Importance of Mitochondrial Oxidative Electron Transport in Optimizing Photosynthesis under Light, Osmotic or Temperature Stress

Thesis submitted to the University of Hyderabad

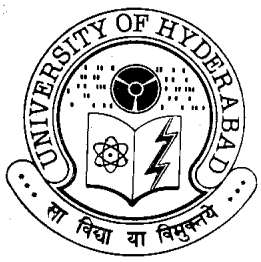
for the Degree of

DOCTOR OF PHILOSOPHY

By

Ch. Dinakar

Supervisor**: Dr. K.P.M.S.V. Padmasree**



Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046, INDIA

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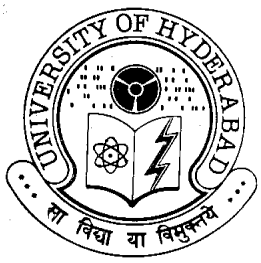

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July 2008

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Importance of Mitochondrial Oxidative Electron Transport in Optimizing Photosynthesis under Light, Osmotic or Temperature Stress"**, has been carried out by me under the supervision of Dr. K.P.M.S.V. Padmasree in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

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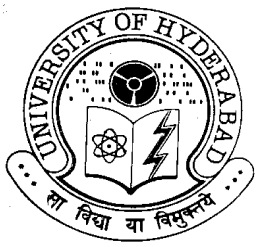

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CERTIFICATE

This is to certify that **Mr. Ch. Dinakar** has carried out the research work embodied in the present thesis entitled "**Importance of Mitochondrial Oxidative Electron Transport in Optimizing Photosynthesis under Light, Osmotic or Temperature Stress"**, for the degree of **Doctor of Philosophy** under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

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Ch. Dinakar

Abbreviations

AA	=	antimycin A
AOX	=	alternative oxidase
APX	=	ascorbate peroxidase
Asc	=	ascorbate (reduced form)
Asc-GSH	=	ascorbate-glutathione
CAT	=	catalase
COX	=	cytochrome oxidase
DHA	=	dehydroascorbate (oxidized form of ascorbate)
DHAP	=	dihydroxyacetone-3-phosphate
GR	=	glutathione reductase
GSH	=	reduced glutathione
GSSG	=	oxidized glutathione
H_2DCF-DA	=	2, 7, -dichlorofluorescein diacetate
KCN	=	potassium cyanide
Mal-OAA	=	malate-oxaloacetate
MDAR	=	monodehydroascorbate reductase
NADP-MDH	=	NADP dependent malate dehydrogenase
OAA	=	oxaloacetic acid
PGA	=	3-phosphoglyceric acid
ROS	=	reactive oxygen species
SHAM	=	salicylhydroxamic acid
SOD	=	superoxide dismutase
Triose-P	=	triose phosphate
UCP	=	uncoupling protein

All the remaining abbreviations are standard ones, as indicated by the journal *Plant Physiology* on their website: http://www.plantphysiol.org, under ‘Instructions for Authors’.

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Chapter 1

Introduction and Review of Literature

Chapter 1

Introduction and Review of Literature

Photosynthesis and respiration are the most important metabolic processes associated with carbon and energy metabolism in higher plants. Photosynthesis is a reductive process involving chloroplasts and respiration is an oxidative process involving mitochondria. During photosynthesis, light energy is transformed through photochemical reactions into biochemically usable chemical potential energy (ATP) and redox potential energy (NADPH) by various electron transport chain (ETC) components of the thylakoid membrane in the chloroplasts. These ATP and NADPH generated during the photochemical reactions are used in the subsequent steps of carbon dioxide fixation and reduction in the Calvin-Benson cycle to generate carbohydrates. During respiration, these carbohydrates are oxidized through glycolysis and tricarboxylic acid (TCA) cycle. The CO_2 generated through decarboxylation reactions of the TCA cycle is released into the atmosphere or recycled to chloroplasts for utilisation in Calvin-Benson cycle. The carbon skeletons of the TCA cycle intermediates are used in various cellular biosynthetic processes, e.g., nitrogen metabolism. On the other hand, NADH generated through TCA cycle is further oxidised by ETC components of the mitochondria and ATP is generated. Though 40-50% of the net carbon gain through photosynthesis are ultimately lost from the plant through respiration, both these processes contribute significantly to the cellular demands of ATP and NAD(P)H (McDonald et al., 2002).

Special features of plant mitochondrial electron transport chain

The energy conserving plant mitochondrial electron transport chain (miETC) exhibits similarities to the miETC of animal mitochondria in containing five respiratory

chain complexes, viz., the rotenone sensitive complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome bc1 reductase), the cyanide-sensitive complex IV (cytochrome *c* oxidase) and complex V (ATP synthase) and two mobile electron carriers ubiquinone and cytochrome c to mediate electron transport from NAD(P)H to molecular O_2. Along with this standard miETC structure, plant mitochondria possess several non-energy conserving pathways of electron transport, both for reduction and oxidation of ubiquinone (Fig. 1.1; Siedow and Umbach, 2000; Millenaar and Lambers, 2003; Vanlerberghe and Ordog, 2002; Rasmusson et al., 2004; McDonald and Vanlerberghe, 2005).

The non energy conserving pathways for reduction of ubiquinone include external and internal NAD(P)H dehydrogenases. On the outer side of the inner membrane facing the inter-membrane space, there is an NADPH dehydrogenase and an NADH dehydrogenase, both regulated by cytosolic Ca^{2+} (Møller, 2001). The external NAD(P)H dehydrogenases can directly oxidize cytosolic NAD(P)H. On the other hand towards matrix side there are two more dehydrogenases, one oxidizing NADH and another NAD(P)H. The second one is Ca^{2+} stimulated. These alternative NAD(P)H oxidizing enzymes are rotenone insensitive, and will reduce the ATP-yield of respiration since they are non-proton pumping and by passes the proton-pumping complex I (Plaxton and Podesta, 2006; Rasmusson et al., 2008).

The non energy conserving pathway for oxidation of ubiquinone includes the alternative oxidase (AOX) pathway. Electron transport through AOX pathway is cyanide resistant and the free energy generated due to electron transport through this pathway is simply released as heat. Hence, reduction of molecular oxygen by electrons passing through AOX pathway dramatically reduces the ATP-yield of respiration as it

circumvents the proton-pumping sites constituted by complexes III and IV of the cytochrome oxidase (COX) pathway in standard miETC (McDonald et al., 2002; Moore et al., 2002).

A more recent addition to the complexity of the plant miETC is the presence of an uncoupling protein (UCP) (Hourton-Cabassa et al., 2004; Plaxton and Podesta, 2006; Navrot et al., 2007; Noguchi and Yoshida, 2008; Nunes-Nesi et al., 2008). UCP is a homologue of thermogenin, an inner membrane protein responsible for non-shivering thermogenesis in mammalian brown fat cells. UCP and thermogenin allow protons to diffuse down their concentration gradient from the intermembrane space into the matrix, circumventing the ATP synthase complex and thus uncoupling electron transport from ATP production. All these non-energy conserving bypass mechanisms provide flexibility to the standard miETC to adapt or acclimatize to the changes in the carbon and energy metabolism during photosynthesis or under stress conditions.

Respiration during light

A strong debate persisted for a long time about the nature and even occurrence of dark respiration in leaves under light (Raghavendra et al., 1994; Raghavendra and Padmasree, 2003; Møller and Gardeström, 2007; Nunes-Nesi et al., 2007). Different workers have reported that respiration is either stimulated or unaffected or inhibited to varying extents in the presence of light (Graham, 1980, Raghavendra et al., 1994; Krömer, 1995, Hoefnagel et al., 1998; Padmasree et al., 2002). Such variation in reports could be due to different factors: the component of dark respiration being monitored (CO_2 efflux or O_2 uptake), the experimental technique being used and the system/tissue used. It is difficult to assess the operation of photosynthesis or respiration in leaves

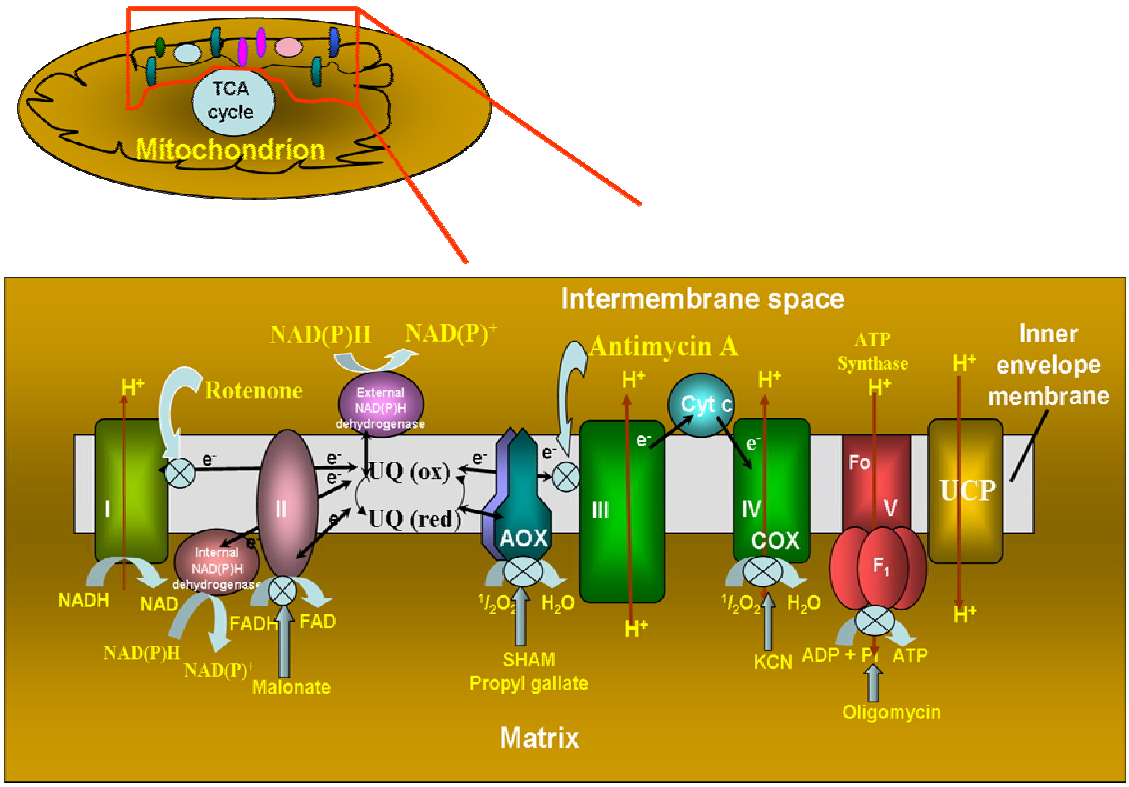

Figure 1.1. The respiratory chain in higher plant mitochondria. Electron flow through COX pathway to molecular O_2 generates electromotive force for ATP synthesis, and the electron flow through AOX pathway generates heat. Abbreviations: I, complex I or NADH dehydrogenase; II, complex II or succinate dehydrogenase; III, complex III or cytochrome bcI reductase; IV, complex IV or cytochrome oxidase; UQ, ubiquinone; AOX, alternative oxidase pathway and UCP, uncoupling protein. Crosses inside circles indicate sites of inhibition of the miETC by different metabolic inhibitors.

