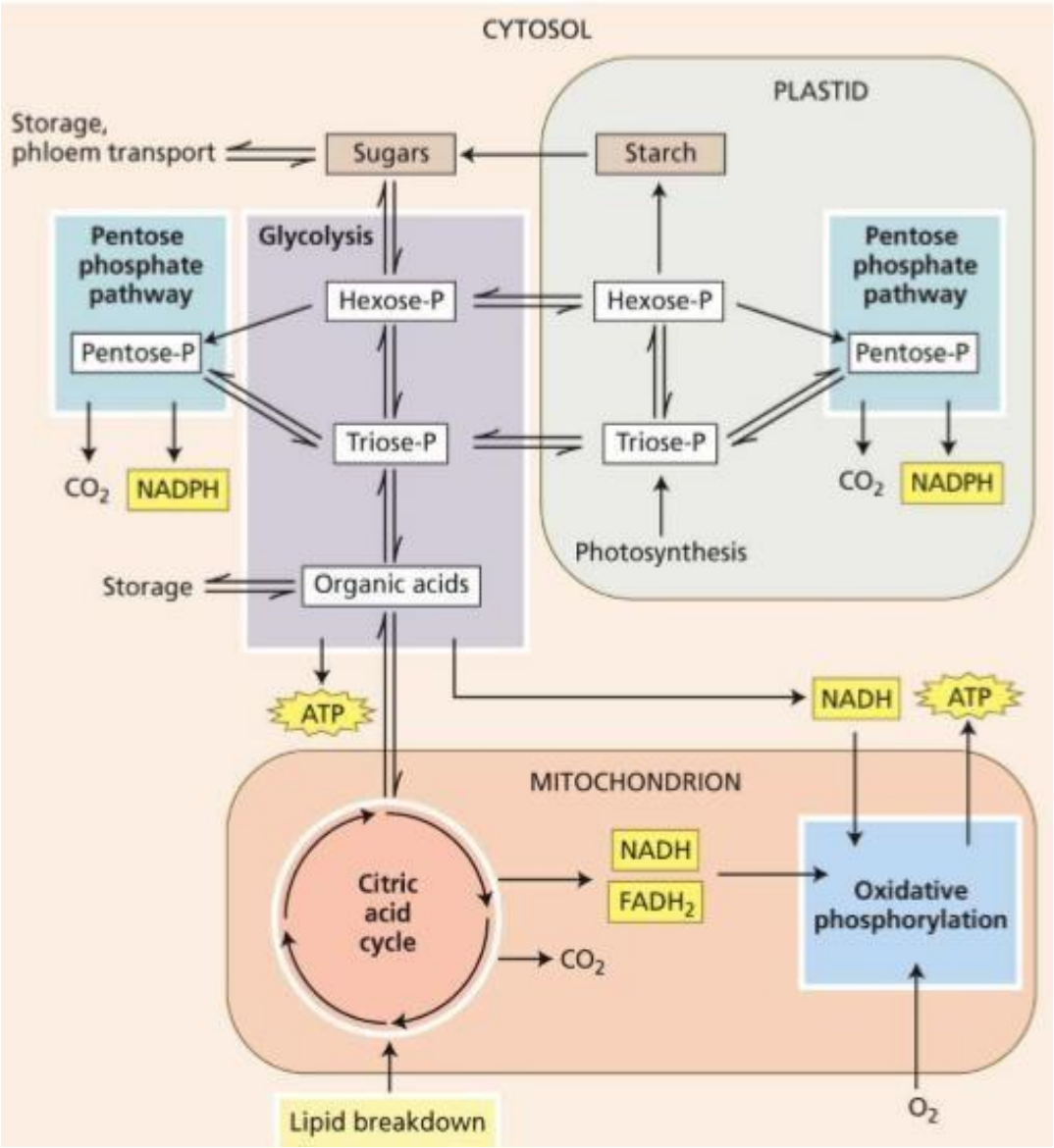

Figure 1.1 Basic steps of cellular respiration pathway and ATP synthesis (Taiz *et al.*, 2014).

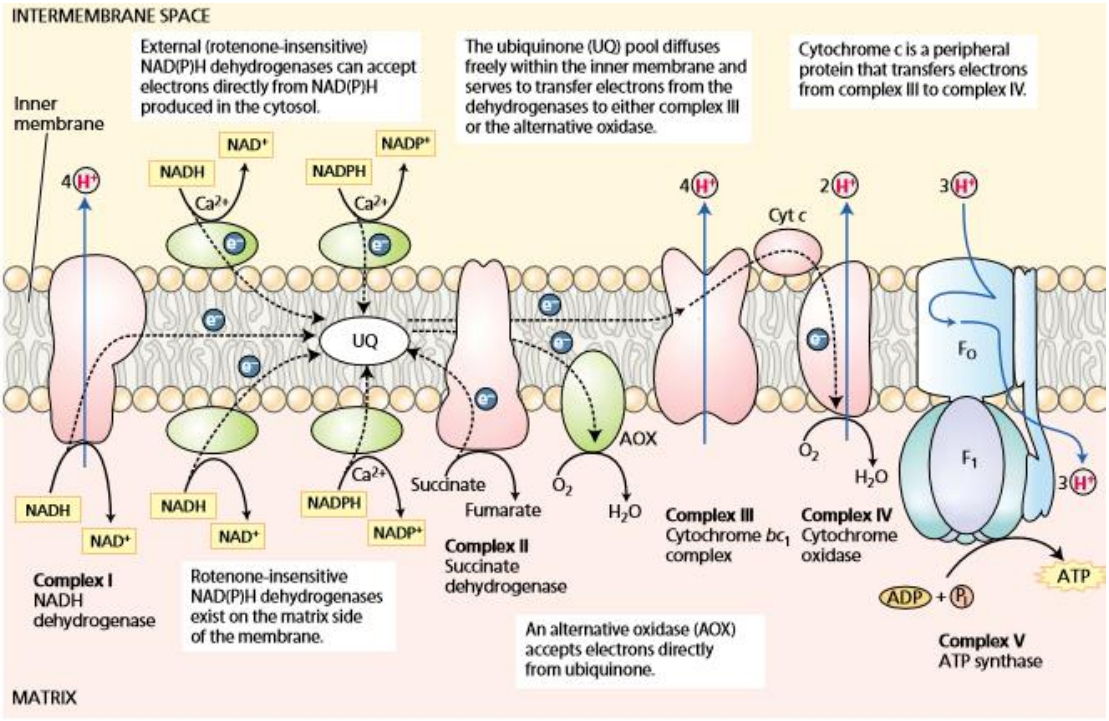

Figure 1.2 Organization of the electron transport chain and ATP synthesis in the inner membrane of plant mitochondria. Besides the five standard protein complexes found in mitochondria, the electron transport chain of plant mitochondria contains five additional enzymes [NAD(P)H dehydrogenases; marked in green] which do not pump protons, but oxidize cytosolic NADH and NADPH and electrons from these external NAD(P)H dehydrogenases enter the main electron transport chain at the level of the ubiquinone pool (Møller 2001; Taiz *et al*., 2014).

1.2 Mitochondrial Electron Transport Pathways: cytochrome pathway and alternative pathway

Cytochrome pathway (CP) is consisted of four major protein complexes; Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome bc_1) and Complex IV (known as cyanide-sensitive cytochrome c

oxidase). These are associated with the inner mitochondrial membrane and facilitate the transfer of electrons from NADH or $FADH_2$ to molecular oxygen, reducing it to water (Figure 1.2). Electron flow along this pathway is coupled to chemiosmotic ATP generation via a proton gradient, that is facilitated by proton pumping at three sites (Complex I, Complex III, and Complex IV) (Figure 1.1). The electrochemical gradient generated by the positively charged protons is dissipated by ATP synthase (Complex V) where the free energy released by the movement of protons back into the matrix from the intermembrane space is used to drive the formation of ATP (adenine triphosphate) from ADP (adenine diphosphate) and inorganic phosphate (Pi) (Taiz *et al.*, 2014).

In addition to the ubiquitous cytochrome pathway, plants, algae, protozoa and some lower invertebrates contain a second respiratory chain called the alternative pathway (AP) where alternative oxidase (AOX) is involved (known as cyanide-insensitive and hydroxamic acid-sensitive terminal oxidase) (Vanlerberghe and McIntosh, 1997; Sluse *et al*., 1998). AOX (32–36 kDa) is an interfacial membrane protein (Sluse *et al.*, 1998). AOX is encoded by five genes of the multigene families *AOX1* and *AOX2* in Arabidopsis (Considine *et al.*, 2002; Borecky, 2006). It contains a non-heme di-iron carboxylate active site and interacts with a single leaflet of the inner mitochondrial membrane (Moore *et al.*, 2013). The AP branches from the cytochrome chain at ubiquinone and transfer electrons from reduced ubiquinone directly to oxygen which provides an alternative route for the passage of electrons (Figure 1.2). Although both routes are involved in transportation of electrons and the reduction of oxygen to form water, the proton translocation at complex I through the AOX protein occurs

without the pumping of protons into the intermembrane space and consequently is not coupled with ATP synthesis or energy conservation (Vanlerberghe and McIntosh, 1997). Thus, it has a lower ATP output, allowing control of cellular redox and energy balance (Vanlerberghe *et al.*, 2009; Dahal *et al.*, 2015).

1.3 Abiotic stress and recovery: anoxia and reaeration (post-anoxia)

Plants are frequently encountered by environmental stresses resulting in altered growth, metabolism and productivity (Ahmad, 2010). Due to climate change, abiotic stress has become a major consequence in worldwide agricultural crop production that lead the scientists to look for solutions to manage them wisely (IPCC, 2007). One of the best ways is to understand the mechanisms that undergo in plants during stress conditions and this knowledge can be used to develop plants that can thrive best in a changing environment. There is still research gap in the understanding of the mechanisms that undergo in cells during abiotic and biotic stresses.

Due to climate change, the incidences of heavy rainfall, flooding and waterlogging are expected to become more frequent (IPCC, 2007). Underground plant organs such as roots or rhizomes suffer especially from the periodical or prolonged oxygen deficiency. The availability of oxygen for plants decreases due to the slower diffusion of oxygen through water and competition for oxygen with respiring microorganisms and the environment become either completely (anoxia or oxygen level below 2%.) or partially (hypoxia) oxygen depleted (Jackson 1985; Drew 1997; Skutnik and Rychter, 2009). This interferes with respiration at the level of electron transport, especially the cytochrome pathway. Plants that are not adapted to flooding and water logging are threatened by energy deficiency and ultimately cell and tissue death under

hypoxic conditions where the concentration of oxygen in the cells becomes too low to support aerobic respiration (Bailey-Serres and Voesenek 2008; Bailey-Serres *et al*., 2012; Licausi 2013). As the major consequence, this creates an energy crisis because of the lack of a suitable electron acceptor. This leads to the accumulation of NADH, NADPH, and decrease the generation of ATP (Kennedy *et al*., 1992; Perata and Alpi, 1993; Crawford and Brandle, 1995). This negatively affects the energy-demanding biological processes (Limami *et al*., 2013; Greenway and Gibbs 2003).

Re-aeration or re-oxygenation during the post-anoxic period occurs when water recedes following flooding or water logging condition (Blokhina *et al*., 2000). Re-exposure to air after a period of oxygen deprivation can cause serious cell damage and injury and it may be more detrimental in some plant species or plant organs than oxygen deficiency itself (Monk *et al*., 1987; Crawford, 1992). Increased reduction state of the cell environment, depletion of ATP and hyper-polarization (increased membrane potential) of the inner mitochondrial membrane during anoxia may develop conditions of enhanced ROS production and oxidative stress after re-exposure to air which is termed as ‘‘postanoxic injury’’ (Blokhina *et al*., 2003). Further, Drew (1997) suggests that consequences of perturbation (an alteration of the function of a biological system by external or internal stimuli) of the cell structure and function during post-anoxia could be far more severe than during the period of uninterrupted anaerobiosis, which means that re-aeration could be more damaging to plant cells and functions than continuous anoxia or hypoxia.

Low oxygen stress (hypoxia or anoxia) poses a considerable threat to crop productivity during flooding and water logging conditions. Different plants show

significantly different levels of tolerance to low oxygen conditions. Therefore, different survival tactics have evolved in plants, with some plants adopting rapid growth avoidance strategies while others only show metabolic shifts to ensure survival (Voesenek *et al*., 2006; Salavati *et al*., 2012). Examples for such molecular responses to low oxygen conditions that have been conserved in plants are the induction of fermentation and glycolysis (Mustroph *et al*., 2010), nitric oxide (NO) and reactive oxygen species (ROS) signaling (Igamberdiev *et al*., 2010; Gupta and Igamberdiev, 2011), rapid changes in transcript and metabolite abundances (Shingaki-Wells *et al*., 2014) and changes in mitochondrial respiratory components facilitating the stress signaling pathways (Blokhina and Fagerstedt, 2010). During post-anoxia, plant shows changes in an array of metabolic pathways to adapt to the condition. However, literature searches reveal that the molecular responses to re-oxygenation are not as well characterized as the response to low oxygen alone (Shingaki-Wells *et al*., 2014).

Different plant species have adapted to different levels of tolerance to anaerobic conditions, whereby several species are able to survive relatively short bursts of hypoxia and recover (Vartapetian *et al*., 1985). Due to their unique carbohydrate metabolism, riparian plant species, such as *Salix variegata*, *Arundinella anomala* and *Althernanthera philoxeroides* (Sairam *et al*., 2009) withstand long-term oxygen deviancy. Moreover, rice (*Oryza sativa*) has flooding resistant genes that helps to survive under anoxia or hypoxia (Xu *et al*., 2006). However, we have observed that tobacco cannot survive under anoxia for a long period of time. Therefore, using tobacco for these experiments would give more detailed molecular level explanation that undergo under the subjected conditions.

1.4 Role of alternative oxidase in plants

AOX has been proposed to play a role in plant growth, reproduction and homeostasis (Hansen *et al*., 2002) and maintains metabolic flexibility for rapid adaptation to stress conditions (Moore *et al*., 2002). AOX plays an important role in pollination in certain families of angiosperms (Seymour, 2001). A high AOX activity in the floral tissue mitochondria helps thermogenesis that facilitates the volatilization of compounds critical for the attraction of insect pollinators (Elthon *et al*., 1989; Moore and Siedow, 1991).

AOX can be induced by a diverse array of biotic and abiotic stresses. This can be due to pathogens (Cvetkovska and Vanlerberghe 2012), nutrient limitation (Noguchi and Terashima, 2006), metal toxicity (Tan *et al*., 2010), salinity (Wang *et al.*, 2010), high light and drought (Giraud *et al*., 2008, Yoshida *et al*., 2011, Zhang *et al*., 2012), low temperature (Wang *et al*., 2011), low oxygen conditions (Clifton *et al*., 2005) and high CO_2 (Gandin *et al*., 2012). The elevation of intracellular oxidative stress, ROS and reactive nitrogen species (RNS) during stress conditions induce the expression of AOX (Vanlerberghe and McIntosh, 1997). Commonly, many of these stressors that increase AOX expression induce the formation of hydrogen peroxide (H_2O_2) which is a key secondary messenger during stress conditions, and it has been proposed as one of the key intermediates in AOX signaling (Maxwell *et al*., 2002; Vanlerberghe, 2013).

In non-thermogenic plants, continued operation of glycolysis and the TCA cycle via the shunting of electrons through the alternative pathway occurs, when cytochrome pathway is impaired or restricted (Lambers, 1982; Vanlerberghe, 2013). This alternative respiration pathway helps to prevent the over-reduction of electron transport

components, particularly the ubiquinone pool, which would in turn reduce the oxidative stress via decreasing the formation of harmful reactive oxygen species (ROS). According to Maxwell *et al.* (1999), the transgenic tobacco cells lacking AOX show increased mitochondrial-derived ROS formation and up-regulation of key genes such as catalase which is involved in antioxidant defense, when compared with wild-type cells. Umbach *et al.* (2005) have used Arabidopsis (*Arabidopsis thaliana*) to study ROS and AOX in whole plants by using transformed lines; *AtAOX1a* overexpressors, *AtAOX1a* anti-sense plants, and overexpressors of a mutated, constitutively active *AtAOX1a*. Leaf tissue of either mutant or wild-type AOX-overexpressors has not shown an increase in oxidative damage, whereas anti-sense lines had levels of damage greater than those observed for untransformed leaves in the presence of KCN (Umbach *et al*., 2005). Further, ROS production has increased markedly in anti-sense and untransformed, but not in the overexpressor, roots with KCN treatment (Umbach *et al*., 2005). Therefore, AOX functions in leaves and roots to ameliorate ROS production when the cytochrome pathway is chemically inhibited which is similar to the study done on tobacco suspension cells (Maxwell *et al*., 1999). However, they have not observed changes in leaf transcript levels of selected electron transport components or oxidative stress-related enzymes under nonlimiting growth conditions, regardless of transformation type as seen in suspension culture cells (Umbach *et al*., 2005). Pasqualini *et al.* (2007) have used wild-type (WT) Xanthi tobacco plants and Xanthi plants transformed with the Bright Yellow tobacco *AOX1a* cDNA with enhanced (SN21 and SN29), and decreased (SN10) AOX capacity in their study and exposed them to an acute ozone (O_3) fumigation (5 h, 250 nL L^{-1}). They suggest that the AOX-

overexpression decreases mitochondrial ROS level that in turn changes the defensive mitochondrial to nucleus signaling pathway and activates ROS scavenging systems.

It has been recorded that the growth and development of plants is unaffected by altering the AOX expression levels under normal growth conditions. Its ability to manage the energy imbalances in the metabolism has shown to be essential in optimizing photosynthetic performance under drought (Giraud *et al.*, 2008; Dahal *et al*., 2015), high light (Yoshida *et al*., 2011) and high CO_2 (Vishwakarma *et al.*, 2015).

1.5 Reactive oxygen species in plants and their role during stress conditions

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered as common by-products of plant cellular metabolism and stress signaling (Paradiso *et al.*, 2016). Exposure to abiotic stress such as anoxia, triggers the formation of toxic ROS and RNS in plant cells, ultimately resulting in oxidative stress (Shingaki-Wells *et al.*, 2014). In plants, chloroplasts in photosynthetic tissues and mitochondria during mitochondrial electron transport chain (mETC) are the primary sources of reactive oxygen species (ROS) (Møller, 2001; Skutnik and Rychter, 2009).

The reaction centers in photosystems (PSI and PSII) of chloroplast thylakoids are the major generation site of ROS (Asada, 2006). The primary reduced product is superoxide anion (O_2^-), and it disassociate into H_2O_2 and O_2 (Asada *et al*., 1974). In PSII, ground (triplet) state of O_2 ($^3O_2$) is excited to singlet state ($^1O_2$) by the reaction center chlorophyll (Chl) (^{3}P680*) (Telfer *et al.*, 1994; Hideg *et al*., 1998). Mitochondria play a dual role in oxidative stress for being both ROS producers and integrators of the cell antioxidant defense systems (Rhoads *et al*., 2006; Rhoads and Subbaiah, 2007). It

has been estimated that about 1% of metabolically consumed O_2 goes into reactive oxygen species (ROS) generation in aerobic cells (Puntarulo *et al*., 1988).

ROS are generated from molecular oxygen through several reductive steps (Steffens *et al.*, 2013; Das and Roychoudhury, 2014) (Table 1.5.1). Complexes I and III of the mETC produce superoxide anion as a primary ROS compound. Superoxide anions ($O^{\cdot-}_2$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), hydroperoxyl radical ($HO^{\cdot}_2$), and ozone (O_3) are generated by direct energy transfer that excites unpaired electrons and the reductive power is provided by electron carriers in mitochondria and chloroplasts (Blokhina and Fagerstedt, 2010; Chang *et al.*, 2012; Shapiguzov *et al.*, 2012). Excess accumulation of ROS such as superoxide, hydroxyl and peroxide radicals damages the normal metabolism and growth of plants. The production of H_2O_2 increases in several abiotic stress conditions including oxygen deprivation (Blokhina and Fagerstedt, 2010). H_2O_2 is a part of redox signaling pathways activating defense responses in all aerobic organisms (Blokhina *et al.*, 2001). It has the relative stability under different physiological conditions and capability to cross biological membranes (Blokhina *et al.*, 2001). The immediate responses of excess ROS accumulation are oxidation of various cellular molecules including lipids, proteins and chlorophylls and damaging nucleic acids (Blokhina and Fagerstedt, 2010).

According to Crawford (1992), the phenomenon of post-anoxic or posthypoxic injury is created by the generation of reactive oxygen radicals and toxic oxidative products such as acetaldehyde. The formation of toxic oxygen species increases under certain stress conditions, when the equilibrium of formation and detoxification of active oxygen species is imbalanced (Foyer *et al.*, 1994). During abiotic stress the biosynthesis

and accumulation of different molecules having protective functions in the cells is induced. These molecules are thought to mediate their protective function by their interaction with, or stabilizing of, different cellular components such as membrane elements or proteins/enzymes.

ROS production under stress is a detrimental factor for plant cell metabolism, which causes peroxidative damage to proteins, nucleic acids, and lipids (Shewfelt and Purvis, 1995). According to Ursini *et al*. (1991), polyunsaturated fatty acids (PUFA) are the most susceptible molecules for radical attack. Biomembranes have a relatively higher content of PUFA than other types of fatty acids. Therefore, they are the major places where a chain reaction occurs via free radical-mediated lipid peroxidation of unsaturated fatty acids (PfisterSieber and Brandle, 1994). Moreover, both ROS and enzymatic reaction via lipoxygenase (LOX) can promote lipid peroxidation (LP) (Halliwell, 1991; Foyer *et al*., 1994; Siedow, 1991; Rosahl, 1996).). LP is linked to severe membrane damage during anoxia and subsequent reaeration, for instance, changes in lipid content and composition, increased ion permeability, loss of fluidity, leakage and inactivation of membrane enzymes and receptors, decrease in adenylated energy charge and the intracellular membrane damage have detrimental effects on cell organelles (Brown and Beevers, 1987; Pfister-Sieber and Brandle, 1994). Malondialdehyde (MDA), ethane and ethylene are the typical LP products (Pfister-Sieber and Brandle, 1994). MDA is a biologically active molecule that is synthesized mainly via the peroxidation of PUFA with three to six double bonds and it is capable of binding to DNA and proteins (Pryor *et al*., 1976).

Table 1.5.1 Members of ROS family, their reactions and functions (Das and Roychoudhury, 2014)

ROS	$t_{1/2}$	Migration distance	Sources	Mode of action	Reaction with DNA	Reaction with protein	Reaction with DNA	Scavenging systems
Superoxide ($O_2^{\bullet -}$)	1–4 μs	30 nm	Membranes, Chloroplasts, Mitochondria	Reacts with double bond containing compounds such as (Fe-S) proteins	No	Via the Fe-center	Extremely low	SOD
Hydroxyl radical ($OH^{\bullet}$)	1 μs	1 nm	Membranes, Chloroplasts, Mitochondria	Extremely reactive with all biomolecules	Rapidly reacting	Rapidly reacting	Rapidly reacting	Flavonoids and Proline
Hydrogen Peroxide (H_2O_2)	1 ms	1 μm	Membranes, Chloroplasts, Mitochondria, Peroxisomes	Oxidizes proteins and forms $OH^{\bullet}$ via $O_2^{\bullet -}$	No	Attacks the Cys residue	Extremely low	CAT, POXs and Flavonoids
Singlet Oxygen ($^1O_2$)	1–4 μs	30 nm	Membranes, Chloroplasts, Mitochondria	Oxidizes proteins, PUFAs and DNA	Reacts with G residue	Attacks Trp, His, Tyr, Met and Cys residues	PUFA	Carotenoids and α -Tocopherol

1.6 Reactive nitrogen species (RNS) and post-translational modifications of proteins in plants during stress conditions

Nitric oxide (NO) is a short-lived, highly reactive signaling molecule (free radical) which regulates several developmental and stress responses in plants (Astier *et al*., 2011; Manjunatha *et al*., 2012; Farnese *et al*., 2016). NO is widely synthesized by several reductive and oxidative pathways in plants (Vishwakarma *et al*., 2015). Nitrite production is highly dependent on enzymatic and non-enzymatic reduction of nitrate (Planchet *et al*., 2005; Mur *et al*., 2013; Chamizo-Ampudia *et al*., 2017). Cytosolic nitrate reductase (NR), mitochondrial nitrite: NO reductase activities are associated with the components of electron transport chain. Plasma membrane nitrite: NO reductase (PM Ni-NOR), xanthine oxidoreductase (XOR), NO synthase like enzyme, polyamine (PA) and hydroxylamine (HA)-mediated pathways have been identified as NO producing reactions (Crawford, 1995; Guo *et al*.,2003;Huang *et al*., 2005; Stöhr and Stremlau, 2005; Planchet and Kaiser, 2006; Gupta *et al*., 2011; Alber *et al*., 2017; Vishwakarma *et al*., 2018). Plant mitochondria are one of the main sites of nitric oxide (NO) production via mechanisms that is still under investigation (Gupta *et al*., 2011). They produce higher levels of NO (1–20 nmol g^{-1} FW h^{-1}) under hypoxic conditions using nitrite as a terminal electron acceptor (Gupta *et al*., 2005).

NO plays an important role during several developmental stages (seed germination, root growth, flowering, growth of pollen tube and leaf senescence), stomatal movement and signaling (Yu *et al*., 2014; Domingos *et al*., 2015; Fancy *et al*., 2017). NO helps in conferring tolerance or eliciting programmed cell death in plants (Siddiqui *et al*., 2011; Sidana *et al*., 2015; Arora *et al*., 2016; He *et al*., 2014). NO can confer tolerance via the induction of AOX (Vishwakarma *et al*., 2015). In recent years, the evidence has been revealed on the important role(s) of NO under low oxygen stress

(Stoimenova *et al*., 2007; Gupta *et al*., 2011). Several studies have attempted to elucidate the role of NO under low oxygen (Planchet *et al*., 2005; Stoimenova *et al*., 2007; Gupta and Igamberdiev, 2011).

NO participates in oxidative metabolism with oxygen or ROS and forms intermediates and other derivatives which are termed as reactive nitrogen species (RNS) such as NO_2, N_2O_3, peroxynitrite ($ONOO^-$) (Nathan and Shiloh, 2000; Delledonne *et al*., 2001; Chamizo-Ampudia *et al*., 2017) (Table 1.6.1). These RNS are harmful for cells and damage cell via modification of macromolecules, including proteins via their Cys thiol groups (Birben *et al*., 2012). Therefore, plants regulate endogenous NO levels via the control of biosynthesis or scavenging by various side reactions. NO along with other RNS modifies proteins via reaction with cysteine thiols forming nitrosothiols (*S*-nitrosylation) which is termed as NO-based post-translational modification (Sehrawat and Deswal, 2014; Sun *et al*., 2006). *S*-nitrosylation is a covalent attachment of NO and reactive cysteine residues in a protein as a reversible modification to form nitrosothiols (-SNO) (Sehrawat and Deswal, 2014). Further, proteins that are related to stress, redox balance, metabolism and photosynthesis have been identified as *S*-nitrosylated targets during abiotic stress (salinity, cold, high light, and cadmium) signaling, regulating stress response, metabolism and respiration (Lindemayr *et al*., 2005; Asteir *et al*., 2011; Sehrawat and Deswal, 2014; Leon *et al*., 2016). Moreover, in the presence of O_2^-, the NO^-derived metabolites ($ONOO^-$) react with tyrosine residues of proteins forming protein 3-nitrotyrosine (Souza *et al*., 2008; Radi, 2013). Nitrotyrosine is a biomarker of nitrosative stress and an oxidative posttranslational modification in cells. The incorporation of nitro group into protein tyrosine can significantly change the structure and function of protein (Sun *et al*., 2006; Souza *et al*., 2008; Radi, 2013). Some of the side reactions are harmful to the cell (e.g. tyrosine nitrosylation of proteins), while *S*-

nitrosylation of proteins or glutathione function in a regulatory manner for instance, promoting the initiation of programmed cell death (Mur *et al*., 2006). Under physiological conditions, protein *S*-nitrosylation and SNOs provide protection preventing further cellular oxidative and nitrosative stress (Sun *et al*., 2006). Another specific NO-scavenging reaction is linked to the operation of hypoxically induced hemoglobin (Hb) called phytoglobin (Pgb) (Igamberdiev and Hill, 2004).

Table 1.6.1 Major RNS generated in biological systems (Griendling *et al*., 2016)

Formula	Name	Comment
·NO	Nitric oxide	Nitrogen-centered free radical
N_2O	Nitrous oxide	"Laughing gas," reacts with oxygen to generate nitric oxide
$ONOO^-$	Peroxynitrite	Product of the reaction between superoxide and nitric oxide
$^{\cdot}NO_2$	Nitrogen dioxide	Free radical, derived from peroxynitrite
N_2O_3	Dinitrogen trioxide	Product of reaction between nitric oxide and nitrogen dioxide
ONOOH	Peroxynitrous acid	Protonated form of peroxynitrite
NO^-	Nitroxyl anion	Conjugate base of nitroxyl
NO^+	Nitrosyl cation	Also, nitrosonium cation
HNO_2	Nitrous acid	Weak monobasic acid
NO_2Cl	Nitrosyl chloride	Derived from nitrite and hypochlorous acid
NO_2^-	Nitrite	Anion, generated from nitric oxide
NO_2^+	Nitronium ion	Also nitryl ion, generated from the removal of e^- from nitrogen dioxide or protonation of nitric acid
RSNOs	Nitrosothiols	Formed by covalent addition of nitric oxide to cysteine and protein or nonprotein sulfhydryl residues

NO production is sensitive to myxothiazol (Myxo) and cyanide which inhibit complex III and COX (complex IV) respectively (Vishwakarma *et al*., 2015). Antimycin is a specific and highly potent inhibitor of electron transfer in the cytochrome bc_1 which binds specifically to the quinone reduction site (Q_i site) of the cytochrome bc_1 complex near the heme b_H (Huang *et al*., 2002). This blocks the reaction at the Q_i site, at which cytochrome *b* equilibrates directly with the ubiquinone/ubiquinol couple, masking the oxidant-induced reduction (Huang *et al*., 2002). Further,

salicylhydroxamic acid (SHAM) and propyl gallate (PG) inhibit AOX and can be used to quantify the activity and capacity of the AOX by measuring the decrease in respiration rate by assuming that electrons are not redirected from the AOX to the cytochrome pathway after addition of these inhibitors (Bahr and Bonner, 1973; Merller *et al*., 1988). AOX has been identified as the enzyme responsible for this oxygen uptake and a cyanide-resistant oxidase component of the plant mitochondrial electron transport chain (Vanlerberghe and McIntosh 1997).

1.7 Cell energy production during low oxygen stress

In the event of a transition from aerobic to hypoxic/anoxic conditions, plant cells switch from aerobic respiration to lactic acid fermentation (Roberts *et al*., 1984a, b). After a transient period of lactic acid fermentation, plant cells shift to alcoholic fermentation which allows the continuation of glycolysis (Roberts *et al*., 1984a, b). During this alcoholic fermentation, alcohol dehydrogenase (ADH, EC 1.1.1.1), a Zn binding enzyme catalyzes the reversible conversion of acetaldehyde to ethanol while oxidizing NADH to NAD^+ (Strommer, 2011). This ensures the maintenance of the glycolytic flux by recycling NAD^+ and support prolonged survival during hypoxia (Perata and Alpi, 1993). This also controls toxic acetaldehyde produced by the decarboxylation of pyruvate (Ismond *et al*., 2003). Certain metabolic pathways are moderated to counteract the energy crisis during oxygen deprivation. Aconitase is a Krebs cycle enzyme that interconverts citrate and isocitrate via the intermediate *cis*-aconitate, and its activity depends on the presence of a [4Fe-4S] cluster (Hanson and Leibold, 1999; Cantu *et al*., 2009). Moreover, aconitase is a ROS-sensitive enzyme and it is reversibly inactivated by superoxide because of the release of iron from the active center, which results in the loss of enzymatic activity (Gardner *et al*., 1997; Cantu *et al*., 2009). Thus, the aconitase inactivation serves as a marker of the mitochondrial ROS

production in many species (Cherkasov *et al*., 2007; Sanni *et al*., 2008). Therefore, the production of excess superoxide ($O_2^{-\cdot}$) and other oxidants under stress conditions may account for inhibition of energy production (Gardner *et al*., 1994; Powell and Jackson, 2003).

During anoxia, oxygen concentrations in the cytoplasm are such that COX cannot effectively donate electrons to oxygen, while hypoxia is a condition whereby COX has at least a limited capacity to use oxygen but several other oxidases such as AOX are inhibited (Igamberdiev and Hill, 2009). Under low oxygen environment, mitochondrial oxygenic respiration declines below a certain oxygen level required to saturate terminal oxidases (Igamberdiev and Hill, 2009). COX is considered as an important player in the phytoglobin-NO cycle. The efficiency of COX to act as a terminal oxidase is disturbed by the production of NO under hypoxia due to irreversible inhibition by NO (Parihar *et al*., 2008). This lowers the affinity of COX for oxygen (Cooper, 2002). The oxygen Km for the alternative oxidase (AOX) is 10 mM (Millar *et al*., 1994; Affourtit *et al*., 2001b) that limits the AOX function under low oxygen conditions (Igamberdiev and Hill, 2009). According to Gupta and Igamberdiev (2011), in the absence of oxygen, COX can produce NO from nitrite. However, AOX plays a role in NO tolerance under normoxic and moderately hypoxic conditions as NO does not inhibit its activity and up-regulates AOX synthesis (Huang *et al*., 2002). AOX transcript and protein amounts have been shown to increase in response to NO and during hypoxia (Gupta *et al*., 2012). There seems to be an increased role of AOX respiration under hypoxic conditions. However, there is no interconnection between NO production and AOX, and it operates independently of NO turnover, perhaps by maintaining respiration under conditions of NO accumulation (Gupta *et al*., 2012;

Cochrane *et al*., 2017). Hence, under conditions in which NO is accumulated, the behavior and role of AOX is likely much different than that of COX.

When the intensity of a detrimental factor is high in plants, they induce the programmed cell death (PCD) which is an active, genetically controlled process initiated to isolate and remove damaged tissues thereby ensuring the survival of the organism (Petrov *et al*., 2015). Due to increased production of RNS and ROS in mitochondria (Dordas *et al*., 2003) and energy deficiency in plant cells during low oxygen stress plant cells undergo necrotic and programmed cell death (Limami *et al*., 2013; Petrov *et al*., 2015). To alleviate stress conditions, plants have developed different morphological adaptations and physiological pathways to improve ATP production and diminish the stress. Phytoglobin-nitric oxide (Pgb-NO) cycle is one of the important biochemical pathways where plants substitute oxygen with nitrite as the terminal electron acceptor for respiration, allowing the respiratory chain to continue generation of the proton motive force required to phosphorylate ADP (Stoimenova *et al*., 2007; Igamberdiev and Hill, 2004; Cochrane *et al*., 2017) (Figure 1.7.1). In this cycle, nitrite is reduced to NO which can then readily escape the mitochondria (Cochrane *et al*., 2017). These NO is scavenged by hypoxically induced phytoglobin (Pgb) (Taylor *et al*., 1994) to form nitrate, which can be converted back into nitrite via nitrate reductase and then transported into the mitochondria for continued NO production (Igamberdiev and Hill, 2004). This leads to significantly lower NO emissions in hypoxic situations where oxygen levels are below 0.3% v/v in Arabidopsis and barley (Hebelstrup *et al*., 2014).

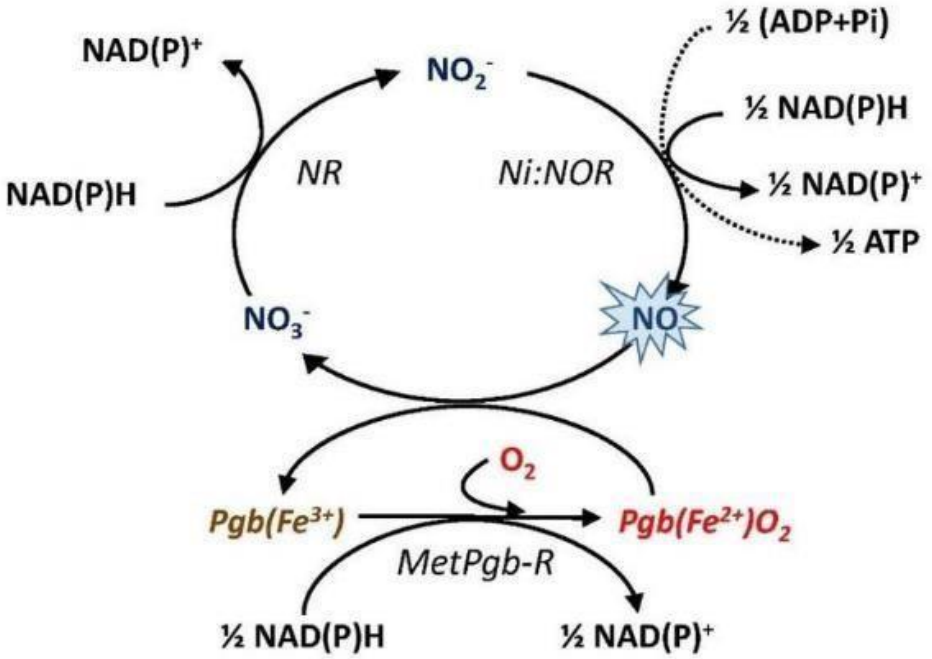

Figure 1.7.1 Operation of the phytoglobin-NO cycle between the hypoxic mitochondrion and cytosol (Ma *et al.*, 2017).

Although AOX does not directly participate in the Pgb-NO cycle, its expression is important for balancing ROS and RNS levels and supply fluxes of glycolysis, TCA cycle and amino acid metabolism both under normoxia and in the conditions of oxygen deficiency (Gupta and Igamberdiev, 2011) (Figure 1.7.2). Moreover, inhibitors such as CN^-, SHAM, nPG also reveal that nitrite-driven ATP synthesis is sensitive to uncouplers (Bendall and Bonner, 1971; Lambowitz and Slayman, 1971; Siedow and Girvin, 1980). Therefore, both terminal oxidases appear to possess nitrite: NO reducing activity (Gupta *et al*., 2010; Gupta and Igamberdiev, 2011).

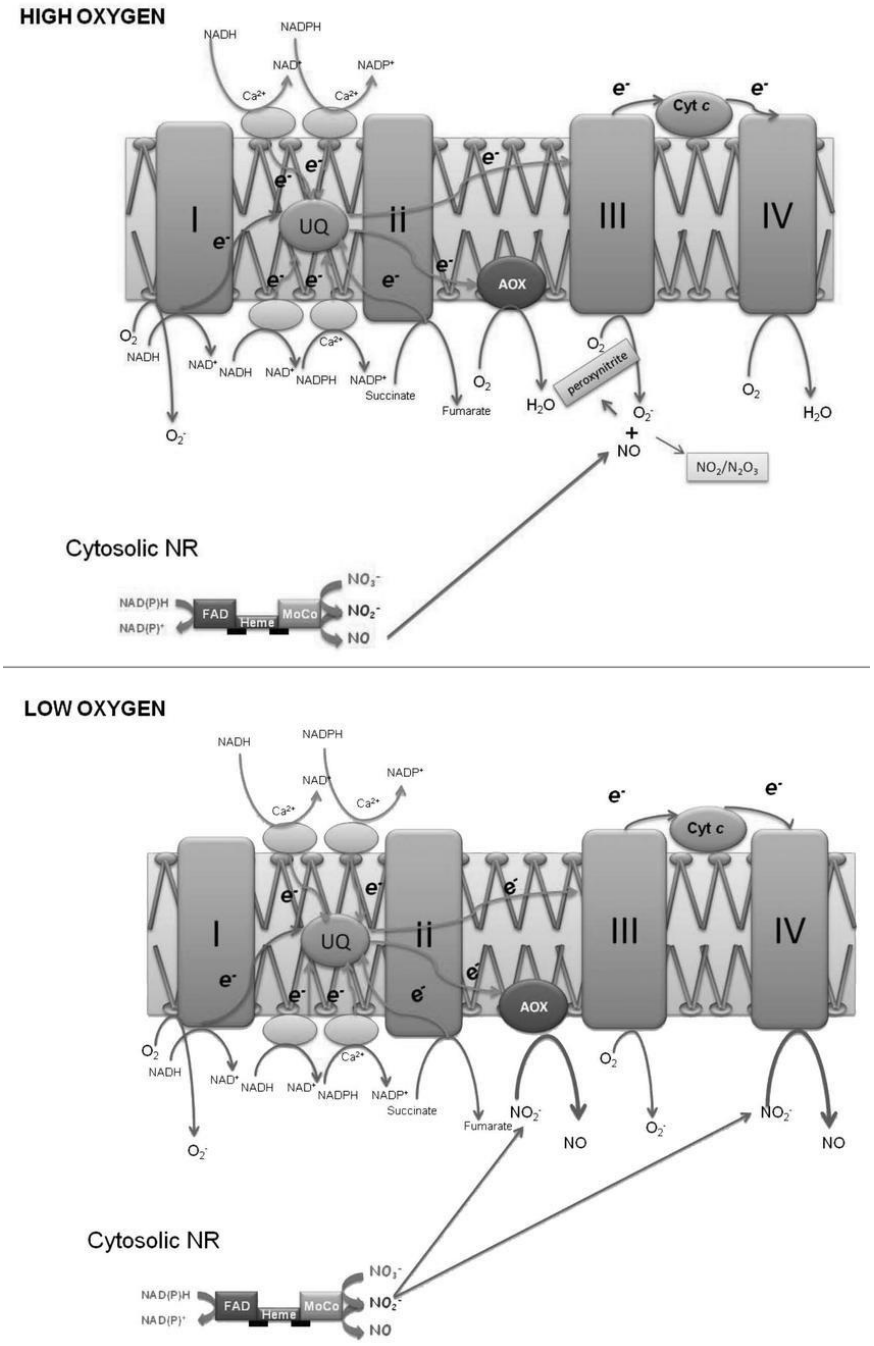

Figure 1.7.2 Upper panel: NO is scavenged by plant mitochondrial electron transport chain components during normoxia. Superoxide produced in mitochondria reacts with NO forming peroxynitirte and NO also easily react with oxygen to form gaseous intermediates (N_2O_3 and NO_2). Lower panel: During hypoxia, nitrite is reduced to NO by the electron transport chain (Gupta *et al*., 2010).

1.8 Antioxidative system in plants

All aerobic organisms show increased levels of both enzymatic and non-enzymatic antioxidant activities in countering the hazardous effects of oxygen radicals by avoiding ROS generation or detoxifying ROS to overcome stress conditions (Walker and McKersie, 1993; Foyer *et al.*, 1995; Mishra *et al*., 1995; Biemelt *et al.*, 1998).

Plant mitochondria exhibit numerous enzymatic and non-enzymatic antioxidant activities to avoid ROS generation or to detoxify ROS (Skutnik and Rychter, 2009). AOX, alternative NAD(P)H dehydrogenases and plant uncoupling proteins (transporters) in mitochondrial inner membrane are the first line of defense to prevent the formation of ROS production due to over-reduction of mETC (Møller, 2001; Czarna and Jarmuszkiewicz, 2005; Umbach *et al.*, 2005). Activation of AOX lowers ROS production whereas, inhibition results in an opposite effect (Czarna and Jarmuszkiewicz, 2005; Umbach *et al*., 2005).

Further, various low-mass ROS scavengers involve in the detoxification of ROS (Das and Roychoudhury, 2014) (Table 1.8.1). This antioxidant machinery have two ways to scavenge ROS (Figure 1.8.1), (i) enzymatic components like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) (ascorbate–glutathione cycle); (ii) non-enzymatic antioxidants like ascorbic acid (AA), reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids, and the osmolyte proline (Noctor and Foyer, 1998; Chew *et al.*, 2003; Das and Roychoudhury, 2014). However, ascorbate and glutathione are considered as the main redox cell buffers and redox sensors (Foyer and Halliwell, 1976; Foyer and Noctor, 2005) (Figure 1.8.1). Chloroplasts are the major source of ROS in

leaves (Møller, 2001) and ascorbate is considered as the main antioxidant (Noctor and Foyer, 1998).