based on uptake/evolution of either O_2 or CO_2, since the measurements are compromised by inter- and intracellular recycling of gases, technical difficulty of monitoring precisely the different types of oxidative reactions besides respiration (e.g. photorespiration, Mehler reaction, chlororespiration) (Raghavendra et al., 1994).

Mass spectrometry was suggested to be a promising solution to resolve this problem, because it can distinguish between the uptake/efflux of O_2 or CO_2 occurring simultaneously during respiration, photosynthesis and related cellular processes. Mass spectrometric studies using $^{13/12}CO_2$ and $^{18/16}O_2$ have revealed that light has a differential effect on respiration (Avelange et al., 1991; Raghavendra et al., 1994; Xue et al., 1996; Atkin et al., 2000b). Later, a radiogasometric method was introduced by Parnik and Keerberg (2007) to measure leaf CO_2 exchange in plants. Using this method they determined the rates of CO_2 fixation, photorespiration and respiration simultaneously in the light under conditions of steady state photosynthesis. Further, this method was useful to discriminate between primary and stored photosynthates as substrates of both photorespiratory and respiratory decarboxylations and estimate the extent of inhibition of respiration in the light. The light inhibition of respiration was found to be larger in species that accumulate starch when compared to the species that do not accumulate starch.

The operation of the Krebs cycle in the light is affected by a combination of atleast two factors: the reversible inactivation of the mitochondrial pyruvate dehydrogenase complex in the light (Tovar-Mendez et al., 2003) and the rapid export of TCA cycle intermediates out of the mitochondria (Hanning and Heldt, 1993) for utilization in glutamate synthesis (Hodges, 2002). It is now clear that while some of the reactions of TCA cycle are inhibited, oxidative electron transport and phosphorylation

continue to be active in light and benefit chloroplast metabolism (Padmasree et al., 2002; Raghavendra and Padmasree, 2003). However, variability in mitochondrial O_2 consumption, indicative of mitochondrial oxidative metabolism in light may depend on several factors, especially, metabolic (substrate supply, redox status of the cell and cellular demands for ATP) and environmental (e.g. light intensity, temperature, water and nutrient availability) factors were identified to be important (Hoefnagel et al., 1998; Nunes-Nesi et al., 2007).

Light has been known to regulate the gene expression of several key respiratory enzymes which include cytochrome oxidase (Hilton and Owen, 1985; Welchen et al., 2002; Curi et al., 2003), phosphoenolpyruvate carboxylase (Sims and Hague, 1981), NADP malic enzyme (Collins and Hague, 1983), and isocitrate dehydrogenase (Igamberdiev and Gardeström, 2003). The light dependent expression of these mitochondrial genes suggest a close association between photosynthesis and mitochondrial metabolism (Svensson and Rasmusson, 2001; Nunes-Nesi et al., 2007; Rasmusson and Escobar, 2007). On the other hand, microarray analysis showed a clear trend of reduced expression of the genes associated with respiratory processes in the light (Urbanczyk-Wochniak et al., 2006). However, this may not necessarily lead to downregulation in the metabolic flux through the pathway. The decreased rate of photosynthesis under photorespiratory conditions in the cytoplasmic male-sterile mutant (CMSII) of *Nicotiana sylvestris*, which exhibits a loss of function of mitochondrial complex I also suggests the close interaction between light and respiration (Dutilleul et al., 2003a).

Importance of mitochondrial respiration in dissipating chloroplastic reducing equivalents

Redox reactions are the fundamental metabolic processes through which cells convert and distribute the energy that is necessary for growth and maintenance. Chloroplasts always have a tendency to get over reduced as the rate of photochemical reaction and utilization of reducing potential in metabolism have been estimated to differ by at least 15 orders of magnitude (Huner et al., 1998). Under various abiotic stress conditions like e.g., sub-optimal CO_2 or O_2, and excess light levels, accumulation of redox equivalents occur in chloroplasts and lead to photoinhibition. It is essential that the excess reduced equivalents are taken out or dissipated quickly to prevent damage to the thylakoid membranes (Gilmore, 1997; Niyogi et al., 1998; Niyogi, 1999). Plants have different outlets to dissipate excess reducing equivalents from chloroplasts and minimize the damage caused by photoinhibition. The dissipatory mechanisms include thermal dissipation capacity, operation of xanthophyll cycle, water-water cycle, PS I cyclic electron transport, and rapid turnover of the D1 protein of PS II. Apart, mitochondrial oxidative metabolism and photorespiration also play a role in preventing overreduction of chloroplastic redox carriers (Raghavendra and Padmasree, 2003).

The malate-oxaloacetate (Mal-OAA) shuttle

The Mal-OAA shuttle is considered to be an important system for reductant transport (Scheibe, 2004). Results presented by Hatch et al. (1984) indicated that a highly active Mal–OAA exchange exists in the chloroplasts. Similar to several Calvin cycle enzymes, NADP dependent malate dehydrogenase (NADP-MDH) in the chloroplast stroma is activated by the light-dependent Fd-thioredoxin (Fd-Trx) system (Miginiac-Maslow et al., 2000). The activated NADP-MDH produces Mal and NADP+

from OAA and NADPH in chloroplasts. Mal can be exported to the cytosol in exchange for OAA via Mal transporters on the chloroplast membrane (Fig.1.2). It has been demonstrated that the acceptor side of PS I was more oxidized in NADP-MDH over-expressing potato leaves than in wild type and NADP-MDH underexpressing ones (Backhausen et al., 1998). In potato leaves under high CO_2 conditions, NADPH is more efficiently consumed by the CO_2 assimilation process, thereby decreasing the NADP-MDH activation state and the reduced fraction of the acceptor side of PSI (Backhausen and Scheibe, 1999). These results support the possibility that NADP-MDH plays a key role in recycling NADPH, especially when its production is in excess of the requirement for the photosynthetic CO_2 assimilation (Scheibe et al., 2005).

The triose phosphate-PGA shuttle

The triose phosphate-phosphate transporter (TPT) exchanges triose-P (e.g., dihydroxyacetone phosphate, DHAP) with inorganic phosphate (Pi). In illuminated leaves, triose-P is exported from the chloroplasts to the cytosol and used for sucrose synthesis. TPT imports Pi into the chloroplast, and this is essential for optimal photo-phosphorylation in the chloroplasts of illuminated leaves (Sharkey and Vanderveer, 1989). TPT can also indirectly exchange triose-P with 3-phosphoglyceric acid (3-PGA). In this case, excess reductants are exported from the chloroplasts in the form of triose-P (Gardeström et al., 2002). The exported triose-P can be oxidized by the cytosolic non-phosphorylating NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (NADP-G3PDH), which produces 3-PGA and NADPH. Alternatively, 3-PGA, ATP and NADH can be produced from triose-P via the phosphorylating NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (NAD-G3PDH) and phosphoglycerate kinase (PGK) in the cytosol (Gardeström et al., 2002, Plaxton and Podesta, 2006).

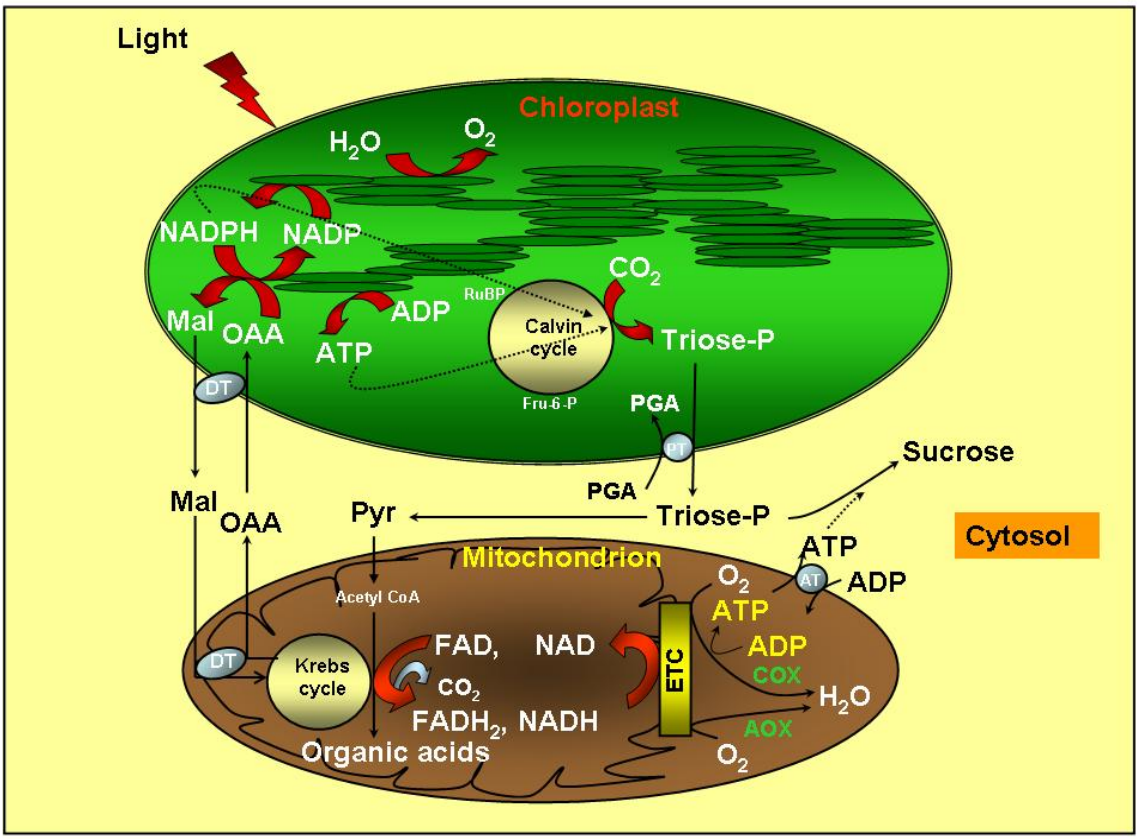

Figure 1.2. The malate-OAA shuttle transfers reducing equivalents from chloroplasts to mitochondria through dicarboxylate translocator (DT). Triose-P-PGA shuttle plays a dual role in exporting reducing equivalents from chloroplasts to mitochondria as well as supplying substrate for sucrose synthesis in cytosol through Pi-translocator (PT). ATP required for sucrose synthesis is translocated from mitochondria to cytosol through adenylate translocator (AT). Mitochondria dissipate chloroplastic reducing equivalents in excess of the requirement of calvin cycle through malate-OAA and triose-P-PGA shuttles in mesophyll cells.

However, NADP-G3PDH has a higher affinity for triose-P than NAD-G3PDH. Although NADP-G3PDH is inhibited by high NAD(P)H concentrations (Scagliarini et al., 1990), the cytosolic NAD(P)H pool is more oxidized under light than in dark (Igamberdiev and Gardeström, 2003). Thus, excess reductants in the chloroplasts can be exported to the cytosol via TPT. In leaves of an *Arabidopsis* TPT-lacking mutant, starch accumulated in the chloroplasts, and both photosynthesis and growth decreased (Walters et al., 2004).

Mitochondria appear to play a significant role in maintaining optimal levels of redox equivalents in the chloroplasts to keep up the Calvin cycle activity (Woodson and Chory, 2008), possibly by coordinating with peroxisomes and cytosol. The reductants in excess of the requirements of the Calvin cycle are exported out of chloroplasts through the shuttling of Mal-OAA, by dicarboxylate translocator and PGA-DHAP by Pi-translocator (Fig. 1.2; Scheibe et al., 2005; Noguchi and Yoshida, 2008). The relative levels of triose-P/PGA and malate/OAA reflect the redox state of cytosol and the cell. Any limitation on the mitochondrial metabolism lead to a marked rise in the redox state of cells, as indicated by the rise in the ratios of malate/OAA or triose-P/PGA. These results support the importance of mitochondrial oxidative electron transport through COX and AOX pathways in dissipating excess chloroplastic reducing equivalents and thereby optimize chloroplastic photosynthesis (Padmasree and Raghavendra 1999c).

AOX: regulation and significance to photosynthesis

Alternative oxidase (AOX) exists as a dimer in the inner mitochondrial membrane (Fig. 1.3). When the two units of the dimer are covalently bound by disulfide bonds, the AOX is in an inactive state. When the disulfide bonds are reduced, the non-

covalently bound dimer becomes active (Umbach et al., 1994). AOX is encoded by a multigene family (Whelan et al., 1996; Saisho et al., 1997). At least three isoforms of AOX are known in soybean (Djajanegara et al., 2002), Complete genome sequencing revealed five genes, classified as four *Aox1* type and one *Aox2* type in *Arabidopsis* plants (Clifton et al., 2006). One reason that plant mitochondria do not produce more superoxide could be due to the presence of an AOX that catalyzes the tetravalent reduction of O_2 by ubiquinone. The activity of AOX is increased by certain organic acids, notably pyruvate, and by reduction of the protein (Fig. 1.3; Millar et al., 1993; Vanlerberghe et al., 1995; Umbach et al., 2006). Transgenic tobacco are being utilized to critically assess the role of AOX in balancing carbon metabolism and electron transport under different physiological conditions such as phosphate-limitation (Parsons et al., 1999).

AOX plays an important role in integrating the processes of carbon metabolism and mitochondrial electron transport, particularly when there is an accumulation of reduced equivalents and pyruvate, e.g. under phosphate deficiency (Vanlerberghe and Ordog, 2002). However, the physiological function of the AOX pathway in green tissues is not clear, although it is known to increase under stress conditions (Vanlerberghe and Ordog, 2002). Experiments with inhibitors suggested that the mitochondrial pathway through COX and AOX pathways is essential for photosynthesis (Padmasree and Raghavendra, 2001a). The AOX pathway can play an important role in protecting chloroplasts against photoinhibition, by dissipating the excess redox equivalents from chloroplasts (Yoshida et al., 2007). Interestingly, the extent and engagement of AOX seems to increase when the cytosol and mitochondria are over-

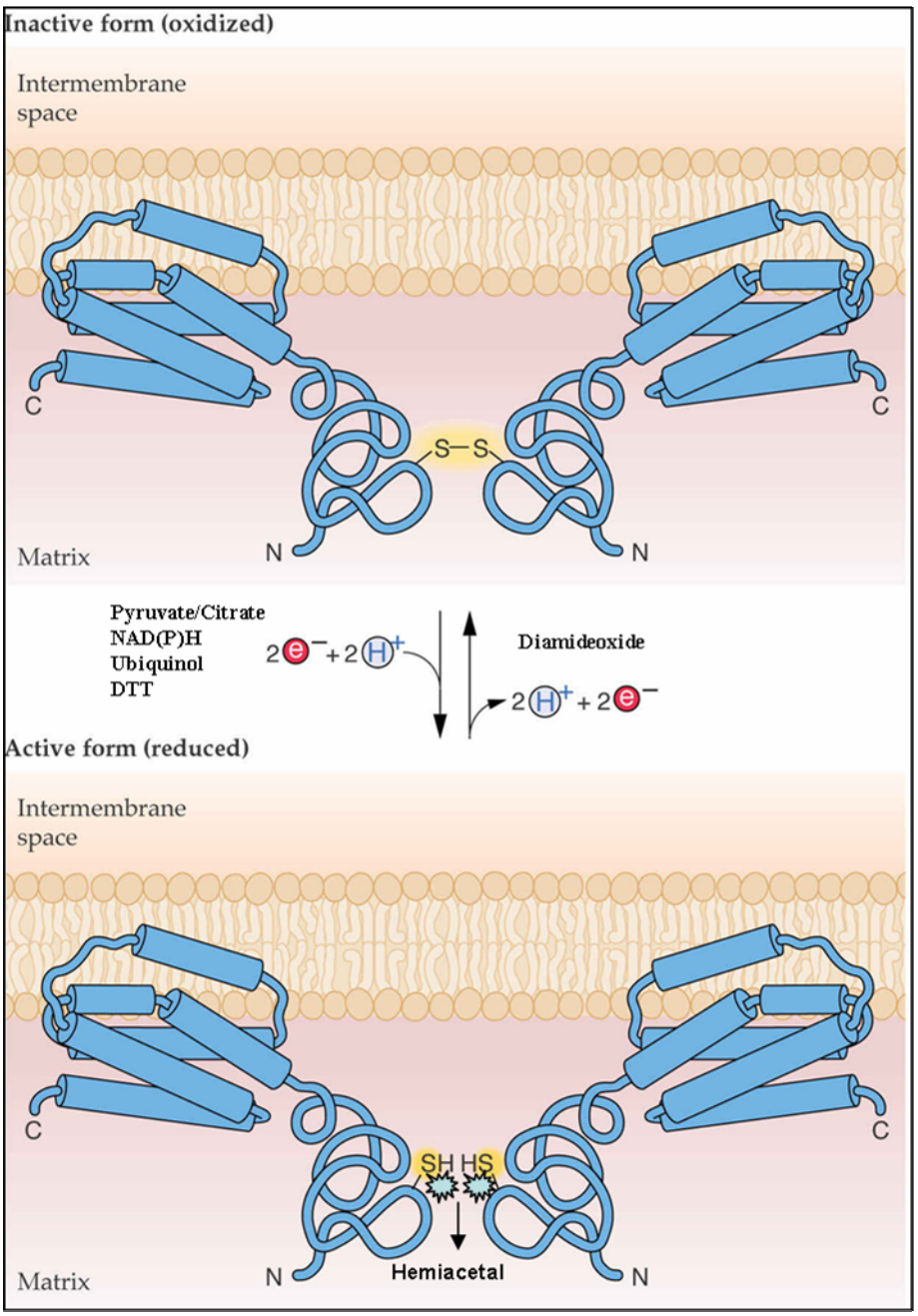

Figure 1.3. Structure of AOX dimer (oxidized form) and monomer (reduced form). In plants, AOX exists in the membrane as a dimer. It is inactive in its oxidized form, and becomes active when the disulfide bridge between the two monomers is reduced and forms a hemiacetal with keto groups. AOX gets activated when there is an accumulation of reducing equivalents, keto acids and also in presence of DTT.

reduced, as happens in the light (Bykova and Møller, 2001). Furthermore, the following evidences suggest the pronounced operation of the AOX pathway in light in green tissues: (i) increased electron flow through the AOX pathway during glycine oxidation (Igamberdiev et al., 2001) (ii) synthesis of AOX protein during greening (Atkin et al., 1993) (iii) increased electron flux through the AOX pathway in light and its decrease on transition to dark (Ribas-Carbo et al., 2000a).