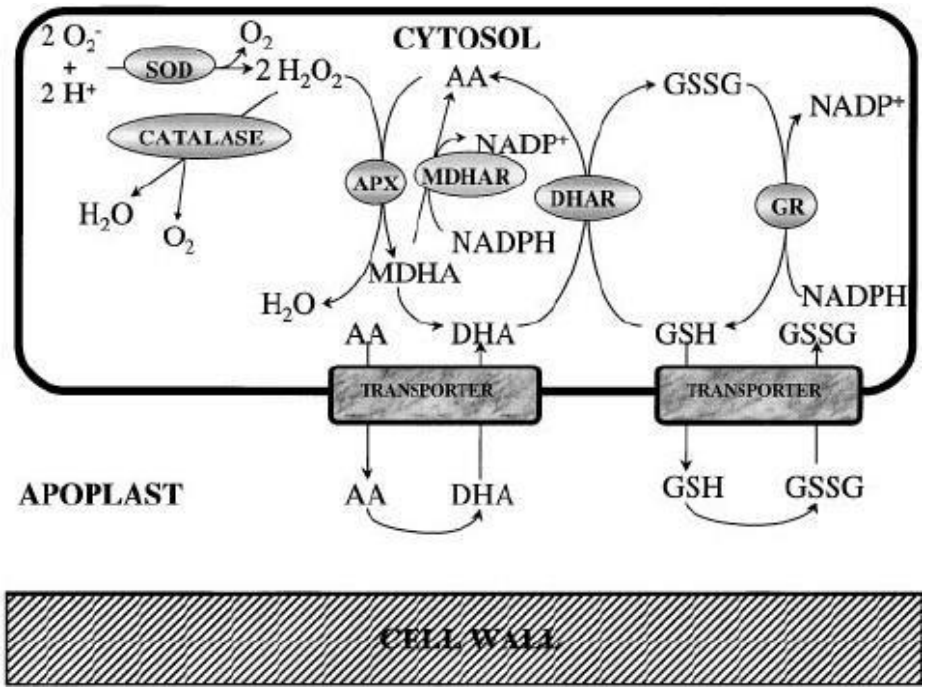

Figure 1.8.1 Reduction of H_2O_2 by the ascorbate-glutathione cycle (Vanacker *et al.*, 1998a)

Reactive OH• radicals are detoxified by superoxide dismutation via SOD. SOD is found in all aerobic organisms and in all subcellular compartments susceptible of oxidative stress (Bowler *et al.*, 1992). Three types of SOD can be identified by their metal cofactor including, the structurally similar FeSOD (prokaryotic organisms, chloroplast stroma), MnSOD (prokaryotic organisms and the mitochondrion of eukaryotes) and structurally unrelated Cu/ZnSOD (cytosolic, chloroplastic and peroxisomal enzyme) (Blokhina *et al.*, 2000). Catalase and peroxidases play a major role in the regulation of intracellular level of H_2O_2 (Blokhina *et al.*, 2000).

Table 1.8.1 List of enzymatic and non-enzymatic antioxidants, their origin and functions (Das and Roychoudhury, 2014)

Enzymatic antioxidants	**Enzyme code**	**Reaction catalyzed**	**Subcellular location**
Superoxide dismutase (SOD)	1.15.1.1	$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow 2H_2O_2 + O_2$	Peroxisomes, Mitochondria, Cytosol, and Chloroplast
Catalase (CAT)	1.11.1.6	$2H_2O_2 \rightarrow O_2 + 2H_2O$	Peroxisome and Mitochondria
Ascorbate peroxidase (APX)	1.11.1.11	$H_2O_2 + AA \rightarrow 2H_2O + DHA$	Peroxisomes, Mitochondria, Cytosol, and Chloroplast
Monodehydroascorbate reductase (MDHAR)	1.6.5.4	2MDHA + NADH → 2AA + NAD	Mitochondria, Cytoplasm, and Chloroplast
Dehydroascorbate reductase (DHAR)	1.8.5.1	DHA + 2GSH → AA + GSSG	Mitochondria, Cytoplasm, and Chloroplast
Glutathione reductase (GR)	1.6.4.2	GSSG + NADPH → 2GSH + $NADP^+$	Mitochondria, Cytoplasm, and Chloroplast
Guaiacol peroxidase (GPX)	1.11.1.7	H_2O_2 + DHA → $2H_2O$ + GSSG	Mitochondria, Cytoplasm, Chloroplast, and ER
Non-enzymatic Antioxidants		**Function**	**Subcellular location**
Ascorbic Acid (AA)		Detoxifies H_2O_2 via action of APX	Cytosol, Chloroplast, Mitochondria, Peroxisome, Vacuole, and Apoplast
Reduced Glutathione (GSH)		Acts as a detoxifying co-substrate for enzymes like peroxidases, GR and GST	Cytosol, Chloroplast, Mitochondria, Peroxisome, Vacuole, and Apoplast
α -Tocopherol		Guards against and detoxifies products of membrane LPO	Mostly in membranes
Carotenoids		Quenches excess energy from the photosystems, LHCs	Chloroplasts and other non-green plastids
Flavonoids		Direct scavengers of H_2O_2 and $^1O_2$ and $OH^\bullet$	Vacuole
Proline		Efficient scavenger of $OH^\bullet$ and $^1O_2$ and prevent damages due to LPO	Mitochondria, Cytosol, and Chloroplast

2. Research objectives

The understanding of the specific role of AOX in the adjustment of energy requirements and metabolic fluxes, balancing the formation of ROS and RNS during anoxia and subsequent post-anoxic recovery is inadequate and controversial. We hypothesize that alternative oxidase in plants has a significant contribution on the controlling of reactive oxygen species and reactive nitrogen species during anoxia and post-anoxic recovery in plants stress conditions in plants, which in turn balance the cellular energy requirements during this oxygen deprivation and recovery. Therefore, the aim of this study was to establish the experimental conditions of tobacco leaves under anoxia and re-aeration to observe the changes in the energy fluxes, formation of ROS and RNS and their balance during in anoxia and post-anoxic recovery in the presence of differentially expressing AOX. We have used transgenic tobacco plants with modified amounts of AOX (knockdown mutants: R19, R29; overexpressors: B7, B8 and wild type control: W) to gain a clear understanding of the role of AOX in the adjustment of metabolic fluxes and in balancing the formation and scavenging of reactive oxygen and nitrogen species (ROS and RNS) during anoxia and subsequent recovery.

3 Methods

3.1 Study species and plant growth

Leaves of tobacco plants (*Nicotiana tabacum* L. cv Petit Havana SR1) in plant family Solanaceae, were used in these experiments. Transgenic lines with suppressed amounts of AOX protein (RI19= R19, RI29= R29) as a result of the presence of an *Aox1a* RNA interference construct, or elevated amounts of AOX protein (B7, B8) as a result of the presence of an *Aox1a* transgene driven by a constitutive promoter, have

been previously characterized (Amirsadeghi *et al*., 2006; Wang *et al*., 2011) and were provided by Dr. Greg C. Vanlerberghe, University of Toronto. The knockdown lines have suppressed amounts of AOX protein relative to the wild type (WT). The amount of AOX protein is little higher in R19 than R29, even under strongly inducing conditions (Amirsadeghi *et al*., 2006; Wang *et al*., 2011; Wang & Vanlerberghe, 2013). Transgenic line B7 and B8 (over-expressing lines) have elevated amounts of AOX (compared with Wt and R lines) (Amirsadeghi *et al*., 2006; Wang *et al*., 2011; Wang & Vanlerberghe, 2013).

Healthy seeds were germinated directly on moist soil in a chamber with a controlled-environment (Model PGR-15, Conviron, St.John's, Canada) with 16 h photoperiod, temperature of 28 : 22°C (light : dark), relative humidity of 60% and photosynthetic photon flux rate (PPFR) of 150 μmol m^{-2} s^{-1} (150 PPFR). Pots were arranged in a completely randomized design. Plants were irrigated daily and fertilized once in every two weeks with 20-20-20 fertilizer as prescribed by the supplier (Plant products, Brampton, Ontario, Canada). Healthy plants were used at one month growing on soil (Figure 2.3.1)

3.2 Treatments: anoxia and re-oxygenation

To test the plants under anoxia, they were placed in a custom-built, sealed, 2 L, dark chamber for 4 h. A steady inflow of nitrogen gas was maintained at 120 mL min^{-1} (Alphagaz 1 grade having ~0.001% oxygen) at one opening and unidirectional air valves facing outwards in the openings to maintain ambient pressure within the chamber while preventing ambient air from entering (Cochrane *et al.*, 2017). The chamber was opaque to obscure any light and contained two small openings on either side. Fully

developed fourth or fifth leaves of the plants were harvested immediately after the treatment, fixed in liquid nitrogen and stored in -80 ^{0}C for subsequent assays.

As the post-anoxia or re-oxygenation treatment, the plants were kept in normal air for 15 min and 2 h after anoxia treatment. The term ‘‘anoxia’’ refers to the first 2–3 min of post-anoxia because extract preparation, which took about 2–3 min, was conducted in air and the subsequent 15 min and 2 h follows the same manner. As the normoxia (control) treatment, the leaves were directly sampled from the growth chamber.

3.3 Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and lipid peroxidation

Superoxide assay was conducted according to Alexou (2013). Fresh leaves (0.1 g) were ground with PVP (equal weight in relation to plant material 1: 1) in 500 µl distilled water and stored at 4 °C for one day. After centrifuging the extract for 20 min at 4 °C, 100-200 µl of the supernatant was combined with 100 µl luminol (3 mM in DMSO) and 700-800 µl distilled water. Absorbance was recorded spectrophotometrically at 425 nm using 900 µl distilled water and 100 µl luminol as the blank. Superoxide content was calculated as nmol g^{-1} fresh weight (FW).

Fresh leaves (600 mg) were homogenized in 3 mL 0.1% (w:v) TCA and centrifuged at 12,000 g for 20 min at 4 ^{0}C. The supernatant was used to estimate lipid peroxidation and H_2O_2 content.

H_2O_2 content was estimated according to Velikova *et al.* (2000) with modifications. To determine H_2O_2, 300 µl of the supernatant was mixed with 500 µl of 2M potassium iodide (KI) and 200 µl of 10 mM potassium phosphate buffer (pH 7.0). The reaction mixture was incubated for 1hr in dark at room temperature. Absorbance was measured

spectrophotometrically at 390 nm. The H_2O_2 was quantified based on a standard curve developed using a known H_2O_2 concentration.

Lipid peroxidation was determined by using thiobarbituric acid (TBA), which determines malondialdehyde (MDA) as a product of lipid peroxidation (Heath and Parker, 1968). One milliliter aliquot of supernatant 1 mL of 10% TCA containing 0.5% (w:v) TBA was added. The mixture was incubated at 95°C for 20 min, quickly cooled in crushed ice for 10 min stop the reaction and centrifuged at 10,000 g for 5 min. The absorbance of supernatant was measured at 532 and 600. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm and the concentration of MDA concentration was calculated per g^{-1} fresh weight (FW), using an extinction coefficient of 155 mM^{-1} cm^{-1}.

3.4 *Antioxidant enzymes*

The extractions for catalase (CAT) and superoxide dismutase (SOD) were performed according to Ma *et al.* (2016) with minor modifications. Leaf samples (0.5 g) was ground in liquid nitrogen and homogenized in 4 ml of ice-cold buffer containing, 50 mM Tris–HCl (pH 8.0), 15 mM (dithiothreitol) DTT and 0.1 M sucrose on ice. The homogenates were centrifuged at 13,000g for 30 min at 4^0C. Supernatants were stored at -80 ^{0}C and used for enzyme activity assays.

3.4.1 Catalase (CAT)

Catalase (EC1.11.1.6) activity was measured according to the Guilbault (1976). The reaction buffer was consisted of 0.05 M phosphate buffer (pH 7) containing 3% H_2O_2 (w/w). The reaction was initiated by adding 20-100 µl of plant extract to the reaction buffer solution (900-980 µl). The absorbance was measured at 240 nm for 3

min. Molar extinction coefficient for H_2O_2 (43.6 M^{-1} cm^{-1}) was used to calculate the equivalent catalase activity per mg protein.

3.4.2 Superoxide dismutase (SOD)

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed according to Beauchamp and Fridovich (1971). This method was based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm. Leaf extract (30-100 µl) was mixed with the assay buffer (900-970 µl) which contains 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM ethylenediaminetetraacetic acid (EDTA), 75 µM NBT, 13 mM methionine, 2 µM riboflavin. Reactions were carried out for 10 min at a light intensity of 300 µmol m^{-2} s^{-1}. The non-irradiated reaction mixture served as the control and the absorbance at 560nm was deducted from the absorbance of the irradiated sample. The amount of enzyme which caused 50% inhibition of NBT reduction was defined as one unit of SOD under the assay condition, and the results were expressed per mg protein.

Enzymes of ascorbate-glutathione cycle were extracted as described by Ma *et al*. (2016). Leaves were homogenized with 50 mM MES-KOH buffer (pH 6.5), containing 40 mM KCl, 2 mM $CaCl_2$, 1 mM ascorbate (for ascorbate peroxidase, added freshly) and then centrifuged at 15000 g for 10 min. Supernatants were collected as a crude enzyme solution for further analysis.

3.4.3 Ascorbate peroxidase (Apx)

Ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to the method described by Ma *et al*. (2016). The assay medium for Apx was 800-880 µl of 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM sodium ascorbate and 20-100 µl sample extract. The reaction was started by adding 100 µl of H_2O_2 (final

concentration 0.01%) and the reaction rate was determined by the change in absorbance at 290 nm. Molar extinction coefficient for ascorbate (2.8 mM^{-1} cm^{-1}) was used to calculate the equivalent catalase activity per mg protein.

3.4.4 Monodehydroascorbate reductase (MDHAR)

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) was measured according to the method described by Ma *et al*. (2016). MDHAR activity was measured in 980-900 µl of 50 mM HEPES buffer (pH 7.6) containing 2.5 mM ascorbate, 0.25 mM NADH, and 20-100 µl of the extract. The assay was initiated by adding 0.4 U cm^{-3} of ascorbate oxidase and the reaction rate was monitored at 340 nm for 3 min. Molar extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate the equivalent MDHAR activity per mg protein.

3.4.5 Dehydroascorbate reductase (DHAR)

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured according to the method described by Ma *et al*. (2016) at 265 nm. The assay buffer contained 980-900 µl of 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, and 20-100 µl of extract. The reaction was initiated by adding freshly prepared DHA (final concentration of 0.8 mM). Molar extinction coefficient of 14 mM^{-1} cm^{-1} was used to calculate the equivalent DHAR activity per mg protein.

3.4.6 Glutathione reductase (GR)

Glutathione reductase (GR, EC 1.8.1.7) activity was monitored at 340 nm for 3min from the rate of conversion of NADPH to NADP (ε_{340} = 6.2 mM^{-1} cm^{-1}) (Ma *et al.*, 2016). The assay mixture contained 980-900 µl of 50 mM HEPES buffer (pH 8.0) containing 0.5 mM EDTA, 0.25 mM NADPH, and 20-100 µl extract. The reaction was

started by adding GSSG to a final concentration of 1 mM and the absorbance was recorded at 340 nm. Molar extinction coefficient of 6.2 mM^{-1} $cm^{-1}$was used to calculate the equivalent GR activity per mg protein.

3.5 Nitric oxide (NO) measurement

NO emission was measured by an efficient method called chemiluminescent described by Cochrane *et al*. (2017). Ozone is chemically oxidized by NO and yields nitrogen dioxide in an excited state where relaxation from this excited state produces distinctive light emission called chemiluminescence (Fereja *et al*., 2013). This is directly proportional to NO concentration (Fereja *et al*., 2013). To measure NO emission from leaves, 10 g of fresh leaves were immediately detached from the plants and placed in an air-tight container with 20 mM HEPES buffer (pH 7.0) and 50 mM sodium nitrate as a nitrogen source (Cochrane *et al*., 2017). The container (500 ml), which contained two openings on each side, has a steady 120 mL min^{-1} inflow of nitrogen gas through one opening, and the other is attached to a chemiluminescent detector (CLD 88 p; Eco-Physics, Dürnten, Switzerland) by a vacuum pump which pulled in air at 120 mL min^{-1} and was connected to an ozone destroyer (Planchet *et al*., 2005). The measuring gas was made NO free by a NO scrubber (EcoPhysics, Switzerland detection limit 20 ppt, 20 s time resolution) and gas flow was regulated by flow controllers (Fisher Scientific). Calibration was routinely carried out with NO free air (0 ppt NO) and with various concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen; Messer Griesheim, Darmstadt, Germany) with NO-free air (Shah *et al*., 2013).

Total NO accumulation was recorded and the rate of NO emission from the leaves was determined per g fresh weight by using chemiluminescent detector (CLD 88 p; Eco-Physics, Dürnten, Switzerland) (Shah *et al*., 2013). Alternative oxidase (AOX) was inhibited by adding 5 mM salicylhydroxamic acid (SHAM) and 0.5 mM propylgallate (PG) separately. The control was carried with only buffer and source of nitrate ($NaNO_3$).

3.6 Total soluble protein, total thiol (SH) groups and S-nitrosylated proteins (SNO)

Protein *S*-nitrosylation was measured according to (Ma *et al*., 2016) by reducing R-SNO to R-SH in the presence of ascorbate and then assaying free thiol groups using 5,5′-dithiol-bis (2-nitrobenzoic acid) (DTNB) (Devin *et al*., 2017). Protein was extracted from 100 mg of fresh leaves that was homogenized using a mortar and pestle in 50 mM HEPES (pH 8.0) containing 1 mM EDTA, 0.1 mM neocuproine, 0.2% (w/v) SDS and 0.5% (w/v) CHAPS. The homogenate was centrifuged (15,000 g, 10 min, 4 °C) and supernatant was added with two volumes of ice-cold acetone and kept overnight at −20 °C to precipitate proteins. The precipitated protein was separated by centrifuging at 15,000 g for 10 min at 4 °C and pellet was washed four times with 70% chilled acetone. The protein was re-suspended in the same volume of extraction buffer. Protein solution was separated into two volumes of 0.9 mL samples and 50 μL of 100 mM ascorbate and 50 μL of distilled water were added to the experimental and control set-up comparatively. After incubating for 1 h at 25 °C, 50 μL of 10 mM DTNB in 75 mM phosphate buffer (pH 7.0) was added and absorbance was measured by spectrophotometer at 412 nm for each set-up. Since the quantity of R-SH generated by ascorbate treatment corresponds to that of R-SNO in proteins (Ma *et al*., 2016), the

difference between the quantity of R-SH between experimental and control was used to calculate the quantity of R-SNO (Ma *et al*., 2016; Devin *et al*., 2017). The total free R-SH groups were quantified in the absence of ascorbate (Ma *et al*., 2016).

Total soluble protein content was determined according to protocol designed by Bradford (1976) using Bovine Serum Albumin (BSA) standard at 595 nm.

3.7 Enzymes and compounds in oxidative metabolism

3.7.1 Alcohol dehydrogenase (ADH)

Extraction of alcohol dehydrogenase (ADH) was performed according to Ma *et al.* (2016) with minor modifications. Leaf samples (0.5 g) were homogenized on ice in 4 ml of ice-cold buffer containing, 50 mM Tris–HCl (pH 8.0), 15 mM (dithiothreitol) DTT and 0.1 M sucrose. The homogenates were centrifuged at 13,000 g for 30 min at 4 ^{0}C. Supernatants were used for ADH assay according to Blandino *et al*. (1997) in ethanol to acetaldehyde direction by using an assay buffer of 50 mM Tris-HCl (pH 8.0), 150 mM ethanol, and 2 mM NAD^{+} at 340 nm. The extinction coefficient of 6.22 mM^{-1} $cm^{-1}$was used to calculate the rate of enzymatic activity per mg protein.

3.7.2 Aconitase

Fresh leaves (100 mg) were extracted into 1ml of extraction buffer [50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$ and 2 mM trisodium citrate] to estimate the activity of aconitase (EC 4.2.1.3) as described by Baumgart and Bott (2011). The homogenate was centrifuged at 10,000 g for 5 min at 4 °C and 40-150 µl of it was mixed with 960-850 µl of assay buffer. The assay buffer contains 50 mM Tris-HCl (pH 7.4) and 40 mM trisodium citrate. The absorbance was measured by spectrophotometer at 240 nm and the extinction coefficient of 3.6 mM^{-1} cm^{-1} was used to calculate total enzymatic activity per g fresh weight (Baumgart and Bott, 2011).

3.7.3 ATP, ADP and ATP/ ADP ratio

Extraction of ATP and ADP was conducted according to Joshi *et al*. (1989) and Yuroff *et al*. (2003) with modifications. Leaf tissue was ground in liquid nitrogen and homogenized in 2.4 M, ice-cold perchloric acid for 60 min on ice and centrifuged for 5 min at 20,000 g at 4 °C. The supernatant was neutralized with 4 M KOH, and the ATP/ADP in the neutralized solution was determined according to supplier's instructions in the EnzyLight^{TM} ADP/ATP Ratio Assay Kit (ELDT-100), Bioluminescent Assay by using a luminometer (FB12 Single Tube Luminometer Titertek-Berthold). ATP and ADP contents were determined using an ATP and ADP standards.

3.8 Statistical analysis

All the experiments were repeated for 3-4 times, the analytical assays in each sample were done in triplicates. Data for all characteristics were subjected to one way-ANOVA using the Minitab software (2017). The data on figures are the means of three biological repeats ± SD. The statistically significant differences at $P < 0.05$ are discussed and the different letters on the graphs designate significant differences at $P < 0.05$.

4 Results

4.1 Plants under treatments

As seen in the figure 4.1, AOX-overexpressing lines grew better than wild type and followed by knockdown lines. Further, under anoxia treatments, the B7 and B8 lines survived better and recovered rapidly than other lines.

Figure 4.1 Tobacco plant lines (R29, R19 = knockdown lines; B7, B8 = overexpressing lines; W = wild type) under normoxia and anoxia (4 h nitrogen gas) treatments. a, b) under normoxia; c, d) under anoxia.

4.2 Superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and lipid peroxidation

These tests give the levels of oxidative stress and damage caused by stresses in the leaves of each plant line in terms of superoxide, hydrogen peroxide and lipid peroxidation.