The function of AOX pathway of mitochondrial electron transport was found to be important during the activation of key chloroplastic enzymes as suggested by the marked decrease in light activation of NADP-malate dehydrogenase, NADP-glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase in presence of SHAM (Padmasree and Raghavendra, 2001a; Raghavendra and Padmasree, 2003). AOX inhibition also resulted in overreduction of the photosynthetic electron transport chain and induction of the cyclic electron flow around PS I at low PPFD in Broad bean leaves (Yoshida et al., 2006). Chemical inhibition of the miETC by antimycin A or the TCA cycle by monofluoroacetate (MFA) caused increased expression of nucleus-encoded AOX genes in plants. The results of Feng et al. (2007) indicated that the role and regulation of AOX in light is determined by the developmental stage of plant photosynthetic apparatus. Thus, there exists a flexible interrelation between AOX respiratory pathway and photosynthesis. Recently a potential role of AOX to initiate new programs of cell development and growth has been proposed, given the fact that chloroplast-mitochondria interaction is crucial for carbon use efficiency and energy transfer (Arnholdt-Schmitt et al., 2006).

Responses of photosynthesis and respiration to abiotic stress

Stress can be defined as any biotic or abiotic factor unfavourable for growth and reproduction of the living organisms, due to either sub-optimal or supra-optimal levels of the factor (Levitt, 1980). Understanding the molecular responses of plants exposed to different abiotic stresses is of much importance as they give hope for developing genetically modified crops to cope up with these stresses in a better way. Higher plants being immobile have a greater need of protection against several stresses, including high or low temperature, water stress, salinity, metal toxicity, light and others. At the whole plant level the effect of stress is usually perceived as a decrease in photosynthesis and growth, and is associated with alteration in carbon and nitrogen metabolism (Cornic and Massacci, 1996; Mwanamwenge et al., 1999). There are many cellular mechanisms by which plants ameliorate the effects of environmental stresses. For instance accumulation of compatible osmolytes such as proline, glycine betaine etc. frequently occurs in plant tissues during various environmental stresses (Ashraf and Foolad, 2007).

High light intensity causes the over-reduction of photosynthetic electron transport chain leading to the production of reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxides (Ledford and Niyogi, 2005), and often result in severe inhibition of the activity of photosystem II (PS II) (Andersson and Aro, 2001). The rate of photosynthesis in higher plants depends on the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) as well as synthesis of RuBP (Ramachandra Reddy, 1996; Chaitanya et al., 2002; Parry et al., 2002). Loss of rubisco activity has been reported in several plants under drought (Parry et al., 2002; Chaitanya et al., 2003). Drought generally reduces the biochemical capacity for carbon assimilation and utilization. Water stress reduces leaf water potential and restricts plant

photosynthesis by inhibition of electron transport/photophosphorylation, inactivation of key assimilatory enzymes and productivity, the problem being particularly severe in arid and semi-arid regions of the world (Boyer, 1982; Kaiser, 1984; Leegood et al., 1985; Sharkey and Seemann, 1989; Jones and Corlett, 1992).

The photosynthetic apparatus has long been recognized as one of the plant components most sensitive to high temperature stress (Berry and Björkman, 1980). Inhibition of the activation of ribulose-1,5-biphosphate carboxylase/oxygenase has been identified as one of the most heat-sensitive components of the photosynthetic apparatus (Law and Crafts-Brandner, 1999; Crafts-Brandner and Salvucci, 2002). The temperature fluctuations will have direct impact on photosynthesis through its effects on the thermally sensitive biochemical and physiological processes. These include (i) sucrose synthesis (ii) photosynthetic carbon reduction (iii) carbon partitioning (iv) inter-system electron transport, which is thought to be at the level of the mobile electron transport carriers, plastoquinone and plastocyanin (Berry and Björkman, 1980; Hikosaka et al., 2006).

Mitochondrial functions contribute to the flexibility, that is essential for plant cells in presence of highly variable environment. Temperature is one of the rapidly fluctuating parameters to which plants must adjust (Atkin and Tjoelker, 2003). Leaf respiration is sensitive to low temperature more in darkness than in the light (Atkin et al., 2000a). Several authors proposed that the AOX pathway may maintain flux through the mitochondrial electron transport during cold conditions (Kiener and Bramlage, 1981; Smakman and Hofstra, 1982; McNulty and Cummins, 1987; Purvis and Shewfelt, 1993; Ribas-Carbo et al., 2000) and in doing so, reduce the production of ROS (Purvis et al., 1995). This ability of AOX to maintain flux in the cold could be due to (i) its

reduced sensitivity to temperature when compared to COX pathways (Kiener and Bramlage, 1981; McNulty and Cummins, 1987; Stewart et al; 1990b) (ii) increase in de novo synthesis of AOX protein after long-term exposure to the cold (Stewart et al., 1990a,b; Vanlerberghe and Mcintosh, 1992a; Gonzalez-Meler et al., 1999; Ribas-Carbo et al., 2000) (iii) increase in AOX protein content invariably occur when plants are exposed to low temperatures (Fiorani et al., 2005; Matos et al., 2007). Weger and Guy (1991) reported that the AOX pathway of *Picea glauca* roots are highly sensitive to the changes in temperature. The results of Rachmilevitch et al. (2007) suggest that maintaining a higher proportion of AOX pathway at high soil temperatures may contribute to root thermo-tolerance and that the AOX pathway may play an important role in adaptation to high soil temperatures in *Agrostis scabra*. Increased expression of AOX and AOX pathway activity in photosynthetic tissues has been reported in plants subjected to low and high temperatures (Vanlerberghe and McIntosh, 1992a, b; Fiorani et al., 2005), water stress (Bartoli et al., 2005; Ribas-Carbo et al., 2005) or pathogen attack (Simons et al., 1999) suggesting that AOX pathway play a role in leaves under stress.

Production of reactive oxygen species (ROS) during abiotic stress

A common feature of different stress factors is their potential to increase the production of reactive oxygen species (ROS) in plant tissues. In plant cells, ROS are continuously produced predominantly in chloroplasts, mitochondria, and peroxisomes (del Rio et al., 2002; Apel and Hirt, 2004; Gadjev et al., 2006) as byproducts of various metabolic pathways (Foyer and Harbinson, 1994; Shao et al., 2008). Generation of ROS is an indispensable process for all aerobic organisms. Every living aerobic cell shows a proper dynamic balance between ROS production and ROS utilization under

optimal conditions of growth. The rapid increase in ROS concentration is defined as "oxidative burst" (Apostol et al., 1989; Foyer and Noctor, 2005). Therefore, the production and removal of ROS in a cell must be strictly controlled. However, the equilibrium between production and scavenging of ROS may be perturbed by a number of adverse abiotic stress factors such as high light, drought, low temperature, high temperature, and mechanical stress (Tsugane et al., 1999; Dat et al., 2000; Apel and Hirt, 2004; Shao et al., 2008).

In photosynthesizing green leaves, the major source of cellular ROS comes from at least three distinct processes. First among these processes is the light-mediated over excitation of chlorophyll leading to the formation of $_1O^2$ at PS II (Fryer et al., 2003; op den Camp et al., 2003). A second process involves the Mehler reaction at PS I in which O_2 is reduced to ROS directly by photosynthetic electron transport (Asada, 1999; Badger et al., 2000; Ort and Baker, 2002). Finally in photorespiration, the recycling of phosphoglycolate formed by the oxygenase reaction of rubisco leads to substantial production of H_2O_2 by a peroxisome-located glycolate oxidase (Willekens et al., 1997; Douce and Neuberger, 1999). Even during optimal conditions, a small portion of the total O_2 is converted to ROS (Asada, 1999).

Mitochondrial electron transport chain is also the major site for the generation of ROS such as superoxide and H_2O_2, which is referred to as mitochondrial ROS (mROS) (Rhoads et al., 2006). Although mROS production is much lower compared to chloroplasts, mROS are important regulators of a number of cellular processes including stress adaptation and programmed cell death (Gechev et al., 2006). Complexes I and III probably represent the primary sites of mROS generation. While the relative importance of these two sites for mROS generation and the factors

influencing their rates of mROS production are largely unknown, an important generalization is that mROS formation increases as the ETC becomes more highly reduced (Møller, 2001). It was shown in animal mitochondria that the flavine mononucleotide (FMN)- containing subunit and an iron-sulphur cluster of the nicotinamide adenine dinucleotide (NADH) dehydrogenase of complex 1 are the sites of O_2^- generation (Chen et al., 2005), especially when this complex is glutathionylated after oxidative stress (Taylor et al., 2003).

Since plants have limited mechanisms of stress avoidance, they require flexible means of adaptation to ever changing environmental conditions. Under nonstressful conditions the antioxidant defence system provides adequate protection against active oxygen and free radicals. AOX participates in the antioxygen defence system, the role of which becomes especially important when the ROS levels increase (Purvis et al., 1995; Purvis, 1997; Skulachev, 1997; Wagner and Moore, 1997). Inhibitors of the AOX {salicylhydroxamic acid (SHAM), propyl gallate and disulfarim} strongly stimulated H_2O_2 production in isolated soybean and pea cotyledon mitochondria suggesting the importance of AOX in ROS detoxification (Popov et al, 1997; Purvis, 1997).

Response of antioxidative system of plants to environmental stress

Plants induce antioxidant defense system in response to ROS generation to diminish cytotoxic functions such as lipid peroxidation, protein modification and DNA damage (Mittler, 2002; Sankhalkar and Sharma, 2005; Møller et al., 2007). These defences are not restricted to the intracellular compartments, but are also found in the apoplast to a limited extent (Neill et al., 2002; Mittler et al., 2004). Higher plants

contain numerous enzymatic, non-enzymatic ROS-scavengers and both water and lipid soluble antioxidants localized in different cellular compartments (Noctor and Foyer, 1998; Asada, 1999; Mittler, 2002). The enzymatic ROS scavenging system consists of several enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), and glutathione reductase (GR). Non enzymatic antioxidants include pigments, tripeptide thiol (glutathione), vitamin C (ascorbate), E (α-tocopherol) etc.

Since biotic and abiotic stresses disrupt the energy balance of plant cells, induction of these antioxidative defences can be assumed to reflect a general strategy required to overcome increased oxidative stress due to imposition of environmental constraints.

Enzymatic Antioxidants

With in the cell, SODs constitute the first line of defense against ROS. SOD, the family of metallo-enzymes catalyses the disproportionation of superoxide ($O_2^{\cdot-}$) to molecular oxygen and H_2O_2. SOD removes superoxide and hence decreases the risk of hydroxyl radical formation from superoxide via the metal-catalyzed Haber–Weiss type reaction. Based on the metal co-factor used by the enzyme, SODs are classified into three groups (Mn-SOD, Fe-SOD and Cu/Zn-SOD) which play a key role in protection against oxidative stress (Santos et al., 2000; Moran et al., 2003) and are located in different compartments of the cell. Mn-SOD is predominantly found in mitochondria and peroxisome, Fe-SOD is located in the chloroplast and Cu/Zn-SOD is located in the chloroplast, cytosol and possibly in the extracellular space (Alscher et al., 2002).

Plants contain monofunctional, tetrameric and heme-containing CATs, which are mostly localized in peroxisomes or glyoxysomes (Willekens et al., 1995; Heinze and Gerhardt, 2002; Feierabend, 2005). CAT itself is inhibited by ROS, such as O_2^- (Kono and Fridovich, 1982), and is generally inactivated by UV or visible light in the presence of O_2. Bacterial, mammalian as well as plant CATs are photoinactivated in the presence of O_2 by blue light which is absorbed by the prosthetic heme's (Cheng et al., 1981; Shang and Feierabend, 1999).

The ascorbate-glutathione (Asc-GSH) cycle that occurs in chloroplasts, cytoplasm, and mitochondria (Noctor and Foyer, 1998) has also been demonstrated in peroxisomes (del Rio et al., 2002, Mittova et al., 2000). This cycle is catalyzed by a set of four enzymes, APX, MDAR, glutathione-dependent DHAR, and GR. The primary peroxidation of ascorbate (Asc) by APX yields the monodehydroascorbate radical (MDA) that is either directly reduced back to Asc by MDAR (Arrigoni et al., 1981) or undergoes non-enzymatic disproportionation to Asc and dehydroascorbate (DHA). Recovery of the Asc from DHA occurs via the glutathione-dependent reaction catalyzed by DHAR, and the oxidized glutathione dimers are re-reduced by the NADPH-dependent GR (Foyer and Halliwell, 1976).

Non enzymatic antioxidants

Non enzymatic antioxidants include the major cellular redox buffers Asc, GSH, as well as secondary metabolites including tocopherol, flavonoids, alkaloids, and carotenoids. Tocopherol, Asc and GSH are central components of plant antioxidant defenses combatting together to limit ROS life and accumulation (Mullineaux and Rausch, 2005). Asc synthesis in leaves is light-dependent (Smirnoff and Wheeler,

2000; Ishikawa et al., 2006). Leaves from high light grown plants tend to have more Asc than those grown at lower light (Gillham and Dodge, 1987). Since high Asc contents tend to be associated with stress tolerance, it is tempting to suggest that its biosynthesis may be regulated by redox triggers and particularly ROS. Perturbation of mitochondrial redox processes in a complex-I-deficient mutant is associated with altered expression of several antioxidant components, both within and outside the mitochondrion (Dutilleul et al., 2003b). Mutants with decreased Asc levels or altered GSH content are hypersensitive to stress (Creissen et al., 1999; Conklin et al., 1996).

GSH is oxidized by ROS, forming oxidized glutathione (GSSG) and Asc is oxidized to monodehydroascorbate (MDA) and DHA through the Asc-GSH cycle. GSSG, MDA, and DHA can be reduced reforming GSH and ascorbate. In response to chilling, heat shock, pathogen attack, and drought stress, plants increase the activity of GSH biosynthetic enzymes (Vanacker et al., 2000, Vernoux et al., 2002) and GSH levels (Noctor et al., 2002). A high ratio of reduced to oxidized Asc and GSH is essential for ROS scavenging in cells. Reduced states of the antioxidants are maintained by GR, MDAR, and DHAR using NADPH as reducing power (Tsugane et al., 1999). In addition, the overall balance among different antioxidants must be tightly controlled. The importance of this balance is evident when cells with enhanced glutathione biosynthesis in chloroplasts show oxidative stress damage, possibly due to changes in the overall redox state of chloroplasts (Creissen et al., 1999). The information on the role of flavonoids and carotenoids in ROS detoxification in plants is very limited. However, overexpression of ß-carotene hydroxylase in *Arabidopsis* lead to increased amounts of xanthophyll in chloroplasts and resulted in enhanced tolerance towards oxidative stress induced by high light (Davison et al., 2002).

Many stresses cause net oxidation of the glutathione pool, often accompanied or followed by increases in total glutathione (Smith et al., 1984; Sengupta et al., 1991; Noctor et al., 2002; Gomez et al., 2004). An important mechanism in perceiving this redox perturbation could be glutathionylation of proteins (Foyer and Noctor, 2003). In this process, glutathione forms a mixed disulphide with a target protein with possible important consequences for activity of enzymes and components such as transcription factors. Glutathionylation can occur as a result of either increase in GSSG concentration or through ROS-mediated oxidation of thiols to thiyl radicals (Starke et al., 2003). Various stress conditions not only cause net oxidation of the glutathione redox couple, but also increases the total glutathione pool.

Importance of mitochondrial respiration in optimizing photosynthesis under abiotic stress conditions

Plants grow using light energy by photosynthetically converting atmospheric CO_2 into carbon-rich compounds (e.g. carbohydrates) in the chloroplasts. These compounds are respired in the cytosol and mitochondria to generate the energy and carbon intermediates necessary for biosynthesis. Thus photosynthesis and respiratory processes are interdependent, with respiration relying on photosynthesis for substrate, and photosynthesis depends on respiration for a range of compounds (e.g. ATP). Though chloroplasts and mitochondria are traditionally considered to be autonomous organelles, several recent studies demonstrated that these two organelles are not only interdependent in their functions, but are also mutually beneficial in their interaction. The following review articles give a detailed information on the interactions between chloroplasts and mitochondria in light and dark using several model systems from lower eukaryotes to higher plants (Azcón-Bieto, 1992; Raghavendra et al., 1994; Gardeström

and Lernmark, 1995; Krömer, 1995; Hoefnagel et al., 1998; Gardeström et al., 2002; Padmasree et al., 2002; Raghavendra and Padmasree, 2003; Yoshida et al., 2006; Noctor et al., 2007; Rasmusson and Escobar, 2007; Sweetlove et al., 2007; Noguchi and Yoshida, 2008; Nunes-Nesi et al., 2008; Woodson and Chory, 2008).

Since plants cannot move away from adverse situations, they adjust quickly to the changing environmental conditions like light, temperature, availability of CO_2, water, through immediate physiological and metabolic adjustments. Photosynthesis under such abiotic stress conditions means modifying all other metabolic functions of a photosynthetic cell to optimise the chloroplast function and to minimize the production of ROS and the consequent oxidative damage. Mechanisms by which mitochondrial function facilitates optimal rates of photosynthesis under stress conditions have become the focus of research in recent years.

Mitochondrial oxidative metabolism has been shown to protect photosynthesis from photoinhibition (Saradadevi and Raghavendra, 1992). Treatment of protoplasts with antimycin A or sodium azide or oligomycin (inhibitors of cytochrome pathway or mitochondrial ATP synthase) aggravated photoinhibition (Saradadevi and Raghavendra, 1992). The inhibition of mitochondrial cytochrome *c* oxidase or ATP synthase enhanced the extent and speed of photoinhibition in *Anacystis nidulans* (Shyam et al., 1993) and *Chlamydomonas reinhardtii* (Singh et al., 1996). Mitochondria might also play a role in the recovery of photosynthesis from photoinhibition, by supplying ATP for D1 protein synthesis (Shyam et al., 1993; Singh et al., 1996). In a CAM fern *Pyrrosia piloselloides*, recycling of respiratory CO_2 helps to keep up rates of photosynthesis under photoinhibitory light (Griffiths et al., 1989). Yoshida et al. (2007) demonstrated AOX as an efficient dissipation system of reducing equivalents from

chloroplasts to protect the chloroplastic machinery from overreduction, and there by protecting photosynthesis. A recent study in broad bean revealed that inhibition of the AOX causes decreases in the rate of photosynthetic oxygen evolution even under low irradiances and suggest that this pathway provides chloroplasts with flexible strategies against photoinhibition (Yoshida et al., 2006). A strong evidence for the critical role of complex I of mitochondrial electron transport chain was provided by the characterization of cytoplasmic male sterile mutants lacking one of the subunit of complex I and displaying markedly reduced rates of photosynthesis (Sabar et al., 2000; Dutilleul et al., 2005).

Alteration in mitochondrial respiration seems to be related to decreased photosynthesis and increased susceptability to photoinhibition under osmotic stress. Mesophyll protoplasts of pea kept in hyperosmotic medium were highly susceptible to photoinhibition when they were exposed to photoinhibitory light. On exposure to hyperosmotic medium at chilling temperature, both photosynthetic and respiratory rates decreased, indicating a correlation between the two processes (Saradadevi and Raghavendra, 1994). However, at 25 ^{o}C, respiration increased, while photosynthesis decreased. More experiments are needed to understand the role of respiration *vis-à-vis* photosynthesis during osmotic stress or varying temperatures.