As seen in figure 4.2, under normoxia, the leaves of AOX-knockdown tobacco plants had a higher level of superoxide than wild type and AOX-overexpressing lines. However, the levels were similar in wild type and AOX-overexpressing lines under normal air condition. Superoxide levels in leaves increased during anoxia and then decreased at 15 min re-exposure to air while it increased again at 2 h post-anoxia. Further, AOX-overexpressing lines showed a significantly lower superoxide level under 2 h post anoxia than in wild type.

In tobacco leaves of knockdown lines (R29 and R19) and B7-overexpressing line had comparatively higher levels H_2O_2 in each treatment than the leaves of B8-overexpressing line and wild type (Figure 4.2). During anoxia, the H_2O_2 levels increased and then declined upon reaeration. At 2 h post-anoxia period, the levels dropped to a similar level in each line.

Under normoxia, level of MDA (as a result of lipid peroxidation) in tobacco leaves was higher in AOX-knockdown lines than other lines, while the levels were similar in both wild type and overexpressing lines (Figure 4.2). However, the levels in all plant lines increased continuously upon anoxia and reaeration. AOX-knockdown lines had significantly higher levels of peroxides than overexpressing lines. Further, those levels were lower in AOX-overexpressing lines than in wild type.

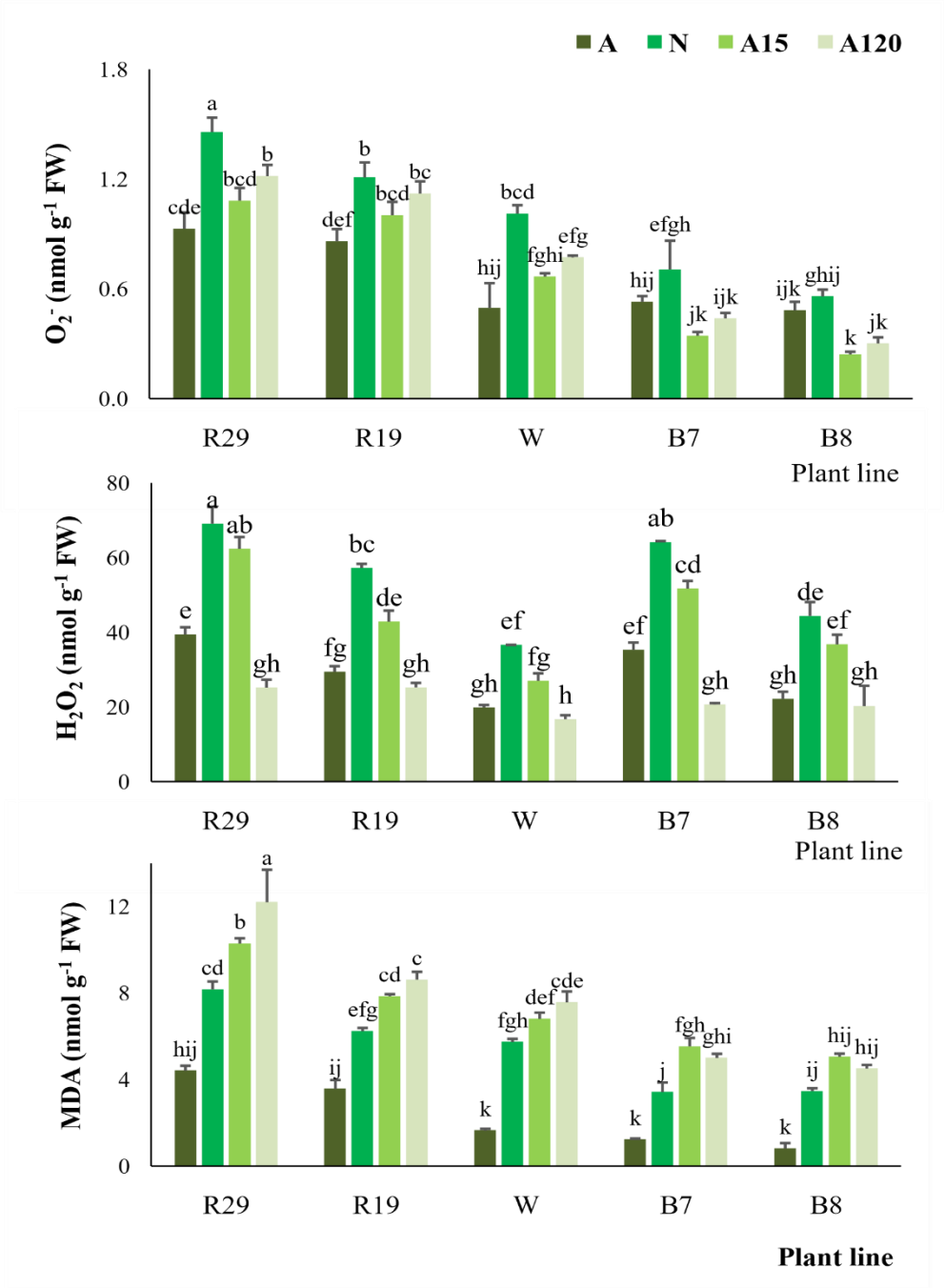

Figure 4.2 Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and lipid peroxidation (as a marker of lipid peroxidation) in the leaves of tobacco plant lines (R29, R19 = knockdown lines; B7, B8 = overexpressing lines; W = wild type) under each treatment: A = normal air or control, N = 4 h exposure to anoxia or nitrogen gas, A15 = 15min post-anoxia, A120 = 2h post-anoxia. Vertical bars represent standard deviations (n = 3). Means that do not share a letter are significantly different at $P \leq 0.05$ (one-way ANOVA test).

4.3 Antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (Apx)

The activities of antioxidant enzymes give an overall idea about the levels of antioxidative responses and whether the plants have a better way to survive or they have induced defense strategies.

As demonstrated in the figure 4.3, AOX-knockdown lines showed significantly higher SOD activity than overexpressing and wild type plants. Normoxia plants have lower value than in other conditions while under anoxia, the levels increased significantly. At 15 min post-anoxic condition, the levels decreased and again increased at 2 h post-anoxic injury to a similar level as seen under anoxia. Catalase activity in AOX-overexpressing lines and wild type were significantly higher than in knockdown lines (Figure 4.3). Leaves under normoxia had a significantly lower activity than the treated leaves, except in B8. Knockdown lines did not show a significant difference between treatments while other lines showed increment under anoxia, decrement at 15 min reaeration and again increment at 2 h post-anoxia.

As depicted in the figure 4.3, wild type and AOX-overexpressing lines show a significantly higher activity of APX than knockdown lines. Though APX activity in R19 remained unchanged under the treatments, others showed a significant decrease during anoxia and reaeration. However, R29 increased the APX activity upon re-exposure to air. The decrement is pronounced more in AOX- overexpressing lines than others.

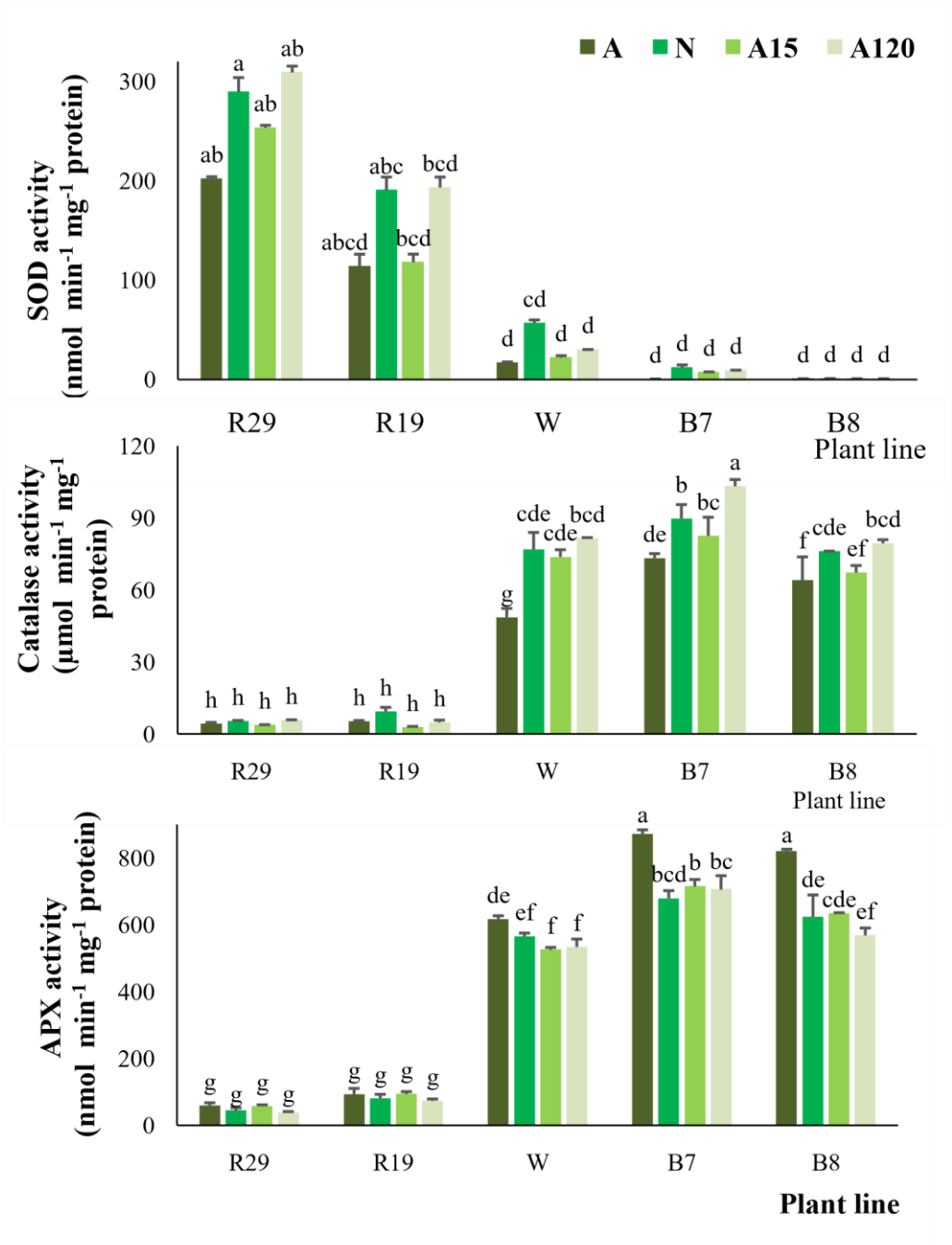

Figure 4.3 Activities of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (Apx) of the leaves of tobacco plant lines (R29, R19 = knockdown lines; B7, B8 = overexpressing lines; W = wild type) under each treatment: A = normal air or control, N = 4h exposure to anoxia or nitrogen gas, A15 = 15min post-anoxia, A120 = 2 h post-anoxia. Vertical bars represent standard deviations (n = 3). Means that do not share a letter are significantly different at $P \leq 0.05$ (one-way ANOVA test).

4.4 Other enzyme activities in ascorbate glutathione cycle: monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR)

These enzyme activities show the levels of oxidative defense in plant leaves, especially to detoxify the levels of hydrogen peroxide via the ascorbate-glutatione cycle.

Activity of MDHAR followed a similar pattern as in APX (Figure 4.4) though knockdown lines also had a significant activity of MDHAR. Except in R29, other lines showed a declining of MDHAR activity under anoxia and then continuous increment upon 15 min to 2 h re-exposure to air.

DHAR activity varied differently between lines (Figure 4.4). When leaves of knockdown lines and wild type did not show a significant change in DHAR activity under the treatment, overexpressing lines showed significant changes. Upon reaeration, AOX-overexpressing lines demonstrated a decline in DHAR activity and again decreased or increased upon reaeration.

According to the figure 4.4, GR activity is higher in AOX-overexpressing lines than in other lines. The activities of each plant line followed a similar pattern under the treatments and AOX-overexpressing lines showed a higher GR activity while knockdown lines had a low level of activity. During anoxia, the GR activity decreased, but the levels in AOX-overexpressing line, especially in B8 is higher than others. In contrast, the GR activity arose significantly during post-anoxic recovery at 15 min and gain decreased upon 2 h re-exposure to normal air.

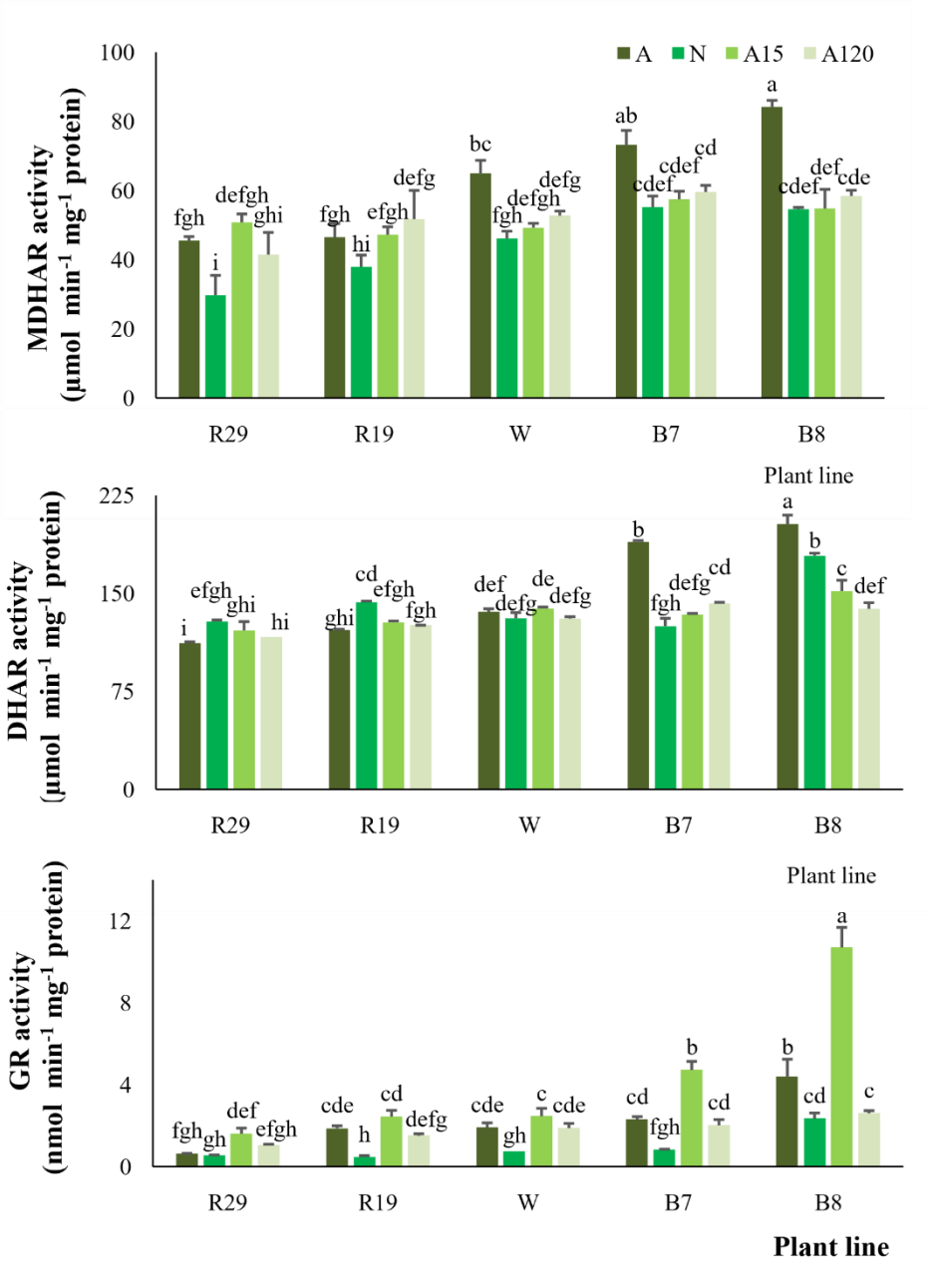

Figure 4.4 Activities of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) of the leaves of tobacco plant lines (R29, R19 = knockdown lines; B7, B8 = overexpressing lines; W = wild type) under each treatment: A = normal air or control, N = 4 h exposure to anoxia or nitrogen gas, A15 = 15 min post-anoxia, A120 = 2h post-anoxia. Vertical bars represent standard deviations (n = 3). Means that do not share a letter are significantly different at $P \leq 0.05$ (one-way ANOVA test).

4.5 Nitric oxide (NO) measurement

This test was performed to estimate the levels of NO in plant leaves after 3 h and 6 h under anoxia. According to the figure 4.5, the NO emission under anoxia is higher in AOX-overexpressing lines than in knockdown lines. When inhibitors of AOX were used, the rate of NO emission decreased in each line and those results are clearer at 6 h of anoxia exposure than at 3 h.

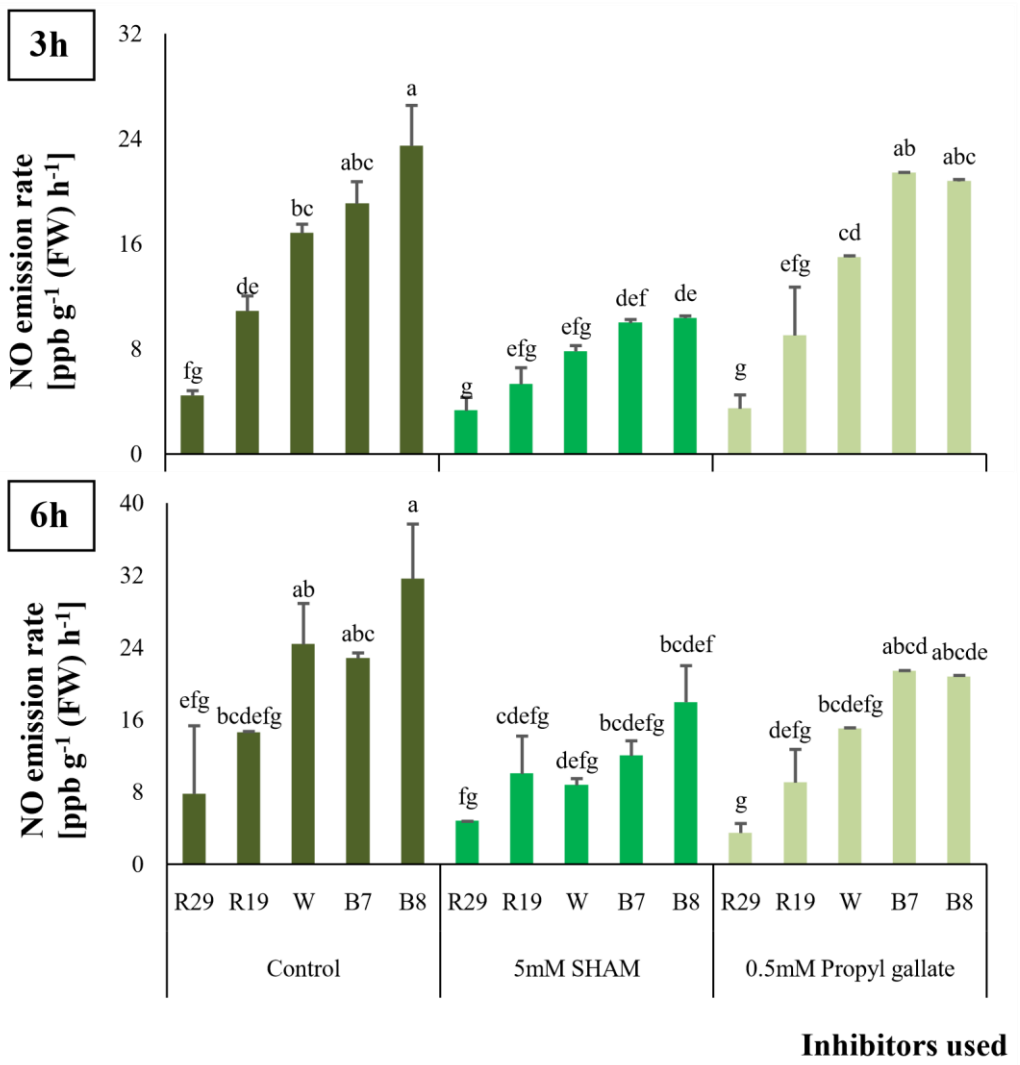

Figure 4.5 Nitric oxide emission under anoxia measured from the leaves of tobacco plant lines (R29, R19 = knockdown lines; B7, B8 = overexpressing lines; W = wild type) under each treatment: A = normal air or control, N = 4 h exposure to anoxia or nitrogen gas, A15 = 15min post-anoxia, A120 = 2h post-anoxia. Vertical bars represent standard deviations (n = 3). Means that do not share a letter are significantly different at $P \leq 0.05$ (one-way ANOVA test).

4.6 Total soluble protein, total thiol (SH) groups and S-nitrosylated proteins (SNO)

These tests demonstrate the levels of proteins, thiol groups and *S*-nitrosylation levels of proteins in plant leaves of each plat line under each treatment. The proteins that have been nitrosylated or exist as free thiols under stress conditions can be compared in relation to the levels of total protein content.

Figure 4.6 shows that the levels of proteins do not vary significantly in tobacco leaves under anoxia or reaeration. However, the total thiol groups are higher in AOX-overexpressing lines than other plant lines (Figure 4.6). Three plant lines showed their own similar variations during treatments. A decline in total thiol groups can be seen in both knockdown and overexpressing lines, while the levels arose during anoxia in wild type. Moreover, total thiol groups increased during 2 h reaeration except in wild type. *S*-nitrosylated proteins in the leaves of tobacco plants under normoxia were higher in AOX-knockdown and wild type lines than in AOX-overexpressing lines. Under anoxia, the levels declined significantly in both AOX-knockdown and wild type lines, while AOX-overexpressing lines showed an increment. Those levels under anoxia in wild type and overexpressing lines were nearly similar to each other. In contrast, the nirosylated protein levels increased during post anoxic recovery at 15 min and 2 h except in wild type at 15 min reaeration.

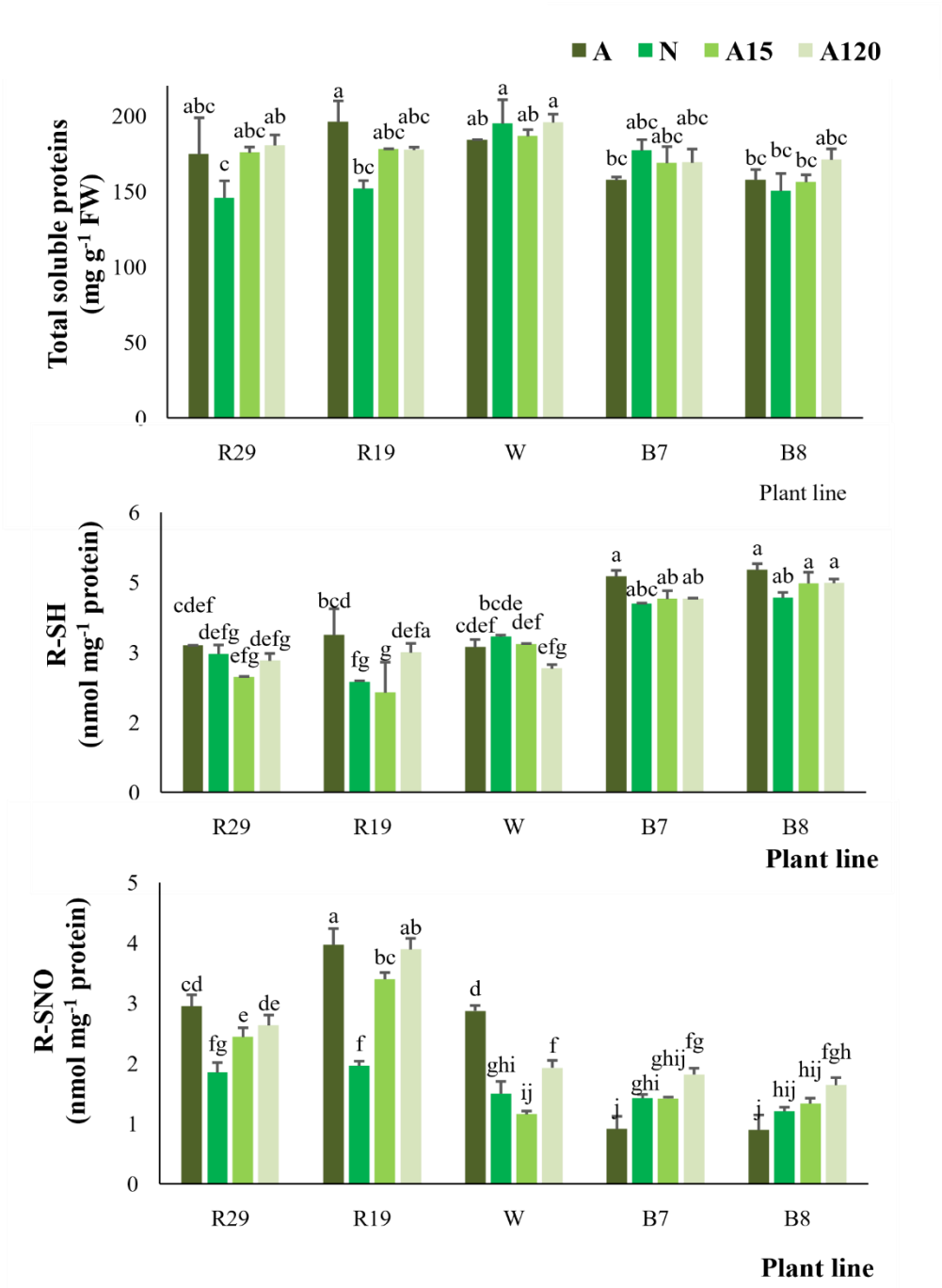

Figure 4.6 Total soluble proteins, total thiol (R-SH) content and R-SNO (*S*-nitrosylated proteins) of the leaves of tobacco plant lines (R29, R19 = knockdown lines; B7, B8 = overexpressing lines; W = wild type) under each treatment: A = normal air or control, N = 4 h exposure to anoxia or nitrogen gas, A15 = 15min post-anoxia, A120 = 2 h post-anoxia. Vertical bars represent standard deviations (n = 3). Means that do not share a letter are significantly different at $P \leq 0.05$ (one-way ANOVA test).

4.7 Enzymes and compounds in oxidative metabolism

These tests were done to estimate the energy levels in the leaves of each plant line under each treatment. ADH and aconitase activities give the overall idea about the fermentation pathway and the TCA cycle.

4.7.1 Alcohol dehydrogenase (ADH) and aconitase

ADH activity in tobacco leaves showed significant variations between lines (AOX-knockdown < overexpressed < wild type) (Figure 4.7.1). Wild type showed a significant increase in ADH activity under anoxia and reaeration. Except B7 line under 2 h post-anoxia, the ADH activity did not show a significant difference under anoxia or during post-anoxic recovery.

As shown in the figure 4.7.1, the aconitase activity of tobacco leaves followed as knockdown < wild type < overexpressed. AOX-knockdown lines did not show a significant difference in aconitase activity under the treatments. Both B7 and B8 overexpressing lines showed a similar pattern of aconitase activity under each condition where the activity increased during anoxia and again declined continuously upon post-anoxic recovery period. In wild type leaves, the activity increased significantly at 15 min post-anoxia and declined to a similar level as seen under anoxia.

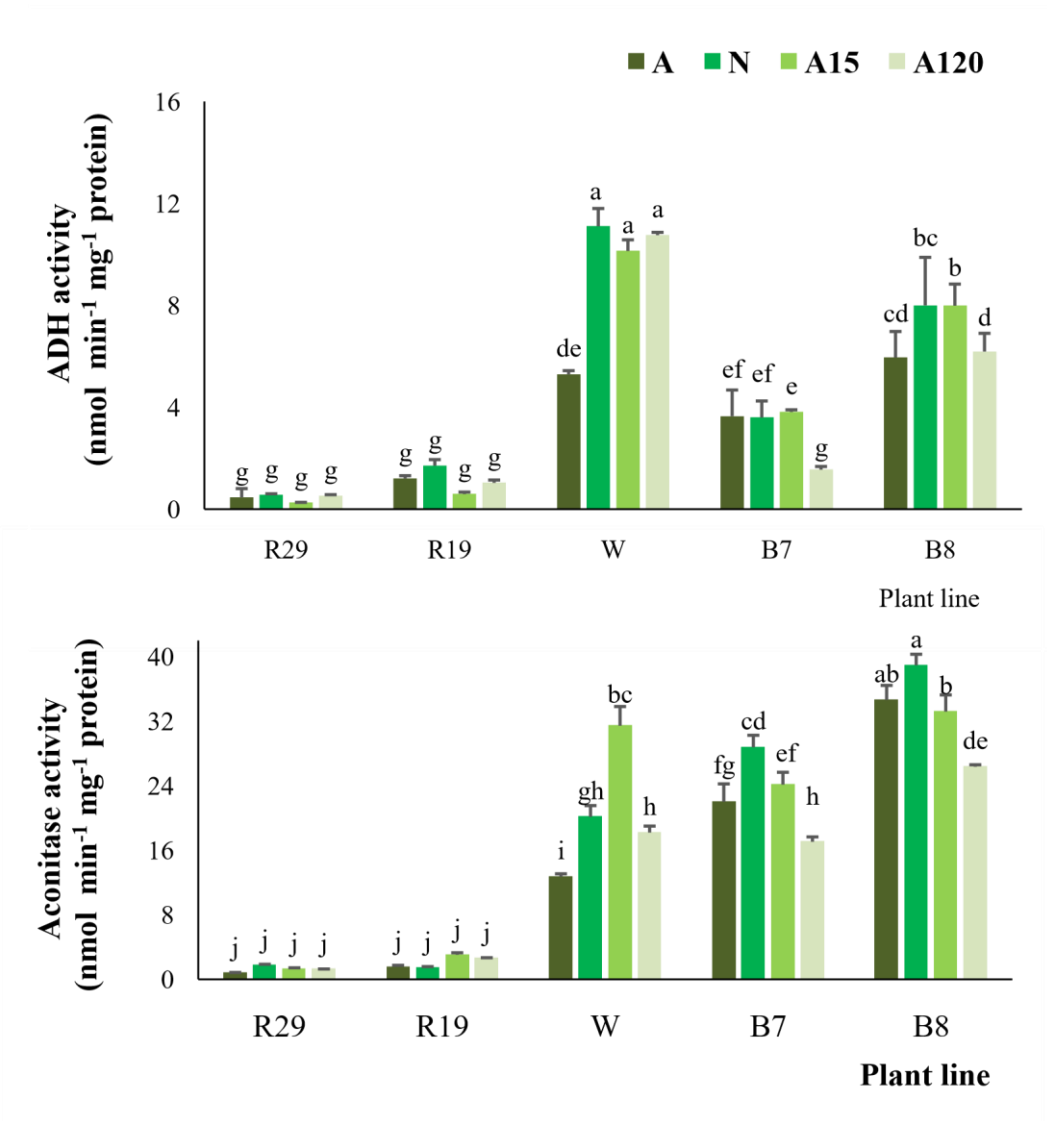

Figure 4.7.1 Alcohol dehydrogenase (ADH) and aconitase activities of the leaves of tobacco plant lines (R29, R19 = knockdown lines; B7, B8 = overexpressing lines; W = wild type) under each treatment: A = normal air or control, N = 4 h exposure to anoxia or nitrogen gas, A15 = 15 min post-anoxia, A120 = 2h post-anoxia. Vertical bars represent standard deviations (n = 3). Means that do not share a letter are significantly different at $P \leq 0.05$ (one-way ANOVA test).

4.7.2 ATP, ADP and ATP/ ADP ratio

As demonstrated in the figure 4.7.2, ATP level under normoxia is higher in AOX-overexpressing lines than others. However, the levels declined during anoxia, where B8 showed a significantly higher ATP amount than others even under anoxia. During post-anoxic recovery period, each line increased the ATP level continuously until 2 h and those levels were significantly higher in AOX-overexpressing lines than others. Except in R19, the ATP levels in leaves of tobacco after 2 h reaeration were higher than they were in normoxia.

Under normoxia, ADP levels in leaves of tobacco were significantly higher in AOX-overexpressing lines than in others (Figure 4.7.2) and each line followed their own patterns in a similar way. Both overexpressing and knockdown lines showed a decrement in ADP levels during anoxia and then arose continuously upon recovery until 2 h.

Total ATP and ADP of tobacco leaves were significantly higher in AOX-overexpressing plant lines under each condition (Figure 4.7.2). ATP and ADP pool declined during anoxia in each line and B8 showed slightly higher amount than others. Same as in ATP, this pool of ATP and ADP inclined upon post-anoxic recovery until 2 h. These levels were similar or significantly higher than those levels under normoxia.

ATP/ADP ratio also showed a similar pattern under each treatment (Figure 4.7.2). However, AOX-overexpressing lines showed a higher ratio under each condition than knockdown and wild type lines. The ratio declined upon anoxia and inclined during post-anoxic recovery. Those levels arose to a similar or significantly higher level at 15 min than seen under normal air condition. In contrast, except in R29, the ratio increased

significantly at 2 h of reaeration. The increment during reaeration was significant in AOX-overexpressing lines and wild type than R19.

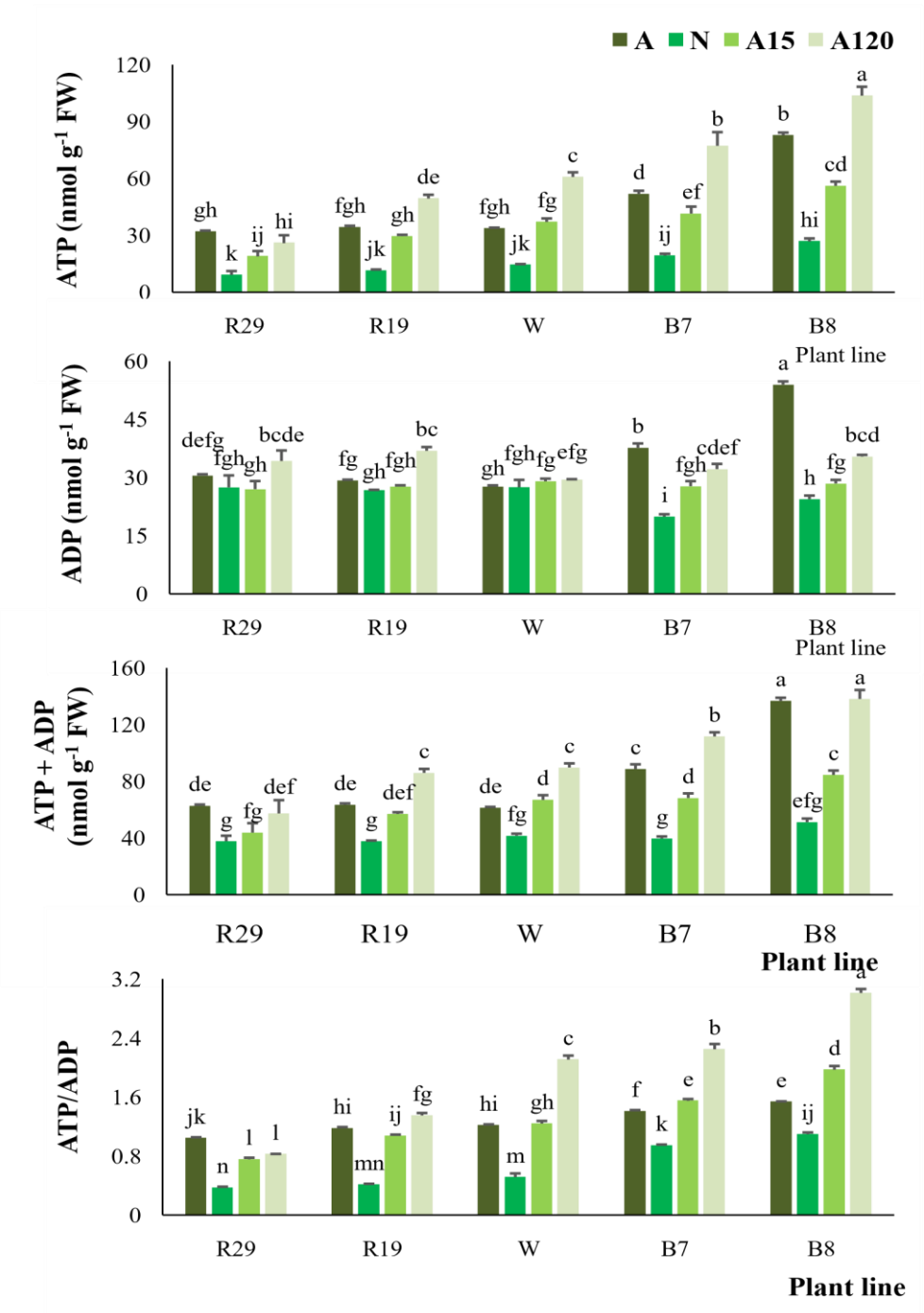

Figure 4.7.2 ATP, ADP, total ATP, ADP and ATP/ATP ratio of the leaves of tobacco plant lines (R29, R19 = knockdown lines; B7, B8 = overexpressing lines; W = wild type) under each treatment: A = normal air or control, N = 4 h exposure to anoxia or nitrogen gas, A15 = 15 min post-anoxia, A120 = 2h post-anoxia. Vertical bars represent standard deviations (n = 3). Means that do not share a letter are significantly different at $P \leq 0.05$ (one-way ANOVA test).

2.4 Discussion

Flooding and submergence create hypoxic or anoxic conditions that reduce oxygen availability for plants to continue photosynthesis and respiration (Banti *et al*., 2013). During the first hours of oxygen shortage the tolerance of the plant to the low oxygen condition increases while prolonged anaerobiosis results in damage and cell death (Drew *et al*., 2000). After water recedes, re-aeration or post-anoxia also result in serious cell damage which is termed as ‘‘post-anoxic injury’’ (Blokhina *et al.*, 2000).

Studies have shown that AOX plays an important role in countering abiotic stress in plants (Li and Xing, 2011; Zhang *et al.*, 2012; Vanlerberghe, 2013; Grabelnych *et al*., 2014; Keunen*et al*., 2016; Saha *et al*., 2016; Vanlerberghe *et al*., 2016). Transcription of gene(s) encoding AOX and levels of AOX proteins increases during stress and aid in regulating ROS to protect the photosystem and maintain cellular homeostasis when the ETC dysfunctions (Li and Xing, 2011; Vanlerberghe *et al*., 2016). Mitochondria of barley leaves have shown a reduced AOX capacity and AOX protein level during post-anoxia, while the AOX capacity in root mitochondria increased under anoxia (Skutnik and Rychter, 2009). Further, an increase in AOX transcript and protein level induction has been recorded in rice seedlings upon reoxygenation (Millar *et al*., 2004; Narsai *et al*., 2009). Increasing evidence shows that under anoxia and reaeration, AOX plays an important role in plant energy metabolism and oxidative stress balance. Therefore, in the present study, we have demonstrated the effect of alternative oxidase (AOX) on the balance of reactive nitrogen species and oxidative metabolism in tobacco leaves during anoxia and subsequent reaeration because, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered as common by-products of plant cellular metabolism and stress signaling

under stress conditions (Paradiso *et al*., 2016). Plant stress signaling and responses are a complex system. Therefore, the exact role of AOX may not be highlighted because, we have used the whole leaf, rather than the mitochondria.

AOX and NADPH dehydrogenase control the production of ROS in the mitochondria (Blokhina and Fagerstedt, 2010). As seen in the present study, superoxide and H_2O_2 levels increased significantly during anoxia in each plant line. The reason for the elevated ROS generation is the high level of reductants accumulated during the period of restricted oxygen availability (Szal *et al*., 2004). Further, tobacco leaves with AOX-knockdown lines showed a higher level of superoxide even under normal conditions than AOX-overexpressing lines which shows that AOX has a protective role. According to Maxwell *et al*. (1999), a constitutive activation of AOX in Arabidopsis or overexpression of Arabidopsi*s AOX1a* in tobacco decreases mitochondrial ROS production. Further, inhibition of AOX increases ROS production (Maxwell *et al*., 1999; Umbach *et al*., 2005).

Anoxia followed by reaeration creates conditions of elevated ROS generation in plants (Skutnik and Rychter, 2009). Arabidopsis cell suspensions have shown that ROS production is high during the 1st and 2nd hours of anoxia and affected almost the whole cell (Paradiso *et al*., 2016). During their study, even under re-oxygenation of 2 h, ROS were still being produced in a lower amount than in anoxia and almost 20 h required in order to restore normal redox homeostasis (Paradiso *et al*., 2016). Our results showed a sudden decrease of superoxide level in each line at 15min of post-anoxic recovery and then it increased again at 2 h of post-anoxia which demonstrates that exposure to air caused a decrease in superoxide level and post-anoxic injury during prolonged exposure

to oxygen may have caused an immediate increase in superoxide level (Figure 4.2). However, H_2O_2 level decreased further at 2 h post-anoxia (Figure 4.2).

Recent evidence shows that Arabidopsis plants expressing AOX in antisense orientation promote ROS production when cytochrome pathway is chemically inhibited with KCN treatment, even though untreated leaf tissues with altered levels of AOX protein did not affect the basal oxidative state (Umbach *et al*., 2005). Since the efficiency of COX to act as a terminal oxidase is disturbed by the production of NO under hypoxia, we suppose the levels of H_2O_2 can increase to a certain level if they are not regulated by the antioxidative system. However, the elevation of H_2O_2 level helps in stimulating the antioxidative system as a defense strategy because, our results showed that the levels of H_2O_2 are higher in both AOX-knockdown and overexpressing lines even under normoxia. Moreover, a study (Pasqualini *et al*., 2007) done by using wild-type (WT) Xanthi tobacco plants and Xanthi plants transformed with the Bright Yellow tobacco *AOX1a* cDNA with enhanced AOX capacity (SN21 and SN29) and decreased AOX capacity (SN10) AOX capacity has shown enhanced H_2O_2 production during post fumigation period of acute ozone (O_3). Further, SN21 and SN29 plant lines have shown more localized leaf damage under fumigation, whereas SN10, similarly to WT plants, was undamaged. Though ozone induced H_2O_2 accumulation in WT and in all transgenic lines, only in transgenic lines with high AOX capacity the H_2O_2 level in the post-fumigation period was high. SN21 plant line has had a higher respiratory capacity across the treatment. They suggest that far from exerting a protective role, the overexpression of AOX triggers an increased O_3 sensitivity in tobacco plants. As stated by Skutnik and Rychter (2009), the AOX capacity depends on the plant species, the plant part and the time of exposure to the stress and the type of stress. Therefore, we suggest that despite

of AOX expression, H_2O_2 levels increase in tobacco leaves when under anoxia and post-anoxia to stimulate the antioxidative system.

ROS production under stress conditions damage plant cell metabolism via peroxidative damage to proteins, nucleic acids, and lipids (Shewfelt and Purvis, 1995). Anoxia has been recorded to have a very limited impact on cell lipids (Brown and Beevers, 1987) when compared with post-anoxia which is regarded as the cause of the hazardous lipid peroxidation (LP) because of the action of ROS (Crawford, 1992; Blokhina *et al*., 1999). According to our results, the LP increased during anoxia and it further increased during reaeration in AOX-knockdown lines and wild type (Figure 4.2). However, the levels of LP are lower in AOX-overexpressing lines which should be due to lower amounts of ROS, especially superoxides than in knockdown lines. Accumulation of various products of LP as a result of reoxygenation has been observed in the roots of the anoxia-intolerant wheat and tolerant rice (Chirkova *et al*., 1998; Blokhina *et al*., 1999). Our results confirm that overexpression of AOX helps in reducing the levels of LP in tobacco leaves under anoxia and upon post-anoxic recovery.