After a period of cold hardening, the leaves of winter rye exhibited an increase in the rates of dark respiration in light along with those of photosynthesis (Hurry et al., 1995). Oligomycin (inhibitor of oxidative phosphorylation) treatment resulted in the inhibition of photosynthesis more in cold hardened leaves than that in nonhardened ones, suggesting that the increase in photosynthetic capacity following cold hardening is due to mitochondrial contribution. A similar situation of increased tolerance to

photoinhibition following cold hardening has been reported in the leaves of winter and spring wheat (Hurry and Huner, 1992). Circumstantial evidence points out to the possible roles of AOX during the maintenance of photosynthesis in low temperature. AOX pathway is up-regulated in leaves of plants experiencing drought, and that this up-regulation involves an increase in not only the overall content of the AOX protein, but also an elevation in the reduced active form of AOX in particular to protect photosynthetic electron transport chain (Bartoli et al., 2005). Further studies and direct evidences are needed to assign any direct role of alternative pathway in the protection of photosynthesis from chilling stress in plants.

The present study has been designed to examine the importance of mitochondrial oxidative electron transport particularly through COX and AOX pathways in optimizing photosynthesis under light, osmotic or temperature stress in mesophyll protoplasts of pea. The approach and objectives of the present work are described in the next chapter.

Chapter 2

Approach and Objectives

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The mutually beneficial interaction between chloroplasts and mitochondria is well studied during the last two decades (Graham, 1980; Krömer, 1995; Xu et al., 1996; Raghavendra and Padmasree, 2003; Yoshida et al., 2006; Noguchi and Yoshida, 2008; Nunes-Nesi et al., 2008). Mitochondria support chloroplast functions in various ways during light and dark (Hoefnagel et al., 1998; Scheibe, 2004). In light, mitochondria play a role in oxidizing photosynthetically generated reducing power to prevent (i) over-energization of chloroplastic electron transport carriers and (ii) oxidative damage to the cellular machinery.

Mitochondrial electron transport through the COX pathway and oxidative phosphorylation have been shown to protect photosynthesis against photoinhibition (Saradadevi and Raghavendra, 1992; Shyam et al., 1993; Singh et al., 1996). The importance and responses of mitochondrial metabolism and photosynthesis during abiotic stress e.g. high light, temperature stress, water stress and nutrient deficiency were indicated in few reports (Saradadevi and Raghavendra, 1994; Bunce et al., 1998; Bailey et al., 2004; Bartoli et al., 2004, 2005; Covey-Crump et al., 2007; Debez et al., 2008; Xu et al., 2008). However the information on the biochemical mechanisms which mediate or coordinate the interactions between chloroplasts and mitochondria during abiotic stress is limited. **The present work is an attempt to elucidate the importance of COX and AOX pathways of mitochondrial oxidative electron transport in optimizing photosynthesis under light, osmotic or temperature stress and identify**

the biochemical factors which mediate the beneficial interactions between chloroplasts and mitochondria during abiotic stress.

The stronger dependence of chloroplast photosynthesis on mitochondrial respiration was demonstrated in different model plants *viz. Arabidopsis thaliana, Brassica napus, Commelina communis, Hordeum vulgare, Lycopersicum esculentum, Nicotiana tabacum, Pisum sativum, Secale cereale* and *Vicia faba* (Gautier et al., 1991; Krömer et al., 1993; Hurry et al.,1995; Padmasree et al., 1999a; Sabar et al., 2000; Carrari et al., 2003; Igamberdiev et al., 2006; Sweetlove et al., 2006; Yoshida et al., 2006; Nunes-Nesi et al., 2008). A wide range of experimental model systems including whole plants, leaf discs, mesophyll cell protoplasts, guard cell protoplasts, *Chlamydomonas reinhardtii*, *Selenastrum minutum*, and *Zostera marina* were used to demonstrate the significance of mitochondrial respiration in not only optimizing photosynthesis, but also protecting photosynthesis from photoinhibition (Saradadevi and Raghavendra, 1992; Xue et al., 1996; Padmasree et al., 2002; Carr and Axelsson, 2008). Bundle sheath cells of NAD-malic enzyme and phosphoenolpyruvate carboxykinase type of C_4 plants are other examples of the extreme dependence of photosynthesis on mitochondria (Gardeström et al., 2002). In the marine angiosperm *Zostera marina*, a major part of the ATP used for the generation of acid zones involved in HCO_3^- utilization was derived from mitochondrial respiration (Carr and Axelsson, 2008). In the present study we have used the system of mesophyll protoplasts isolated from *Pisum sativum* to examine the importance of mitochondrial oxidative electron transport in optimizing photosynthesis under light, osmotic or temperature stress conditions.

Pea (*Pisum sativum*) is a mesophytic plant that is easy and fast to grow. Seeds get germinated within 3 days and the 2nd pair of fully expanded leaves from the seedlings were used in our experiments to isolate mesophyll protoplasts, which can be obtained within 10-15 days from the day of sowing seeds. Pea plants being smaller in size, can be grown in a large number in a relatively small area. Being a typical example for C_3 plants it is a very convenient system for physiological and biochemical studies. Further, isolation of mesophyll protoplasts from pea leaves in comparison to other plants is easy and rapid (Devi et al., 1992). Pea plants offer a very good experimental material in the form of intact leaves, leaf discs/leaf slices, isolated protoplasts and organelles, for the study of physiological and biochemical functions in the presence of added compounds and metabolites (Saradadevi and Raghavendra 1992; Padmasree and Raghavendra, 1999a, 2001b, 2002).

The isolated mesophyll protoplasts are an excellent model to achieve our objective because of certain advantages it offer over whole plant (or) leaf discs. The protoplasts allow free diffusion of O_2 or CO_2 due to the absence of barriers like intercellular spaces and cell walls. Hence, measurements of photosynthesis/respiration are not limited by intercellular recycling of gases. The externally added test compounds like antimycin A and salicylhydroxamic acid (SHAM) are taken in quickly and their effects on photosynthesis/respiration can be observed within 5-10 minutes. In protoplasts the coordination between different compartments/organelles is maintained. Therefore, they are best suited to examine the *in vivo* situation when compared to isolated organelles. Further, using mesophyll protoplasts it is also easy to impose the environmental stresses, e.g., light, osmotic or temperature, to which the plants are frequently exposed. However, protoplasts also have certain disadvantages like limited

stability at room temperature, fragile nature and the tendency to sediment. Since the protoplasts have limited stability, the experiments involving protoplasts should therefore be short and done on the same day of isolation.

We used classic mitochondrial inhibitors, antimycin A and SHAM to restrict the electron transport through COX pathway and AOX pathway respectively. These inhibitors were used at a very low concentration so as to minimize the general perturbation of the metabolic system.

The following are the main objectives of the present work:

1. To examine the role of ROS and antioxidants in an intact cell system in which the beneficial interaction of mitochondrial metabolism with photosynthetic carbon assimilation occurs.
2. To study the importance of mitochondrial oxidative electron transport in optimizing photosynthesis under light, osmotic or temperature stress with emphasis on the changes in the following biochemical components.
   - Adenylates: ATP, ADP and ATP/ADP
   - Malate-OAA shuttle: Mal, OAA
   - Cellular ROS
   - Antioxidant metabolites: Asc, DHA, GSH, GSSG
   - Antioxidant enzymes: SOD, CAT, APX, MDAR, and GR.
3. To examine the contribution of COX and AOX pathway to total respiration under light, osmotic or temperature stress.

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Chapter 3

Materials and Methods

Chapter 3

Materials and Methods

Plant material

Plants of pea (*Pisum sativum* L., cv. Arkel) were raised from seeds, procured from Pocha Seeds, Pune, India (Fig. 3.1). The pea seeds were soaked in water overnight, surface sterilized with 0.2% (v/v) sodium hypochlorite solution for 30 mins and then washed for 1 h under running tap water. Big and round seeds were selected and sown in plastic trays filled with soil and farmyard manure (3:1) and were watered twice daily. The plants were grown outdoors under natural photoperiod of approximately 12 h and average daily temperatures of 30 ^{o}C day / 20 ^{o}C night. The second pair of fully unfolded leaves were picked from 8- to 10-day-old plants and used for isolation of mesophyll protoplasts (Padmasree and Raghavendra, 1999a).

Isolation of mesophyll protoplasts

A typical protoplast preparation is shown in Fig. 3. 2. The mesophyll protoplasts were isolated from pea leaves by minor modifications of the procedure of Devi et al. (1992). The lower epidermis of leaves was stripped off with the help of forceps. The midrib was discarded and the leaves were cut into small pieces of approximately 1 cm^2 size. The leaf pieces devoid of abaxial epidermis were floated on plasmolysis medium containing 0.3 M sorbitol, 10 mM MES-KOH pH 6.0, and 1mM $CaCl_2$ for 30 mins. The preplasmolysis medium was carefully removed and the leaf strips were subjected to enzymatic digestion in a medium containing 0.4 M sorbitol, 10 mM MES pH 5.5, 1 mM $CaCl_2$, 0.25 mM Na_2 EDTA, 2% (w/v) Cellulase Onozuka R-10, 0.2% (w/v) Macerozyme R-10 (Yakult Honsha Co. Ltd, Nishinomiya, Japan),

Figure 3.1. A view of 8 to10 day-old-plants of pea (*Pisum sativum* cv. Arkel) grown in the field (outdoors).