Tobacco cells overexpressing AOX show a lower expression of genes encoding ROS scavenging enzymes, including the superoxide dismutase genes *SodA* and *SodB*, as well as glutathione peroxidase (Maxwell *et al*., 1999). In our results, AOX-overexpressing lines do not show a significant activity of SOD when tested with nitroblue tetrazolium (Figure 4.3). However, the AOX-knockdown lines showed a significantly higher SOD activity which may be due to higher levels of superoxides. Post-anoxia causes the generation of superoxide radicals measured as an induction of SOD activity during re-aeration (Monk *et al*., 1989). High SOD activity can contribute to flooding tolerance by improving detoxification of superoxide upon re-oxygenation

(Monk *et al.*, 1987; VanToai and Bolles, 1991). In the present study, SOD activity decreased during 15 min of reaeration and again increased by 2 h (Figure 4.3). This should be due to higher oxidative stress during 2 h of reaeration than at 15 min reaeration (Figure 4.3). However, catalase activity was higher in AOX-overexpressing lines and wild type than knockdown lines under each condition. Catalase activity increased during anoxia and 2 h post-anoxia and decreased during 15 min of re-exposure to air. (Figure 4.3). Both wild type and AOX-overexpressing lines had nearly similar catalase activity while B8 did not show a significant difference between treatments and normal condition which indicates its tolerance to these anoxic and post-anoxic stresses (Figure 4.3).

Enzyme activity and ascorbate and glutathione contents in rice seedlings gradually increase when anoxically pretreated plants are re-aerated (Ushimaro *et al.*, 1992). During post-hypoxia, the roots of wheat (*Triticum aestivum*) seedlings could cope up with the deleterious effects of oxygen radical generation due to increased GR activity and the content of glutathione (Albrecht and Wiedenroth, 1994). Antioxidant enzymatic defense systems such as, SOD, APX, glutathione and ascorbate concentration and the reduction states of glutathione and ascorbate pools are activated during post-hypoxia (Monk *et al*., 1987; Albrecht and Wiedenroth, 1994; Biemelt *et al.*, 1998). As stated by Blokhina *et al*. (2000), plants with a higher antioxidant concentration and/or redox state have a greater tolerance to abiotic stress, including anoxia. According to our results, although knockdown lines had a higher concentration of SOD, that did not help to stimulate the antioxidative system (Figure 4.3).

Leaf APX in tobacco may contribute to enhanced oxidation of ascorbate rather than scavenging H_2O_2 since anoxia and reaeration resulted in a decline in APX activity

in our study. However, that activity was higher in wild type and AOX-overexpressing lines than in knockdown lines (Figure 4.3). Further, DHAR in AOX-overexpressing lines during anoxia and post-anoxia was lower and similar to the levels in knockdown and wild type plant lines. Therefore, it is likely that regeneration of ascorbate by this pathway was low during anaerobiosis and post-anoxia. However, as stated by Blokhina *et al*. (2000), data on antioxidant levels and the activity of antioxidant enzymes are somewhat contradictory due to the records on both decreases and increases in antioxidative capacity. This diversification is due to the response specificity of a plant species and different experimental conditions for instance, stress treatment, duration of stress, assay procedure and parameters measured (Blokhina *et al*., 2000). A large-scale investigation of 11 plant species with contrasting tolerance to anoxia showed increase of the activities of monodehydroascorbate reductase (MDHAR) and/or dehydroascorbate reductase (DHAR) activities after several days of anoxia treatment and then the intolerant plants have demonstrated very low activities or without any changes of the activities (Blokhina *et al.*, 2000). Reduced glutathione (GSH) content decreased significantly during the post-anoxic period, while ascorbic acid (AA) increased in the tolerant species (Wollenweber-Ratzer and Crawford 1994). Roots of wheat seedlings under root hypoxia or whole plant anoxia has demonstrated a significant increase in the reduced forms of ascorbate and glutathione (Biemelt *et al*., 1998). A rapid decrease in the redox state of both antioxidants was observed during reaeration (Biemelt *et al*., 1998). Further, the activities of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) decreased slightly or remained unaltered under hypoxia, whereas anoxia inhibited enzyme activities significantly (Biemelt *et al*., 1998). Moreover, Yan *et al*. (1996) has

shown that GR, ascorbate peroxidase (Apx), catalase (CAT) and superoxide dismutase (SOD) activities were inhibited in corn leaves under prolonged flooding, while a short-term treatment led to an increase of the activities. Therefore, it is difficult to delineate a universal mechanism for the whole antioxidant system in response to anoxia in plants (Blokhina *et al*., 2000). However, published records on the effect of AOX on the antioxidative system in plants are rare.

A rapid conversion of this reduced GSH into oxidized glutathione or glutathione disulfide (GSSG) can occur during adverse or stress conditions (Hasanuzzaman *et al*., 2017). Glutathione reductase (GR) and glutathione peroxidase (GPX) maintain a balanced state of GSH/GSSG and decreased activity of the glutathione-regenerating enzyme glutathione reductase (GR) can reduce the GSH/GSSG ratio (Mahmood *et al*., 2010; Hasanuzzaman *et al*., 2017). As seen in our results, the GR activity decreased during anoxia and increased during reaeration, which in turn reduces the GSH level during anoxia and increases the level during post-anoxia (Figure 4.4). This pattern is highlighted in AOX-overexpressing lines compared to other lines, that stresses the importance of AOX during anoxia and post-anoxic stress survival. A remarkable drop in the level of reduced glutathione was detected after anoxia and reoxygenation in anoxia tolerant (rice and *Iris germanica*) and wheat and anoxia-intolerant (*I. pseudacorus)* plants (Blokhina *et al.*, 2000). The levels decreased rapidly after short periods (considering anoxia tolerance of the species) of anoxia (Blokhina *et al.*, 2000). However, these changes had not been associated with a corresponding increase in GSSG and the GSSG content declined following the same tendency (Blokhina *et al*., 2000). A decline in APX, DHAR and GR activity during anoxia was also reported by Biemelt *et al.* (1998) in roots of wheat and in leaves of barley during anoxia (Skutnik

and Rychter, 2009). We suppose that the low level of activation of antioxidative system in AOX-overexpressing lines is due to the lower level of ROS than in knockdown lines.

NO is an important signaling molecule in plant stress responses (Astier *et al*., 2011; Manjunatha *et al*., 2012; Farnese *et al*., 2016) and it might induce *AOX* expression indirectly (Vishwakarma *et al*., 2015). NO treatment of Arabidopsis cell suspension has been shown an increase of the capacity of alternative respiratory pathway to counteract the toxicity of NO (Huang *et al*., 2002). Furthermore, a pre-treatment of barley seedlings with NO increased both *AOX1* expression and antioxidant enzyme activities which confers better growth and adaptability during arsenic stress (Shukla *et al*., 2015). In contrast, recent evidence suggests that AOX plays a role in regulation of nitric oxide (NO) production and signaling (Kumari *et al*., 2019). When AOX is induced under aerobic conditions in response to various stresses, electron transfer through complexes III and IV can be reduced and thus prevents the leakage of electrons to nitrite and the subsequent accumulation of NO (Kumari *et al*., 2019). Further, excess NO inhibits complex IV and thus, the AOX pathway minimizes nitrite-dependent NO synthesis in the cytochrome pathway (Kumari *et al*., 2019). However, under hypoxia, AOX has a specific role and AOX facilitates nitrite-dependent NO production through the phytoglobin-NO cycle to increase energy efficiency under hypoxia (Kumari *et al*., 2019). According to our results, AOX is involved in NO production from tobacco leaves under anoxia because, AOX-overexpressing plant lines showed a significant increase of NO production than the knockdown lines during anoxia (Figure 4.5). Arabidopsis cell suspensions have shown that NO production seemed to be particularly high during the 1st hours of anoxia (Paradiso *et al*., 2016). During re-oxygenation of 2 h, NO was

still producing and almost 20 h were required in order to recover redox homeostasis (Paradiso *et al.*, 2016).

This phenomenon was further confirmed by AOX inhibitors during our experiments. In the present study, we used salicylhydroxamic acid (SHAM) and propyl gallate (PG) to inhibit AOX to quantify the specific activity and capacity of the AOX (Bahr and Bonner, 1973; Merller *et al*., 1988). When SHAM and PG were used, a reduction of NO emission was observed during anoxia in tobacco leaves in all plant lines (Figure 4.5). This highlights the contribution of AOX in NO production in cells. However, we suppose that AOX might not be the only contributor of NO from tobacco leaves under anoxic stress because, in all plant lines the NO production almost got halved despite of its level of AOX expression (Figure 4.5).

AOX has been identified a cyanide-resistant oxidase component of the plant mitochondrial electron transport chain (Vanlerberghe and McIntosh 1997). However, 2 mM KCN inhibited the production of NO completely (not shown) during our experiments and 0.05 mM antimycin A did not work efficiently to demonstrate a clear co-relation of NO production (data not presented). This can be due to different levels of tolerance of the tobacco cells to the inhibitors or inefficient diffusion or absorption of those inhibitors into cells since we used tobacco leaves during the experiments. However, in actively respiring animal tissues, cyanide (1mM) has been able to inhibit cytochrome c oxidase and drop the rate of respiration to less than 1% of its initial level (Taiz *et al*., 2014). Moreover, mitochondria isolated from several plant tissues have shown incomplete inhibition of respiration by cyanide (Schonbaum *et al*., 1971). Most of the plant tissues has shown that the level of cyanide-resistant respiration representing 10 to 25% (in some tissues up to 100%) of the uninhibited control rate (Taiz *et al*.,

2014). As an example, mitochondria isolated from the spadices of members in plant family Araceae, such as *Arumma culatum* (Bendall and Hill, 1956; Bendall, 1957) and *Symplocarpus foetidus* (Bendall, 1971; Hackett, 1957; Hackett and Haas, 1958; Yocum and Hackett, 1957), show only little sensitivity to cyanide inhibition, while mitochondria from the hypocotyls of etiolated mung beans (*Phaseolus aureus*) show partial sensitivity under cyanide or Antimycin-A which is approximately 70% of rate of inhibition (Ikuma and Bonner, 1967). Further, KCN or Antimycin-A nearly or completely inhibit mitochondrial respiration of potato tubers (*Solanum tuberosum*) (Schonbaum *et al.*, 1971). Therefore, the NO production from plants or any type tissue are governed by a complex system.

NO and other RNS formed during stress conditions are responsible for *S*-nitrosylation of proteins (Sehrawat and Deswal, 2014). According to our results, the concentration of *S*-nitrosylated proteins in tobacco leaves are lower in AOX overexpressing lines than in the knockdown lines under each condition (Figure 4.6). This indicates that despite of less production of NO, its turnover is higher in knockdown lines in terms of *S*-nitrosylation. However, during anoxia, the level of nitrosylated proteins decreased in other lines, except in AOX-overexpressing lines which may be due to comparatively higher NO production from overexpressing lines (Figure 4.6). Further it is shown that the free thiol groups in AOX-overexpressing lines are comparatively higher than other plant lines (Figure 4.6). As seen in our observations, Albrecht and Wiedenroth (1994) have recorded that anaerobic treatment followed by reaeration resulted in a significant decrease of total thiol-groups in wheat roots. In our results, during anoxia, the total thiol groups of tobacco leaves have decreased in AOX-overexpressing lines at 15 and 2 h, and further decreased in AOX-knockdown lines at

15 min, but again increased by 2 h of post-anoxia (Figure 4.6). During reaeration, the nitrosylation of proteins increased in each line which may be due to high oxidative stress created during re-exposure of plants to air that will form more RNS. Further, as stated by Romero-Puertas *et al*. (2013), the *S*-nitrosylation pattern of proteins involved in a specific stress and the nitrosylation levels can be controversial and depends on the concentration of proteins, period under stress exposure, sources of ROS and antioxidant defense.

Plants under hypoxia/anoxia, undergo fermentation to compensate the energy requirements to some extent (Roberts *et al*., 1984a, b). According to our results, ADH activity increased significantly in wild type and overexpressing line (B8) under anoxia and reaeration (Figure 4.7.1). However, ADH activity is lower in knockdown lines than other plant lines which may be due to high oxidative stress inactivating the enzyme activity as described by Crow *et al*. (1995) and Men and Wang (2007). Since NO donors can inactivate ADH activity (Crow *et al*., 1995; Gergel and Cederbaum, 1996), this must be the cause for overexpressing lines having less ADH activity than in wild type. Along with re-exposure to air, the ADH activity in all plant lines remained unchanged as in anoxia or decreased to a certain level because, the expression of ADH is sensitive to oxygen concentrations (van Dongen *et al*., 2009). However, according to Salavati *et al*. (2012), ADH and pyruvate dehydrogenase complex (PDC) exhibit a significant elevation during reoxygenation than the aerated controls in rice coleoptiles (Salavati *et al*., 2012).

Aconitase in the Krebs cycle interconverts citrate to isocitrate (Hanson and Leibold, 1999). Nitric oxide (NO) production under low oxygen condition in Arabidopsis, has resulted in the inhibition of aconitase activity and subsequent citrate

accumulation, which in turn, appears to result in AOX induction (Gupta *et al.*, 2012). Accumulation of superoxide under stress conditions hinders aconitase activity (Gardner *et al*., 1997). According to our findings we suppose that the high oxidative stress in knockdown plant lines may have caused a lower activity of aconitase than the other lines (Figure 4.7.1). Further, Powell and Jackson (2003) have found that the inhibition of these enzyme activities during reoxygenation are due to excess $O_2^{-\cdot}$ produced in lung epithelial mitochondria, because inactivation of these three enzymes could be prevented by overexpression of MnSOD in these cells during reoxygenation. Therefore, in our results, high oxidative stress during reaeration may also have reduced aconitase activity. However, during anoxia we could observe an increase in aconitase activity in overexpressing plant lines which may be due to removal of reactive oxygen species effectively by the activation of antioxidant system (Figure 4.7.1). Though NO were higher in AOX-overexpressing lines, its effect on aconitase activity in AOX-overexpressing lines were not seen. Therefore, the aconitase activity under low oxygen stress appears to be more dependent on oxidative stress than NO production.

Under low oxygen conditions, plant cells produce ATP by anaerobic glycolysis and phytoglobin-mediate $NAD(P)^+$ regeneration via Phytoglobin-NO cycle (Igamberdiev *et al*., 2005; Mancuso and Marras, 2006). As seen in our results, during anoxia, the total ATP, total ATP and ADP and ATP/ADP ratio decreases (Figure 4.7.2). However, those levels are comparatively higher in AOX-overexpressing lines than others. Further, NO production in AOX-overexpressing lines is higher than others during anoxia which helps in driving the Phytoglobin-NO cycle under anoxia. This further confirms that AOX is involved in ATP production under low oxygen conditions

and subsequent post-anoxic recovery period via this cycle, to sustain cellular energy status.

Narsai *et al*. (2009) have observed a rapid shift towards aerobic growth in rice that had been germinated 24 h under anoxia. However, decreased ATP and increased cytoplasmic acidity during anoxia can hinder recovery upon reoxygenation (Menegus *et al*., 1991; Felle, 2005). In another study, rice has shown a rapid response to reinstate the aerobic TCA cycle after grown in anoxia for 24 h (Narsai *et al*., 2009). A rapid and significant induction of genes encoding respiratory chain components and alternative pathway components has been recorded within 3 h of aerobic treatment after 24 h of anaerobic germination of rice coleoptiles (Narsai *et al*., 2009). Further, genes encoding glycolytic enzymes and TCA cycle components have significantly been induced (Narsai *et al*., 2009). Carbohydrates, arabinose and trehalose accumulates during reoxygenation, suggesting a restoration of carbohydrate pools when the energy crisis is relieved (Shingaki-Wells *et al*., 2014). Reoxygenated rice coleoptiles respire in a KCN-insensitive manner because of AOX transcript and protein level induction upon reoxygenation (Millar *et al*., 2004; Narsai *et al*., 2009). In our results, the amount of ATP, total ATP and ADP and ATP/ ADP ratio increases during reaeration (15 min < 2 h), which further confirms rapid restoration of energy requirements during post-anoxic recovery and AOX-overexpressing lines had more recovery ability than other plant lines (Figure 2.3.8.2).

6 Conclusions, recommendations and future directions

Recently, mitochondria have emerged as an important player in nitric oxide (NO) production in plants. Recent records suggest that AOX also plays a role in

regulation of nitric oxide production and signaling and NO is needed to induce AOX pathway under phosphate-limiting conditions in *Arabidopsis thaliana* seedlings (Kumari *et al*., 2019). However, the exact role of AOX under stress conditions, especially under reaeration or whether AOX is directly involved in NO production is still not fully known and controversial. Therefore, the focus of this work was to investigate the role of AOX during anoxia and reaeration by using leaves of tobacco plants that are differentially expressing AOX.

According to the results of the present study, AOX helps to create little amount of ROS in tobacco leaves during low oxygen environment and during post-anoxic recovery. Further, the levels of superoxide in tobacco leaves decrease during post-anoxic recovery period and increases as the time of exposure increases. However, as nitric oxide levels are comparatively higher in AOX-overexpressing lines, it could reduce the efficiency of COX and increase H_2O_2 in AOX-overexpressing lines. This helps in elevating antioxidative responses in tobacco leaves. Due to increased levels of ROS, lipid peroxidation increased especially, when AOX is downregulated because, the antioxidative system (especially catalase and APX) is not fully activated to reduce ROS levels in tobacco leaves of AOX-downregulating plants. Moreover, elevated levels of MDA during post-anoxia confirms that post anoxic injury has a significant role in ROS production in tobacco leaves than under anoxia. However, exact plant stress responses in the presence of AOX in mitochondria may not be highlighted during our study since we have used leaves rather than mitochondria alone.

AOX plays a major role in NO production in tobacco leaves under anoxia. This was confirmed by the chemiluminescence method during this study in the presence of

inhibitors of AOX (5mM SHAM and 0.5mM PG) under anoxia. However, the major turnover of NO is not *S*-nitrosylation of proteins in tobacco leaves.

Significantly higher NO production in the presence of overexpressing AOX helps to drive the phytoglobin-NO cycle to sustain the energy balance (ATP, ADP) during anoxia and post anoxia in tobacco leaves. Further, AOX-overexpression helps the tobacco leaves to restore the energy status more efficiently during post anoxia than in other plant lines. ADH activity is low when AOX is downregulated and the levels increase during anoxia and post-anoxia, while overexpression of AOX do not show a significant difference even under post-anoxia. AOX-overexpression creates a significantly higher activity of aconitase and despite of the higher NO content the levels increase during anoxia and decreased during reaeration. Activities of ADH and aconitase are disturbed by higher levels of ROS, despite of high NO production, especially in knockdown lines. Therefore, aconitase activity is not governed by NO alone. Further, AOX helps driving the fermentation and TCA cycles under anoxia. Although fermentation is not affected during post anoxia, the aconitase activity decreased which slows down the TCA cycle upon post-anoxic recovery.

Downregulation of AOX creates an environment of higher ROS which in turn activates antioxidative system in tobacco leaves. However, overexpression of AOX helps to activate the antioxidative system and reduce the ROS as a defense mechanism. During anoxia and post- anoxia, AOX- overexpressed line B8, has lower levels of ROS formation. Ascorbate peroxidase (APX) is not efficiently involved as an antioxidant enzyme to remove H_2O_2 during anoxia or post-anoxia in tobacco leaves.

Other antioxidant enzymes in ascorbate and glutathione cycle (DHAR, MDHAR) do not significantly contribute in anoxia and post-anoxia in tobacco leaves

in any plant line. Conversion of GSSG (oxidized form) to GSH (reduced form) is low during anoxia and this is more pronounced when AOX is overexpressing. However, at 15 min of reaeration, a significantly higher level of GR is observed and again decrease at 2hr. This conversion is higher during short periods of post-anoxia and again reduce during prolonged post-anoxia.

We suppose that expression of AOX and the activation of antioxidative system and post-translational modifications depend on the plant species, plant part used, type of stress used and the period of stress exposure. Because the activation of antioxidative system can be different when the plants are under acclimation period or they are more tolerant to a specific stress condition. Therefore, a model for plant metabolic pathways cannot be attained only by using one species, one plant line or one specific exposure time for a stress condition. It is advisable to use several concentrations of oxygen to introduce hypoxia also. Another important aspect is that the plant that we use for these experiments need to be anoxia or hypoxia intolerant species to see the cellular level mechanisms when they are exposed to stress rather than using intolerant species. Roots (in contrast to leaves), are often exposed to hypoxic or anoxic conditions during soil flooding periods (Gupta *et al*., 2005). They suggest that plant roots act differently during hypoxia than shoots. Therefore, we recommend using roots as they are the underground parts affected by anoxia and post-anoxic recovery than above ground parts. It would be more efficient if the mitochondrial suspensions are used. Moreover, molecular level studies will give a good insight in the level of gene expressions of these experiments, because some experiments such as nitrobluetetrazolium method to test superoxide levels (especially in AOX-overexpressing lines) were very difficult, hemoglobin method (data not presented) to detect NO were not effective during our experiments.

Luminol- chemiluminescense (Luminol kit Sigma-Aldrich CS 1000-1KT) or microscopic method can be used to detect superoxide levels. Since RNS are produced during abiotic stress, it would be useful to measure other RNS and $ONOO^-$ because, ROS react with NO to produce $ONOO^-$.

According to our findings, we suppose that AOX is involved in NO production in tobacco leaves under anoxia and reaeration. However, AOX will not be the only contributor of NO production and there may be other pathways and mechanisms that involves in this process. However, we could see that AOX-overexpression helped tobacco leaves to sustain the energy production and metabolic pathways during anoxia and post-anoxia. Moreover, this AOX expression decreased the levels of ROS formation while activating the antioxidative system during anoxia and post-anoxic recovery.

7 References

Affourtit, C., Krab, K., & Moore, A.L. (2001b). Control of plant mitochondrial respiration. *Biochimica et Biophysica Acta,* 1504, 58–69.

Ahmad, P. (2010). Growth and antioxidant responses in mustard (*Brassica juncea* L.) plants subjected to combined effect of gibberellic acid and salinity. *Archives of Agronomy &Soil Science*,56, 575–588.

Albrecht, G., & Wiedenroth, E.M. (1994). Protection against activated oxygen following re-aeration of hypoxically pre-treated wheat roots, The response of the glutathione system. *Journal of Experimental Botany*., 45, 449–455.

Alber, N. A., Sivanesan, H., & Vanlerberghe, G. C. (2017). The occurrence and control of nitric oxide generation by the plant mitochondrial electron transport chain. *Plant cell & environment*, 40 (7), 1074-1085.

Alexou, M. (2013). Development-specific responses to drought stress in Aleppo pine (Pinus halepensis Mill.) seedlings, *Tree Physiology*, 33, 1030–1042.

Amirsadeghi, S., Robson, C.A., McDonald, A.E., & Vanlerberghe, G.C. (2006). Changes in plant mitochondrial electron transport alter cellular levels of reactive oxygen species and susceptibility to cell death signaling molecules. *Plant & Cell Physiology*, 47, 1509–1519.

Arora, D., Jain, P., Singh, N., Kaur, H., & Bhatla, S.C. (2016). Mechanisms of nitric oxide crosstalk with reactive oxygen species scavenging enzymes during abiotic stress tolerance in plants, *Free Radical Research*, 50, 291–303.

Asada, K., Kiso, K., & Yoshikawa, K. (1974). Univalent reduction of molecular oxygen by spinach chloroplasts on illumination. *Journal of Biological Chemistry*, 249, 2175–2181

Asada, K. (2006). Production and Scavenging of Reactive Oxygen Species in Chloroplasts and Their Functions. Plant Physiology, 141 (2), 391-396; DOI: 10.1104/pp.106.082040

Astier, J., Rasul, S., Koen, E., Manzoor, H., Besson-Bard, A., Lamotte, O., Jeandroz, S., Durner J., Lindermayr, C., & Wendehenne, D. (2011). S-Nitrosylation: an emerging post-translational protein modification in plants. *Plant Science*, 181, 527–533

Bahr, J.T., & Bonner, M.I. (1973). Cyanide-insensitive respiration. The steady stdtes of skunk cabbage spadix and bean hypocotyl mitochondria. *Journal of Biological Chemistry*, 248, 3441-3445.

Bailey-Serres, J., & Voesenek, L.A. (2008). Flooding stress: acclimations and genetic diversity. *Annual Review of Plant Biology*, 59, 313–339.

Bailey-Serres, J., Fukao, T., Gibbs, D.J., Holdsworth, M.J., Lee, S.C., Licausi, F., Perata, P., Voesenek, L.A., & van Dongen, J.T. (2012). Making sense of low oxygen sensing. *Trends Plant Science*, 17, 129–138.

Banti, V., Giuntoli, B., Gonzali, S., Loreti, E., Magneschi. L., Novi,G., Paparelli, E., Parlanti, S., Pucciariello, C., Santaniello, A., &Perata, P. (2013). Low oxygen response mechanisms in green organisms. *International Journal of Molecular Sciences*, 14(3), 4734–4761. doi:10.3390/ijms14034734

Baumgart, M., & Bott, M. (2011). Biochemical characterisation of aconitase from *Corynebacterium glutamicum*, *Journal of Biotechnology*, 154, 163–170.

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: improved assays and assay applicable to acrylamide gels. *Annals of Biochemistry*, 44, 276–278.

Bendall, D. S., & Hill, R. (1956). Cytochrorne components in the spadix of Arum maculatum. *New Phytologist*, 55, 206-212.

Bendall, D. S. (1957). Cytochromes and some respiratory enzymes in mitochondria from the spadix of Arum maculatum. *The Biochemical Journal*, 70(3), 381–390. doi:10.1042/bj0700381

Bendall, D. S., & Bonner, W. D. (1971). Cyanide-insensitive respiration in plant mitochondria. *Plant Physiology*. 47, 236-245.

Biemelt, S., Keetman, U., & Albrecht, G. (1998). Re-Aeration following Hypoxia or Anoxia Leads to Activation of the Antioxidative Defense System in Roots of Wheat Seedlings. *Plant physiology*, 116(2), 651–658.

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *The World Allergy Organization Journal*, 5(1), 9–19. doi:10.1097/WOX.0b013e3182439613

Blandino, A., Caro, I., & Cantero, D. (1997). Comparative study of alcohol dehydrogenase activity in flor yeast extracts. *Biotechnology Letters,* 19, 651–654. doi: 10.1023/A:1018386731116

Blokhina, O.B., Fagerstedt, K.V., & Chirkova, T.V.. (1999). Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. *Physiologia Plantarum,* 105, 625–632.

Blokhina, O.B., Virolainen, E., Fagerstedt, K.V., Hoikkala, A., Wähälä, K., & Chirkova, T.V. (2000). Antioxidant status of anoxia-tolerant and -intolerant plant species under anoxia and reaeration. *Physiologia Plantarum*, 109, 396–403.

Blokhina, O.B., Chirkova, T.V., & Fagerstedt, K.V. (2001). Anoxic stress leads to hydrogen peroxide formation in plant cells. *Journal of Experimental Botany*, 52, 1179–1190.

Blokhina, O., Virolainen, E., & Fagerstedt, K.V. (2003). Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Annals of Botany*, 91, 179–194.

Blokhina, O., & Fagerstedt, K. V. (2010). Reactive oxygen species and nitric oxide in plant mitochondria: origin and redundant regulatory systems. *Physiologia Plantarum,* 138, 447–462. 10.1111/j.1399-3054.2009.01340.x

Borecky, J., Nogueira, F. T., de Oliveira, K. A., Maia, I. G., Vercesi, A. E., & Arruda, P. (2006). The plant energy-dissipating mitochondrial systems: depicting the genomic structure and the expression profiles of the gene families of uncoupling protein and alternative oxidase in monocots and dicots. *Journal of Experimental Botany*, 57, 849–864. 10.1093/jxb/erj070

Bowler, C., Van Montagu, M. & Inze, D. (1992). Superoxide dismutase and stress tolerance. *Annual Review of Plant Physiology & Plant Molecular Biology*, 43, 83116.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Annals of Biochemistry*, 72, 248-254.

Brown, D.J., & Beevers, H. (1987). Fatty acid of rice coleoptiles in air and anoxia. *Plant Physiol*ogy, 84, 555-559.

Cantu, D., Schaack, J., & Patel, M. (2009). Oxidative inactivation of mitochondrial aconitase results in iron and H2O2-mediated neurotoxicity in rat primary mesencephalic cultures. *PloS one, 4*(9), e7095. doi:10.1371/journal.pone.0007095

Chamizo-Ampudia, A,, Sanz-Luque, E., Llamas, A., Galvan, A., & Fernandez, E. (2017). Nitrate reductase regulates plant nitric oxide homeostasis. *Trends in Plant Science*, 22, 163–174.

Chang, R., Jang, C.J., Branco-Price, C., Nghiem, P., & Bailey-Serres, J. (2012). Transient MPK6 activation in response to oxygen deprivation and reoxygenation is mediated by mitochondria and aids seedling survival in Arabidopsis. *Plant Molecular Biology*, 78, 109–122. 10.1007/s11103-011-9850-5

Cherkasov, A., Overton, R.A. Jr., Sokolov, E.P., & Sokolova, I.M. (2007). Temperature dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, Crassostrea virginica:

a role of temperature in oxidative stress and allosteric enzyme regulation. *Journal of Experimental Biology*, 210, 46-55. doi:10.1242/jeb.02589

Chew, O., Whelan, J., & Millar, A.H. (2003). Molecular definition of the ascorbateglutathione cycle in Arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants. *Journal Biological Chemistry*, 278, 46869–46877.

Chirkova, T.V., Novitskaya, L.O., & Blokhina, O.B. (1998). Lipid peroxidation and antioxidant systems under anoxia in plants differing in their tolerance to oxygen deficiency. *Russian Journal of Plant Physiology*, 45, 55–62.

Cochrane, D. W., Shah, J. K., Hebelstrup, K. H., & Igamberdiev, A. U. (2017). Expression of phytoglobin affects nitric oxide metabolism and energy state of barley plants exposed to anoxia. *Plant Science*, 265, 124-130. https://doi.org/10.1016/j.plantsci.2017.10.001

Cooper, C.E. (2002). Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? *Trends in Biochemical Sciences*, 27, 33–39.

Considine, M. J., Holtzapffel, R. C., Day, D. A., Whelan, J., & Millar, A. H. (2002). Molecular distinction between alternative oxidase from monocots and dicots. *Plant Physiol*ogy. 129, 949–953. 10.1104/pp.004150

Clifton, R., Lister, R., Parker, K.L., Sappl, P.G., Elhafez, D., Millar, A.H., Day, D.A., &Whelan, J. (2005). Stress-induced co-expression of alternative respiratory chain components in Arabidopsis thaliana. Plant Molecular Biology, 58, 193.

Crawford, R. M. M. (1992). Oxygen availability as an ecological limit to plant distribution. *Advances in Ecological Research*, 23, 93-185.

Crawford, N. M. (1995). Nitrate: nutrient and signal for plant growth. *The plant cell*, 7(7), 859.

Crow, J.P., Beckman, J.S., &McCord, J.M. (1995) Sensitivity of the essential zincthiolate moiety of yeast alcohol dehydrogenase to hypochlorite and peroxynitrite. *Biochemistry*, 34(11), 3544–52.

Cvetkovska, M., & Vanlerberghe, G.C. (2012). Coordination of a mitochondrial superoxide burst during the hypersensitive response to bacterial pathogen in Nicotiana tabacum. *Plant, Cell & Environment*, 35, 1121–1136.

Czarna, M., & Jarmuszkiewicz, W. (2005) Activation of alternative oxidase and uncoupling protein lowers hydrogen peroxide formation in amoeba Acanthamoeba castellanii mitochondria. *FEBS Lett.* 579, 3136–3140.

Dahal, K., Martyn, G.D., &Vanlerberghe, G.C. (2015). Improved photosynthetic performance during severe drought in Nicotiana tabacum overexpressing a nonenergy conserving respiratory electron sink. *New Phytology*, 208, 382–395.

Das, K., & Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environment & Science*, 2, 53.

Delledonne, M., Zeier, J., Marocco, A., & Lamb, C. (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive

disease resistance response. *Proceedings of the National Academy of Sciences of the USA*, 98, 13454–13459.

Domingos, P., Prado, A. M., Wong, A., Gehring, C., & Feijo, J. A. (2015). Nitric oxide: a multitasked signaling gas in plants. *Molecular plant*, 8(4), 506-520.

Dordas, C., Hasinoff, B.B., Igamberdiev, A.U., Manac'h, N., Rivoal, J., & Hill, R.D. (2003). Expression of a stress-induced haemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. *The Plant Journal*, 35, 763– 770.

Drew, M.C. (1997). Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annual Review of Plant Physiology and Plant Molecular Biology*, 48, 223–250

Drew, M.C., He, C.J., & Morgan, P.W. (2000). Programmed cell death and aerenchyma formation in roots. *Trends in Plant Science*, 5, 123–7.

Elthon, T.E., Nickels, R.L., &McIntosh, L. (1989). Mitochondrial events during development of thermogenesis in *Sauromatum guttatum* (Schott). *Planta*, 180, 82–89.

Fancy, N. N., Bahlmann, A. K., & Loake, G. J. (2017). Nitric oxide function in plant abiotic stress. *Plant, cell & environment*, 40(4), 462-472.

Farnese, F.S., Menezes-Silva, P.E., Gusman, G.S., &Oliveira, J.A. (2016). When bad guys become good ones: the key role of reactive oxygen species and nitric oxide in the plant responses to abiotic stress. *Frontiers in Plant Science*, 7, 471.

Felle, H.H. (2005). pH regulation in anoxic plants. *Annals of Botany*, 96, 519– 532.

Fereja, T.H., Hymete, A., and Gunasekaran, T. (2013). "A Recent Review on Chemiluminescence Reaction, Principle and Application on Pharmaceutical Analysis," ISRN Spectroscopy, 230858, 12.

Foyer, C.H., &Halliwell, B. (1976). The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta,* 133, 21–25.

Foyer, C.H., Descourvieres, P., & Kunert, K.J. (1994). Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. *Plant Cell Environment*, 17, 507–523.

Foyer, C.H., Souriau, N., Perret, S., Lelandais, M., Kunert, K.J., Pruvost, C., &Jounanin, L. (1995). Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. *Plant Physiology*, 109, 1047–1057.

Foyer, C.H., &Noctor, G. (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell*, 17, 1866–1875.

Gandin, A., Duffes, C., Day, D.A., & Cousins, A.B. (2012). The absence of alternative oxidase AOX1A results in altered response of photosynthetic carbon assimilation to increasing CO2 in *Arabidopsis thaliana*. *Plant Cell Physiology,* 53, 1627–1637.

Gardner, P.R., Nguyen, D.H., & White, C.W. (1994). Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. *Proc Natl Acad Sci USA*, 91, 12248–12252.

Gardner, P. R., Costantino, G., Szabo, C., & Salzman, A. L. (1997). Nitric oxide sensitivity of the aconitases. *Journal of Biological Chemistry*, 272, 25071-25076. doi:10.1074/jbc.272.40.25071

Griendling, K.K., Touyz, R.M., Zweier, J.L., Dikalov, S., Chilian, W., Chen, Y.R., Harrison, D.G., & Bhatnagar, A. (2016). Measurement of Reactive Oxygen Species, Reactive Nitrogen Species, and Redox-Dependent Signaling in the Cardiovascular System: A Scientific Statement From the American Heart Association. *Circulation Research*, 119 (5), e39–e75.

Gergel, D., & Cederbaum, A.I. (1996). Inhibition of the catalytic activity of alcohol dehydrogenase by nitric oxide is associated with *S*-nitrosylation and the release of zinc. *Biochemistry*, 35, 16186-16194.

Giraud, E., Ho, L.H.M., Clifton, R., Carroll, A., Estavillo, G., Tan, Y.F., Howell, K.A., Ivanova, A., Pogson, B.J., Millar, A.H., &Whelan, J. (2008). The Absence of Alternative Oxidase1a in Arabidopsis Results in Acute Sensitivity to Combined Light and Drought Stress. Plant *Physiology*, 147, 595–610.

Grabelnych, O.I., Borovik, O.A., Tauson, E.L., Pobezhimova, T.P., Katyshev, A.I., Pavlovskaya, N.S., Koroleva, N.A., Lyubushkina, I.V., Bashmakov, V.Y., Popov, V.N., Borovskii, G.B., & Voinikov, V.K. (2014). Mitochondrial energy-dissipating systems (alternative oxidase, uncoupling proteins, and external NADH dehydrogenase) are involved in development of frost-resistance of winter wheat seedlings. *Biochemistry (Mosc)*, 9(6),506-19.

Greenway, H., & Gibbs, J. (2003). Mechanisms of anoxia tolerance in plants. II. energy requirements for maintenance and energy distribution to essential processes. *Functional Plant Biology*, 30, 999–1036.

Guilbault, G.G. (1976). Glucose Oxidase. In: Handbook of Enzymatic Methods of Analysis, M.K. Schwartz, ed., *New York: Marcel Dekker Inc*., 106-109.

Guo, F. Q., Okamoto, M., & Crawford, N. M. (2003). Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science*, 302(5642), 100-103.

Gupta, K.J., Stoimenova, M., & Kaiser, W.M. (2005). In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ, *Journal of Experimental Botany*, 56, 2601–2609.

Gupta, K.J., Igamberdiev, A.U., & Kaiser, W.M.(2010). New insights into the mitochondrial nitric oxide production pathways. *Plant Signal. Behav.*, 5, 999–1001.

Gupta, K.J., Fernie, A.R., Kaiser, W.M., & van Dongen, J.T. (2011). On the origins of nitric oxide, *Trends Plant Sci.*, 16, 160–168.

Gupta, K. J., & Igamberdiev, A. U. (2011). The anoxic plant mitochondrion as a nitrite: NO reductase. *Mitochondrion*, 11, 537–543. 10.1016/j.mito.2011.03.005

Gupta, K.J., Hebelstrup, K.H., Mur, L.A., & Igamberdiev, A.U. (2012). Plant hemoglobins: important players at the crossroads between oxygen and nitric oxide. *FEBS Lett.*, 585, 3843–3849.

Hackett, D. P. (1957). Respiratory mechanisms in the aroid spadix. *Journal of Experimental Botany*, 8, 157-171.

Hackett, D. P. & Haas, W.D. (1958). Oxidative phosphorylation and functional cytochromes in skunk cabbage mitochondria. *Plant Physiology*, 33.

Halliwell, B. (1991). Reactive oxygen species in living sys- tems: source, biochemistry and role in human disease. *American Journal of Medicine Supplement* 3C, 91, 14-22.

Hansen, L.D., Church, J.N., Matheson, S., McCarlie, V.W., Thygerson, T., Criddle, R.S. & Smith, B.N. (2002) Kinetics of plant growth and metabolism. *Thermochimica Acta*, 388, 415– 425.

Hanson, E. S., & Leibold, E. A. (1999). Regulation of the iron regulatory proteins by reactive nitrogen and oxygen species. *Gene expression*, 7(4-6), 367–376.

Hasanuzzaman, M., Nahar, N., Hossain, M.S., Mahmud, J.A., Rahman, A., Inafuku, M., Oku, H., & Fujita, M. (2017). Coordinated actions of glyoxalase and antioxidant defense systems in conferring abiotic stress tolerance in plants. *International Journal of Molecular Science*, 1, 200. doi: 10.3390/ijms18010200.

Hattori, Y., Nagai, K., Furukawa, S., Song, X.-J., Kawano, R., Sakakibara, H., Wu, J., Matsumoto, T., Yoshimura, A., Kitano, H., Matsuoka, M., Mori, H., & Ashikari, M. (2009). The ethylene response factors *SNORKEL1* and *SNORKEL2* allow rice to adapt to deep water. *Nature*, 460, 1026–1030.

Heath, R.L., & Parker, L. (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys*, 125, 189-198

Hebelstrup, K.H., Shah, J.K., Simpson, C., Schjoerring, J.K., Mandon, J., Cristescu, S.M., Harren, F.J., Christiansen, M.W., Mur, L.A., & Igamberdiev, A.U. (2014). An assessment of the biotechnological use of hemoglobin modulation in cereals. *Physiologia Plantarum*, 150, 593–603.

He, H.Y. Gu, M.H., & He, L.F. (2014). The role of nitric oxide in programmed cell death in higher plants, in: M.N. Khan, M. Mobin, F. Mohammad, F.J. Corpas (Eds.), Nitric Oxide Plants Metab. Role Stress Physiol, *Springer International Publishing, Cham*, 281–296.

Hideg, E., Kalai, T., Hideg, K., & Vass, I**.** (1998). Photoinhibition of photosynthesis in vivo results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves. *Biochemistry*, 237, 11405–11411

Huang, X., von Rad, U.,& Durner, J. (2002). Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in Arabidopsis suspension cells. *Planta*, 215, 914–923.

Huang, L. S., Cobessi, D., Tung, E. Y., & Berry, E. A. (2005). Binding of the respiratory chain inhibitor antimycin to the mitochondrial bc1 complex: a new crystal structure reveals an altered intramolecular hydrogen-bonding pattern. *Journal of Molecular Biology*, 351(3), 573–597. doi:10.1016/j.jmb.2005.05.053

Igamberdiev, A.U. & Hill, R.D. (2004). Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. *Journal of Experimental Botany*, 55, 2473–2482.

Igamberdiev, A.U., Baron, K., Manac'h-little, N., Stoimenova, M., & Hill, R.D. (2005). The Haemoglobin/nitric oxide cycle: Involvement in flooding stress and effects on hormone signalling. *Annals of Botany,* 96, 557-564.

Igamberdiev, A.U. & Hill, R.D. (2009). Plant mitochondrial function during anaerobiosis. *Annals of Botany*, 103, 259-268.

Igamberdiev, A.U., Bykova, N.V., Shah, J.K,. & Hill, R.D. (2010) Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms. *Physiologia Plantarum,* 138, 393–404.

Ikuma, H. & Bonner, W.D.(1967). Properties of higher plant mitochondria. III. Effects of respiratory inhibitors. *Plant Physiology,* 42, 1535

IPCC. (2007). Climate Change 2007: The Physical Science Basis. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M. and Miller, H.L., Eds., Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge University Press, Cambridge, UK and New York, USA, 996.

Ismond, K.P., Dolferus, R., De Pauw, M., & Dennis, E.S., (2003). Good
AG. Enhanced low oxygen survival in Arabidopsis through increased metabolic flux in the fermentative pathway. *Plant physiology*, 132(3), 1292–302. 10.1104/pp.103.022244

Jackson, M. (1985) ethylene and responses of plants to soil waterlogging and submergence. *Annual Review of Plant Physiology and Plant Molecular Biology*, 36, 145–174.

Joshi, A. K., Ahmed, S. & Ames, G. F. L. (1989). Energy coupling in bacterial periplasmic transport systems. *Journal of Biology & Chemistry*, 264, 2126–2133.

Kennedy, R.A., Rumpho, M.E., & Fox, T.C. (1992). Anaerobic metabolism in plants. *Plant Physiology*, 100, 1–6.

Keunen, E., Florez-Sarasa, I., Obata, T., Jozefczak, M., Remans, T., Vangronsveld, J., & Cuypers, A. (2016). Metabolic responses of Arabidopsis thaliana roots and leaves to sublethal cadmium exposure are differentially influenced by alternative oxidase1a. *Environmental & Experimental Botany*, 124, 64-78. http://dx.doi.org/10.1016/j.envexpbot.2015.11.015

Kumari, A., Pathak, P.K., Bulle, M., Igamberdiev, A.U., & Gupta, K.J. (2019). Alternative oxidase is an important player in the regulation of nitric oxide levels under normoxic and hypoxic conditions in plants. *Journal of Experimental Botany*. 70(17), 4345-4354. doi: 10.1093/jxb/erz160.

Lambers, H. (1982). Cyanide-resistant respiration: A non-phosphorylating electron transport pathway acting as an energy overflow. *Physiologia Plantarum,* 55, 478–485.

Lambowitz, A. M. & Slayman, C. W.(1971). Cyanide-resistant respiration in Neurosporacrassa. *Journal of Bacteriology*, 108, 1087-1096.

Limami, A.M., Diab, H. & Lothier, J. (2013). Nitrogen metabolism in plants under low oxygen stress, *Planta*, 239, 531–541.

Licausi, F. (2013). Molecular elements of low-oxygen signaling in plants. *Physiologia Plantarum*, 148, 1–8.

Li, Z., & Xing, D. (2011). Mechanistic study of mitochondria-dependent programmed cell death induced by aluminium phytotoxicity using fluorescence techniques. *Journal of Experimental Botany*, 62(1), 331-43.

Leon, J., Costa, Á., & Castillo, M.C. (2016). Nitric oxide triggers a transient metabolic reprogramming in Arabidopsis. *Scientific Report*, 6, 37945.