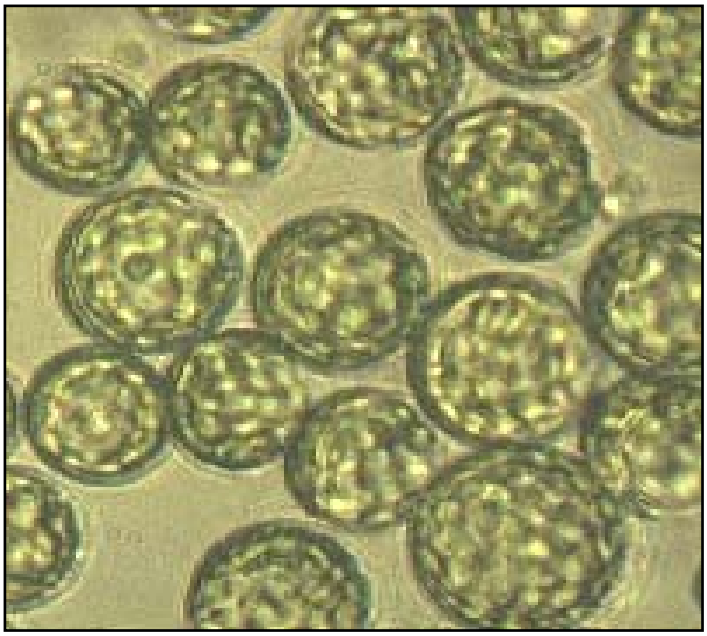

Figure 3.2. Photomicrograph of mesophyll protoplasts isolated from leaves of pea (*Pisum sativum*) suspended in reaction medium containing 0.4 M sorbitol (iso-osmoticum).

10 mM sodium ascorbate and 0.25% BSA under low light intensities of 50-100 μE m^{-2} s^{-1} at 30 ^{o}C (Saradadevi and Raghavendra, 1992). After 30 min, the digestion medium was gently removed with a pasture pipette and washing medium containing 0.4 M sorbitol, 10 mM MES pH 6.0, 1 mM $CaCl_2$ was added to the petridish containing the digested leaf pieces. The petridish was tapped and swirled gently to release the protoplasts into the medium. All further operations were done at 4 ^{o}C. The suspension was filtered through a nylon filter of 60 μm pore size to remove the debris and the filtrate was centrifuged at 500 rpm for 5 mins. The supernatant was discarded and the pellet was washed thrice using washing medium and centrifuging at the same speed for 3 min to remove the broken protoplasts. Finally, the mesophyll protoplasts were washed with suspension medium containing 0.4 M sorbitol, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, in 10 mM HEPES-KOH pH 7.0, and left on ice in a small aliquot of the suspension medium for further use.

Purity/Intactness of protoplasts

The viability and intactness of protoplasts was checked with neutral red and Evans blue. The purity of protoplast preparation normally ranged from 90-97%.

Estimation of chlorophyll

Chlorophyll was estimated in mesophyll protoplasts by extracting into 80% (v/v) acetone (Arnon, 1949). An aliquot of 12.5 μl of protoplast suspension was added to 5 ml of 80 % (v/v) acetone and mixed well using a cyclo-mixer. The absorbance of acetone extract was measured at 652_{nm} (A_{652} - to determine chlorophyll) and 710_{nm} (A_{710} - to correct for turbidity), using a spectrophotometer (Shimadzu UV-1700). The

chlorophyll concentration in the protoplast preparation was calculated using the following formula:

Chl (mg ml^{-1} of protoplast suspension) = (A_{652} - A_{710}) x 11.11

Stress treatment to protoplasts

The isolated mesophyll protoplasts of pea were subjected to high light, hypo-/ hyper-osmoticum, sub-/supra-optimal temperature stress as follows.

Light stress

The mesophyll protoplasts equivalent to 10 μg Chl ml^{-1} in 1 ml of reaction media (0.4 M sorbitol, 1mM $CaCl_2$, 1mM $MgCl_2$, 10 mM HEPES-KOH buffer, pH 7.5 were exposed to different light intensities, up to 3000 μE m^{-2} s^{-1}, for 10 min in a perplex chamber at 25 ^oC (Fig. 3.3). Bicarbonate was not added to the reaction medium in the perplex chamber. Different light intensities were provided by simultaneously operating one, two (or) three tungsten bulbs (Philips, Comptalux, 225 V/75 W), arranged around the thermo-jacketed pre-incubation chamber, as described in Saradadevi and Raghavendra (1992). Light from these bulbs was focused, by passing it through water filled round bottomed flasks, which acts as focusing lens. These water filters arranged between the illuminating bulbs and pre-incubation chamber also help to dissipate the heat generated through halogen bulbs. Flow of air with the help of fan between the lamps and incubation chamber ensured that the chamber was not heated up by the lamps. During incubation, the protoplast suspension was stirred gently. Mesophyll protoplasts pre-incubated in darkness at 25 ^oC were used as control samples. When required an appropriate volume of mitochondrial inhibitors: Antimycin A (0.1 μM) or salicylhydroxamic acid (SHAM, 0.5 mM) were included in protoplasts

suspension during incubation. The stocks of antimycin A and SHAM were prepared in absolute ethanol.

Osmotic Stress Treatment

Mesophyll protoplasts were subjected to osmotic stress by varying the concentration of sorbitol in the reaction media in a step wise from 0.4 M to 0.1 M (hypo-osmotic stress) or from 0.4 M to 1.0 M (hyper-osmotic stress) respectively, for 10 min at 25 ^{o}C (Saradadevi and Raghavendra, 1994). To evaluate the immediate effect of osmotic stress on protoplasts equivalent to 10 μg Chl, the concentration of sorbitol alone was increased in the reaction medium, while other components in the reaction medium were same. Protoplasts preincubated under darkness at 25 ^{o}C and 0.4 M sorbitol were treated as controls.

Temperature Stress Treatment

Aliquots of 10 μg Chl ml^{-1} of the protoplast suspension in 1 ml of reaction media (0.4 M sorbitol, 1mM $CaCl_2$, 1mM $MgCl_2$, 10 mM HEPES-KOH buffer pH 7.5) were subjected to different temperatures by decreasing or increasing the temperature of the medium (H_2O) around the thermo-jacketed pre-incubation chamber from 25 ^{o}C to 10 ^{o}C (sub-optimal temperature) or from 25 ^{o}C to 50 ^{o}C (supra-optimal temperature stress) using a refrigerated circulatory water bath (Julabo F10) for 10 min (Fig. 3.3). Protoplasts preincubated under darkness at 25 ^{o}C and 0.4 M sorbitol were treated as controls.

Treatment of mesophyll protoplasts with mitochondrial electron transport inhibitors

Mitochondrial electron transport inhibitors, antimycin A and SHAM (procured from Sigma Aldrich, U.S.A) were added to the reaction medium in the preincubation chamber after light, osmotic or temperature stress treatments, to give the required final concentrations and subsequently illuminated (1000 μE m^{-2} s^{-1}) for 10 min. These samples were further used for metabolite analysis and enzyme assays.

Monitoring Photosynthesis and Respiration

The photosynthetic O_2 evolution and respiratory O_2 uptake rates of the mesophyll protoplasts subjected to various stresses: light, osmoticum or temperature (with and without mitochondrial inhibitors) were monitored polarographically using a Clark type oxygen electrode system, controlled by Hansa-Tech software at 25 ^oC (Fig. 3.3). While the Calvin cycle activity of the mesophyll protoplasts was measured as rate of bicarbonate dependent O_2 evolution, PS II activities of the mesophyll protoplasts was measured as the rate of *p*-benzoquinone dependent O_2 evolution. After various stress treatments, the mesophyll protoplasts equivalent to 10 μg Chl were transferred from pre-incubation chamber to oxy-graph chamber containing (0.4 M sorbitol, 1mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES-KOH buffer pH 7.5). An illumination of 1000 – 1200 μE m^{-2} s^{-1} was provided by a 35 mm slide projector (with xenophot [halogen] lamp, 24 V/150 W) to monitor $NaHCO_3$ / *p*-BQ-dependent photosynthetic O_2 evolution (Fig. 3.3). While 1.0 mM $NaHCO_3$ was added to the reaction medium before measurement of respiratory O_2 uptake, 1.0 mM *p*-BQ was added to the reaction medium, just before light was switched on after measurement of

dark respiratory O_2 uptake. Oxygen content in the electrode chamber was precalibrated at 25 ^oC with air saturated water using sodium dithionate. A typical recorder trace of respiratory O_2 uptake and photosynthetic O_2 evolution is shown in Fig. 3.4.

Analysis of the engagement of alternative pathway and the extent of cytochrome and alternative pathways

After exposure of mesophyll protoplasts to light, osmotic or temperature stress, the activities of the cyanide sensitive cytochrome oxidase (COX) and cyanide resistant alternative oxidase (AOX) pathways of mitochondrial electron transport were determined by modifying the procedure suggested by Laties (1982). The respiratory O_2 uptake was monitored in the presence of SHAM (to inhibit the AOX pathway) with and without KCN or antimycin A (to inhibit COX pathway) (Fig. 3.5). The relative expressions of these two pathways is calculated by the method described in Vani and Raghavendra (1994) as follows:

$$\% \text{ Cytochrome pathway activity: } \frac{V_{SHAM} - V_{KCN + SHAM}}{V_T} \times 100 \qquad \text{Equation: 1}$$

$$\% \text{ Alternative pathway activity: } \frac{V_T - V_{SHAM}}{V_T} \times 100 \qquad \text{Equation: 2}$$

% Residual respiration: 100 — (% cytochrome pathway activity + % alternative pathway activity) Equation: 3

The engagement of AOX pathway (ρ) was determined by plotting the rates of respiration of mesophyll protoplasts in the presence of SHAM alone against those in the presence of both SHAM and KCN (Fig. 3.6), as described by Lambers (1985).