Ma, Z., Marsolais, F., Bykova, N.V., & Igamberdiev, A.U. (2016). Nitric Oxide and Reactive Oxygen Species Mediate Metabolic Changes in Barley Seed Embryo during Germination. *Frontiers in Plant Science,* 7, 138. doi: 10.3389/fpls.2016.00138

Ma, Z., Bykova, N.A., & Igamberdiev, A.U. (2017). Cell signaling mechanisms and metabolic regulation of germination and dormancy in barley seeds. *The Crop Journal*, 5, 459–477.

Mahmood, Q., Ahmad, R., Kwak, S.S., Rashid, A., & Anjum, N.A. (2010). Ascorbate and glutathione: protectors of plants in oxidative stress. In: Mahmood Q, Ahmad R, Kwak SS, Rashid A, Anjum NA, editors. Ascorbate–glutathione pathway and stress tolerance in plants. *Berlin: Springer*, 209–229.

Mancuso, S., & Marras, A.M. (2006). Adaptative Response of Vitis Root to Anoxia. *Plant & Cell Physiology*, 47 (3),401–409.

Manjunatha, G., Gupta, K.J., Lokesh, V., Mur, L.A., & Neelwarne, B. (2012). Nitric oxide counters ethylene effects on ripening fruits. *Plant Signaling & Behaviour*, 7, 476–483.

Maxwell, D.P., Wang, Y., & McIntosh, L. (1999). The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *PNAS*, 96, 8271–8276.

Maxwell, D.P., Nickels, R., & McIntosh, L. (2002). Evidence of mitochondrial involvement in the transduction of signals required for the induction of genes associated with pathogen attack and senescence. *The Plant Journal*, 29, 269– 279.

Men, L., & Wang, Y. (2007). The oxidation of yeast alcohol dehydrogenase-1 by hydrogen peroxide in vitro. *Journal of proteome research*, 6(1), 216–25. 10.1021/pr0603809

Menegus, F., Cattaruzza, L., Mattana, M., Beffagna, N., & Ragg, E. (1991). Response to anoxia in rice and wheat seedlings. Changes in pH of intracellular components, glucose-6-phosphate level, and metabolic rate. *Plant Physiology*, 95, 760–767.

Millar, A.H., Bergensen, F.J., & Day, D.A. (1994). Oxygen affinity of terminal oxidases in soybean mitochondria. *Plant Physiology & Biochemistry*, 32, 847–852.

Millar, A.H., Trend, A.E., & Heazlewood, J.L. (2004). Changes in the mitochondrial proteome during the anoxia to air transition in rice focus around cytochromecontaining respiratory complexes. *The Journal of Biological Chemistry*, 279, 39471–39478

Mishra, P.N., Fatma, T., & Singhal, G.S. (1995). Development of antioxidative defence system of wheat seedlings in response to high light. *Physiolgia Plantarum*, 95,72–82.

Møller, I.M. (2001). Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annual Review of Plant Physiology and Plant Molecular Biology*, 52, 561–591.

Monk, L.S., Fagerstedt, K.V., & Crawford, R.M.M. (1987). Superoxide dismutase as an anaerobic polypeptide. A key factor in recovery from oxygen deprivation in *Iris pseudacorus*? *Plant Physiol*ogy, 85, 1016–1020.

Moore, A.L., & Siedow, J.N. (1991). The regulation and nature of the cyanideresistant alternative oxidase of plant mitochondria. *Biochimica et Biophysica Acta (BBA) – Bioenergetics,* 1059, 121–140.

Moore, A.L., Albury, M.S., Crichton, P.G., & Affourtit, C. (2002) Function of the alternativeoxidase: is it still a scavenger? *Trends Plant Science*, 7, 478–81.

Moore, A.L., Shiba, T., Young,L., Harada, S., Kita,K., & Ito, K. (2013). Unraveling the heater: new insights into the structure of the alternative oxidase. *Annual Review of Plant Biology*, 64, 637-663

Mur, L.A.J., Mandon, J., Persijn, S., Cristescu, S.M. Moshkov, I.E., Novikova, G.V., Hall, M.A., Harren, F.J.M., Hebelstrup, K.H., & Gupta, K.J. (2013). Nitric oxide in plants: an assessment of the current state of knowledge. *AoB PLANTS*, 5, 052.

https://doi.org/10.1093/aobpla/pls052

Mustroph, A., Lee, S.C., Oosumi, T., Zanetti, M.E., Yang, H., Ma K., YaghoubiMasihi, A., Fukao, T., & Bailey-Serres, J. (2010). Cross-kingdom comparison of transcriptomic adjustments to low-oxygen stress highlights conserved and plantspecific responses. *Plant Physiology*, 152, 1484–1500.

Narsai, R., Howell, K.A., Carroll, A., Ivanova, A., Millar, A.H. & Whelan, J. (2009). Defining core metabolic and transcriptomic responses to oxygen availability in rice embryos and young seedlings. *Plant Physiology*, 151, 306– 322.

Nathan, C., & Shiloh, M.U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proceedings of the National Academy of Science USA*, 97, 8841–8848.

Noctor, G., & Foyer, C.H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology & Plant Molecular Biology*, 49, 249-279.

Noguchi, K., & Terashima, I. (2006). Responses of spinach leaf mitochondria to low N availability. *Plant, Cell & Environment*, 29, 710–719.

Paradiso, A., Caretto, S., Leone, A., Bove, A., Nisi, R., & De Gara, L. (2016). ROS Production and Scavenging under Anoxia and Re-Oxygenation in Arabidopsis Cells: A Balance between Redox Signaling and Impairment. *Frontiers in Plant Science*, 7, 1803. doi:10.3389/fpls.2016.01803

Parihar, A., Vaccaro, P., & Ghafourifar, P. (2008b). Nitric oxide irreversibly inhibits cytochrome oxidase at low oxygen concentrations: evidence for inverse oxygen concentration-dependent peroxynitrite formation. *IUBMB Life*, 60, 64–67.

Pasqualini, S., Paolocci, F., Borgogni, A., Morettini, R., & Ederli, L. (2007). The overexpression of an alternative oxidase gene triggers ozone sensitivity in tobacco plants. *Plant, Cell & Environment*, 30, 1545–1556.

Perata, P., & Alpi, A. (1993). Plant responses to anaerobiosis. *Plant Science*, 93(1–2), 1–17.

Petrov, V., Hille, J., Mueller-Roeber, B., & Gechev, T. S. (2015). ROS-mediated abiotic stress-induced programmed cell death in plants. *Frontiers in plant science*, *6*, 69. doi:10.3389/fpls.2015.00069

Pfister-Sieber, M., & R. Brändle. (1994). Aspects of plant behaviour under anoxia and postanoxia. *Proceedings of the Royal Society, Edinburgh 102 B*, 313–324.

Planchet, E., Gupta, K.J., Sonoda, M., & Kaiser, W.M. (2005). Nitric oxide emission from tobacco leaves and cell suspensions: rate-limiting factors and evidence for the involvement of mitochondrial electron transport, *Plant Journal*, 41, 732–743.

Planchet, E., & Kaiser, W. M. (2006). Nitric oxide (NO) detection by DAF fluorescence and chemiluminescence: a comparison using abiotic and biotic NO sources. *Journal of Experimental Botany*, 57(12), 3043-3055.

Powell, C.S., & Jackson, R.M. (2003). Mitochondrial complex I, aconitase, and succinate dehydrogenase during hypoxia-reoxygenation: modulation of enzyme activities by MnSOD. *American Journal of Physiology-Lung Cellular Molecular Physiology*, 285, L189-L198.

Pryor, W. A., Stanley, J. P., & Blair, E. (1976). Autooxidation of polyunsaturated fatty acids: a suggested mechanism for the formation of TBA-reactive materials from prostaglandine like endoperoxides. *Lipids*, 11, 370-379.

Puntarulo, S., Sánchez, R. A., & Boveris, A. (1988). Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. *Plant Physiology*, 86, 626–630. 10.1104/pp.86.2.626

Radi, R. (2013). Protein tyrosine nitration: biochemical mechanisms and structural basis of its functional effects. *Accounts of Chemical Research*, 46, 550-9.

Rhoads, D.M., Umbach, A.L., Subbaiah C.C., &Siedow, J.N.(2006). Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. *Plant Physiol.* 141, 357–366.

Rhoads, D.M., & Subbaiah, C.C. (2007). Mitochondrial retrograde regulation in plants *Mitochondrion,* 7, 177-194.

Roberts, J.K., Callis, J., Wemmer, D., Walbot, V., & Jardetsky, O. (1984a). Mechanisms of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. *Proceedings of the National Academy of Science USA*, 81,6029–6033.

Roberts, J.K., Callis, J., Jardetsky, O., Walbot, V., & Freeling, M. (1984b). Cytoplasmic acidosis as a determinant of flooding intolerance in plants. *Proceedings of the National Academy of Science USA*. 81, 6029–6033.

Romero-Puertas, M. C., Rodriguez-Serrano, M., & Sandalio, L. M. (2013). Protein Snitrosylation in plants under abiotic stress: an overview. *Frontiers in Plant Science,*4, 373. doi: 10.3389/fpls.2013.00373

Rosahl, S. (1996). Lipoxygenases in plants: their role in de- velopment and stress response. *Zeitschrift für Naturforschung*, 51, 123-138.

Royo, B., Moran, J. F., Ratcliffe, R. G., & Gupta, K. J. (2015). Nitric oxide induces the alternative oxidase pathway in Arabidopsis seedlings deprived of inorganic phosphate. *Journal of experimental botany*, *66*(20), 6273–6280. doi:10.1093/jxb/erv338

Saha, B., Borovskii, G., & Panda, S. K. (2016). Alternative oxidase and plant stress tolerance. *Plant signaling & behavior*, 11(12), e1256530. doi:10.1080/15592324.2016.1256530

Sairam. R.K., Dharmar, K., Chinnusamy, V., & Meena, R.C. (2009). Waterlogging-induced increase in sugar mobilization, fermentation, and related gene expression in the roots of mung bean (*Vigna radiata*) *Journal of Plant Physiology*, 166, 602–616.

Salavati, A., Khatoon, A., Nanjo, Y. & Komatsu, S. (2012) Analysis of proteomic changes in roots of soybean seedlings during recovery after flooding. *Journal Proteomics*, 75, 878–893

Sanni, B., Williams, K., Sokolov, E. P., & Sokolova, I.M. (2008). Effects of acclimation temperature and cadmium exposure on mitochondrial aconitase and LON protease from a model marine ectotherm, Crassostrea virginica. *Comparative Biochemistry & Physiology C*, 147, 101-112. doi:10.1016/j.cbpc.2007.08.005

Schonbaum, G. S., Bonner, W. D., Jr., Storey, B. T., & Bahr, J. T.(1971). Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. *Plant Physiology,* 47, 124–128 .

Sehrawat, A., & Deswal, R. (2014). S-Nitrosylation Analysis in Brassica juncea Apoplast Highlights the Importance of Nitric Oxide in Cold-Stress Signaling. *Journal of Proteome Research*, 13(5), 2599-619. (ISSN: 1535-3907)

Seymour, S. (2001). Biophysics and Physiology of temperature regulation in thermogenic flowers. *Bio Science Reports*, 21(2), 223-236.

Shapiguzov, A., Vainonen, J. P., Wrzaczek, M, & Kangasjärvi, J. (2012). ROS-talk – how the apoplast, the chloroplast, and the nucleus get the message through. *Frontiers in Plant Science*, 3, 29210.3389/fpls.2012.00292

Shewfelt, R.L., & Purvis, A.C. (1995). Toward a comprehensive model for lipid peroxidation in plant tissue disorders. *Horticultural Science*, 30(2), 213-218.

Shukla, P., Singh, S., Dubey, P., Singh, A., & Singh, A.K. (2015). Nitric oxide mediated amelioration of arsenic toxicity which alters the alternative oxidase (Aox1) gene expression in *Hordeum vulgare* L, *Ecotoxicology of Environmental Safety*, 120, 59–65.

Sidana, S., Bose, J., Shabala, L., & Shabala, S. (2015). Nitric oxide in drought stress signalling and tolerance in plants, in: M.N. Khan, M. Mobin, F. Mohammad, F.J. Corpas (Eds.), Nitric Oxide Action Abiotic Stress Responses Plants. *Springer International Publishing*, Cham, 95–114.

Siddiqui, M.H., Al-Whaibi, M.H., & Basalah, M.O. (2011). Role of nitric oxide in tolerance of plants to abiotic stress. *Protoplasma*, 248, 447–455.

Siedow, J. N., & Girvin, M. E. (1980). Alternative respiratory pathway: its role in seed respiration and its inhibition by propyl gallate. *Plant Physiology*, 65, 669-674.

Siedow, J.N. (1991). Plant lipoxygenase: structure and function. Annual Review of Plant *Physiology & Plant Molecular Biology*, 42, 145-188.

Skutnik, M., & Rychter, A. M. (2009). Differential response of antioxidant systems in leaves and roots of barley subjected to anoxia and post-anoxia. *Journal of plant physiology*, 166, 926-937. doi: 10.1016/j.jplph.2008.11.010

Steffens, B., Steffen-Heins, A., & Sauter, M. (2013). Reactive oxygen species mediate growth and death in submerged plants. *Frontiers in plant science*, 4, 179. doi:10.3389/fpls.2013.00179

Strommer, J. (2011). The plant ADH gene family. *The Plant Journal*. 66(1), 128–42. 10.1111/j.1365-313X.2010.04458.x

Shah, J.K., Cochrane, D.W., De Paepe, R., & Igamberdiev, A.U. (2013). Respiratory complex I deficiency results in low nitric oxide levels, induction of hemoglobin and upregulation of fermentation pathways. *Plant Physiology & Biochemistry*, 63, 185-190

Shingaki-Wells, R., Millar, A.H., Whelan, J., & Narsai, R. (2014). What happens to plant mitochondria under low oxygen? An omics review of the responses to low oxygen and reoxygenation. *Plant, Cell & environment*, 37, 2260–2277.

Sluse, F.E. & Jarmuszkiewicz, W. (1998). Alternative oxidase in the branched mitochondrial respiratory network: an overview on structure, function, regulation, and role. *Brazilian Journal ofMedical & Biological Research*, 31(6), 733-747. https://dx.doi.org/10.1590/S0100-879X1998000600003

Souza, J.M., Peluffo, G., & Radi, R. (2008). Protein tyrosine nitration-Functional alteration or just a biomarker? *Free Radical Biology & Medicine*, 45, 357-66.

Stöhr, C., & Stremlau, S. (2005). Formation and possible roles of nitric oxide in plant roots. *Journal of Experimental Botany*, 57(3), 463-470.

Stoimenova, M., Igamberdiev, A.U., Gupta, K.J., & Hill, R.D. (2007). Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria. *Planta*, 226, 465-474.

Sun, J., Steenbergen, C., & Murphy, E. (2006). S-nitrosylation: NO-related redox signaling to protect against oxidative stress. *Antioxidants & redox signaling*, 8(9-10), 1693–1705. doi:10.1089/ars.2006.8.1693

Szal, B., Drozd, M. & Rychter, A.M. (2004). Factors affecting determination of superoxide anion generated by mitochondria from barley roots after anaerobiosis. *Journal of Plant Physiology*, 161,1339– 1346.

Taiz, L., Zeiger, E. Moller, I.M., & Murphy, A. (2014). Plant physiology and development. 6th Edition, *Sinauer Associates, Inc*., Sunderland, 616-621. Redwood City, Calif: enjamin/Cummings Pub. Co. ISBN: 9781605352558

Tan, Y.F., O'Toole, N., Taylor, N.L, & Millar, A.H. (2010). Divalent Metal Ions in Plant Mitochondria and Their Role in Interactions with Proteins and Oxidative StressInduced Damage to Respiratory Function. *Plant Physiology*, 152, 747–761.

Taylor, E.R., Nie, X.Z., MacGregor, A.W., & Hill, R.D. (1994). A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. *Plant Molecular Biology*, 24, 853–862.

Telfer, A., Bishop, S.M., Phillips, D., & Barber, J. (1994). Isolated photosynthetic reaction center of photosystem II as a sensitizer for the formation of singlet oxygen. *Journal of Biological Chemistry*, 269, 13244–13253.

Umbach, A.L., Fiorani, F.,& Siedow, J.N. (2005). Characterization of transformed Arabidopsis with altered alternative oxidase levels and analysis of effects on reactive oxygen species in tissue. *Plant Physioogyl*, 139, 1806–1820.

Ursini, F., Maiorino, M., & Sevanian, A. (1991). Membrane hydroperoxides. In Sies, H. (Ed.) Oxidative stress: oxidants and antioxidants, *London-New York: Academic Press*, 319-36.

Ushimaro, T., Shibasaka, M., & Tsuji, H.(1992). Development of O_2^- detoxification system during adaptation to air of submerged rice seedlings. *Plant Cell Physiol*ogy, 33,1065–1071.

van Dongen, J.T., Fröhlich, A., Ramírez-Aguilar, S.J., Schauer, N., Fernie, A.R,, Erban, A., Kopka, J., Clarke, J., Langer, A. & Geigenberger, P. (2009). Transcript and metabolite profiling of the adaptive response to mild decreases in oxygen concentration in the roots of Arabidopsis plants. *Annals of Botany*, 103, 269–280.

Vanacker, H., Carver, T.L.W., & Foyer, C.H. (1998a). Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. *Plant Physiology*, 17, 1103–1114.

Vanlerberghe, G.C., & McIntosh, L. (1997). Alternative oxidase: From gene to function. *Annual Review of Plant Physiology & Plant Molecular Biology*, 48, 703-734.

Vanlerberghe, G.C., Cvetkovska, M. & Wang, J. (2009). Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? *Plant Physiology*,137, 392-406

Vanlerberghe, G.C. (2013). Alternative Oxidase: A Mitochondrial Respiratory Pathway to Maintain Metabolic and Signaling Homeostasis during Abiotic and Biotic Stress in Plants. International, *Journal of Molecular Sciences,* 14, 6805– 6847.

Vanlerberghe, G.C., Martyn, G.D., & Dahal, K. (2016). Alternative oxidase: a respiratory electron transport chain pathway essential for maintaining photosynthetic performance during drought stress.*Physiologia Plantarum*., 157(3), 322-37.

Vartapetian, B.B., Zakhmylova, N.A., & Generozova, I.P. (1985). Ultrastructure of wheat coleoptile mitochondria at short-term anoxia and post-anoxia. *Plant, Cell & Environment,* 8, 65–67.

VanToai, T.T., & Bolles, C.S. (1991). Postanoxic injury in soybean (Glycine max) seedlings. *Plant Physiology*, 97, 588–592.

Velikova, V., Yordanov, I., & Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants. *Plant Science,* 151, 59-66.

Vishwakarma, A., Tetali, S.D., Selinski, J., Scheibe, R., & Padmasree, K. (2015). Importance of the alternative oxidase (AOX) pathway in regulating cellular redox and ROS homeostasis to optimize photosynthesis during restriction of the cytochrome oxidase pathway in Arabidopsis thaliana. *Annals of Botany,* 116, 555–569.

Voesenek, L.A.C.J., Colmer, T.D., Pierik, R., Millenaar, F.F., & Peeters, A.J.M. (2006) How plants cope with complete submergence. *The New Phytologist*, 170, 213– 226.

Walker, M.A., & McKersie, B.D. (1993). Role of ascorbate-glutathione antioxidant system in chilling resistance of tomato. *Journal of Plant Physiology*, 141, 234–239.

Wang, H., Liang, X., Huang, J., Zhang, D., Lu, H., Liu, Z., & Bi, Y. (2010). Involvement of Ethylene and Hydrogen Peroxide in Induction of Alternative Respiratory Pathway in Salt-Treated Arabidopsis Calluses. *Plant Cell Physiology*, 51, 1754–1765.

Wang, J., Rajakulendran, N., Amirsadeghi, S., & Vanlerberghe, G.C. (2011). Impact of mitochondrial alternative oxidase expression on the response of Nicotiana tabacum to cold temperature. *Physiologia Plantarum*, 142, 339-351.

Wang, J., & Vanlerberghe, G.C. (2013). A lack of mitochondrial alternative oxidase compromises capacity to 507 recovers from severe drought stress. *Physiolia Plantarum*, 149, 461-473.

Wollenweber-Ratzer, B., & Crawford, R. M. M. (1994). Enzymatic defense against post-anoxic injury in higher plants. *Proceedings of the Royal Society of Edinburgh, 102B*, 381-390.

Xu, K., Xu, X., Fukao, T., Canlas, P., Maghirang-Rodriguez, R., Heuer, S., Ismail, A.M., Bailey-Serres, J., Ronald, P.C., & Mackill, D.J. (2006). *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature, 442, 705–708.

Yan, B., Dai, Q., Liu, X., Huang, S., & Wang, Z. 1996. Flooding-induced membrane damage, lipid oxidation and activated oxygen generation in corn leaves. *Plant & Soil*, 179, 261-268.

Yocum, C. S., & Hackett, D.P. (1957). Participation of cytochromes in the respiration of the aroid spadix. *Plant Physiology*. 32,186–191.

Yoshida, K., Watanabe, C.K., Terashima, I., & Noguchi, K. (2011). Physiological impact of mitochondrial alternative oxidase on photosynthesis and growth in Arabidopsis thaliana. *Plant, Cell & Environment*, 34, 1890–1899.

Yu, M., Lamattina, L., Spoel, S. H., & Loake, G. J. (2014). Nitric oxide function in plant biology: a redox cue in deconvolution. *New Phytologist*, 202(4), 1142-1156.

Yuroff, A. S., Sabat, G., & Hickey, W.J. (2003).Transporter-mediated uptake of 2chloro- and 2-hydroxybenzoate by Pseudomonas huttiensis strain D1. *Applied Environmental Microbiology*, 69,7401–7408. doi:10.1128/AEM.69.12.7401-7408.2003

Zhang, L.T., Zhang, Z.S., Gao, H.Y., Meng, X.L., Yang, C., Liu, J.G., & Meng, Q.W. (2012). The mitochondrial alternative oxidase pathway protects the photosynthetic apparatus against photodamage in Rumex K-1 leaves. *BMC Plant Biology,* 12, 40.