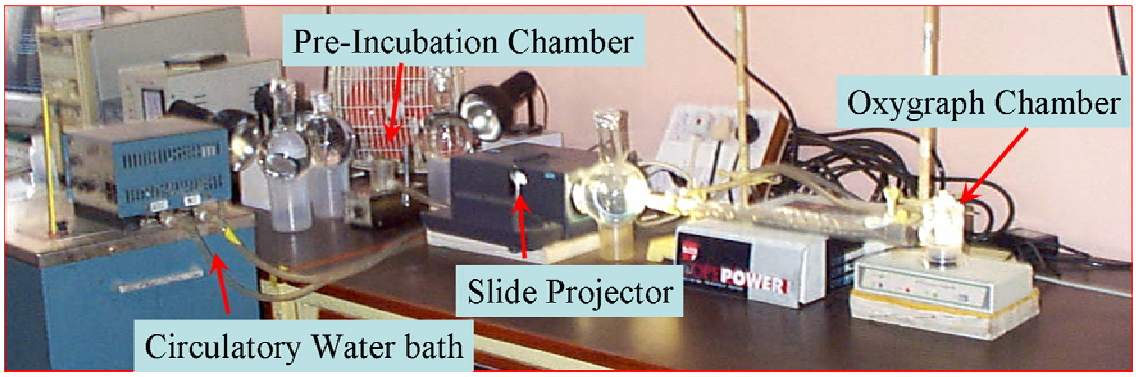

Figure 3.3. Experimental set up to impose light, osmotic or temperature stress to mesophyll protoplasts of pea.

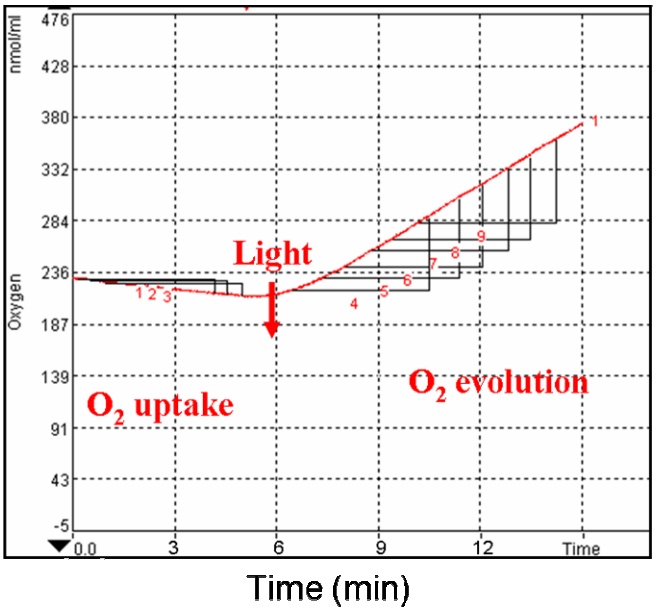

Figure 3.4. A typical recorder trace of respiratory O_2 uptake and photosynthetic O_2 evolution by mesophyll protoplasts of pea at 25 ^{o}C at 0.4 M sorbitol in reaction media.

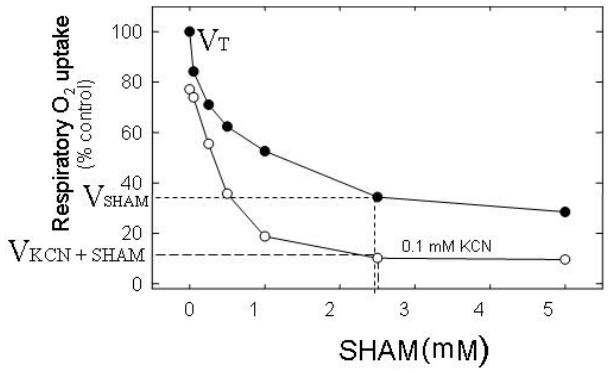

Figure 3.5. Respiratory O_2 uptake in mesophyll protoplasts of pea in the presence of SHAM without KCN (closed circle) and SHAM with KCN (open circle). The contribution of COX and AOX pathways to total respiration are calculated by applying V_T = respiratory activity in the absence of inhibitors, V_{SHAM} = respiratory rate in presence of SHAM and $V_{KCN + SHAM}$ = respiratory rate in the presence of both KCN and SHAM in equations 1 and 2, described elsewhere in materials and methods.

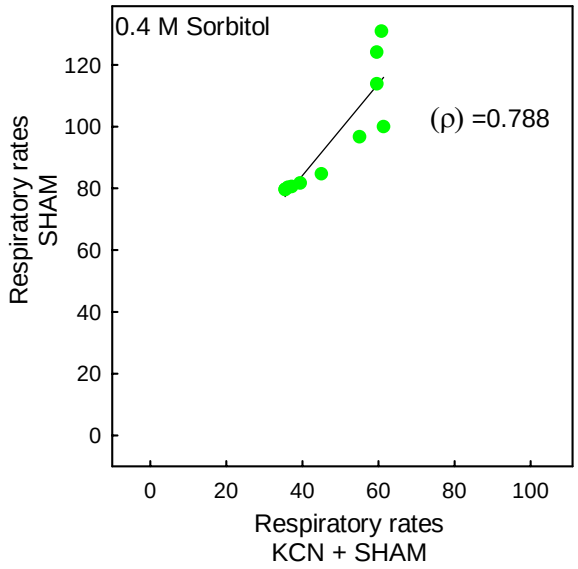

Figure 3.6. Determination of engagement of alternative pathway in mesophyll protoplasts of pea by applying 1 order regression to the respiratory rates (KCN + SHAM) on X-axis against respiratory rates (SHAM) on Y-axis.

Metabolite Analysis

Estimation of ATP, ADP and Pyruvate

Aliquots of protoplast samples containing 100 μg Chl after exposure to light, osmotic or temperature stress treatments under steady state photosynthesis (with or without mitochondrial inhibitors) were withdrawn and 70% $HClO_4$ was added, such that the final concentration of $HClO_4$ would be 3% (v:v). The samples were frozen in liquid nitrogen until used (usually, the next day). When required, the samples were thawed and centrifuged at 7000 *g* for 10 min. The supernatants were neutralized with KOH/ triethanolamine and left on ice for 30 min. The neutralized samples were centrifuged at 7000 *g* and the cleared supernatant was used for estimation of ATP, ADP and pyruvate, by modifying the procedure of Stitt et al. (1989). The levels of ATP were measured using enzymatic assays coupled to NADPH formation, while ADP and pyruvate levels were measured by coupling to NADH utilization, respectively. The reaction medium for the assay of ATP (1 ml) contained 150 mM TEA buffer pH 7.5, 0.5 mM NADP, 10 mM $MgCl_2$, and 100 μl of neutralized sample. The reaction was started by the addition of 0.023 μkat glucose-6-phosphate dehydrogenase (Glc-6-P-DH, E.C. 1.1.1.49) to consume internal Glc-6-P levels. After an equilibration period of 6-8 min after addition of Glc-6-P-DH, ATP levels were monitored by following the net increase in absorbance at 340 nm after the addition of 10 mM glucose and 0.047 μkat hexokinase (HK, E.C.2.7.1.1) (Fig. 3.7A).

The reaction medium (1 ml) for the assay of pyruvate contained 150 mM Tris-HCl pH 8.1, 7.5 mM $MgCl_2$, 0.08 mM NADH, 2 mM PEP, 0.046 μkat lactate dehydrogenase (LDH, E.C. 1.1.1.27) and 100 μl of neutralized sample. After an

equilibration period of 2–3 min after the sample addition, the reaction was started by the addition of LDH. The content of pyruvate was calculated from the net decrease in absorbance at 340 nm after the addition of LDH (Fig. 3.7B).

The reaction medium for the assay of ADP contained 150 mM Tris-HCl pH 8.1, 7.5 mM $MgCl_2$, 0.08 mM NADH, 2 mM PEP, 100 µl of neutralized sample, 0.046 µkat lactate dehydrogenase (LDH, E.C. 1.1.1.27) and 0.067 µkat pyruvate kinase (PK, E.C. 2.7.1.40). After an equilibration of 2–3 min after sample addition the reaction was started by the sequential addition of LDH and PK. The content of ADP was calculated from the net decrease in absorbance at 340 nm after the addition of PK (Fig. 3.7B).

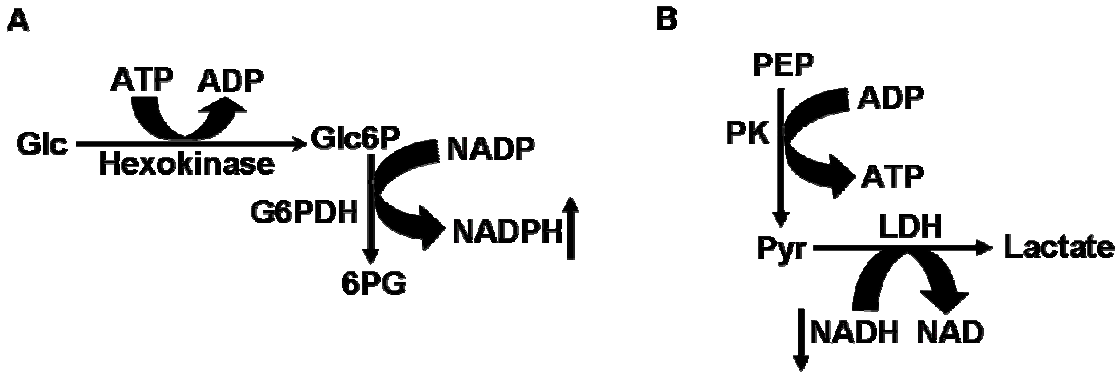

Figure 3.7. A schematic view of ATP (A), ADP and pyruvate (B) estimation in mesophyll protoplasts of pea.

Estimation of malate and oxaloacetate

The neutralized samples after treatment with $HClO_4$ (3% v/v) were used for estimation of malate and oxaloacetate by enzymatic assays coupled to NADH oxidation or NAD[P] reduction at 340 nm using a dual wavelength spectrophotometer (Shimadzu, UV-vis 160A) (Padmasree and Raghavendra 1999c). The cellular level of oxaloacetate was calculated from the equation of [(oxoglutarate) × (aspartate)] /

[(glutamate)× (6.61)], based on the equilibrium of glutamate oxaloacetate transaminase (GOT) (K = 6.61, Veech et al., 1969). The levels of oxoglutarate, aspartate and glutamate were determined according to the method of Bergmeyer (1983).

The levels of oxoglutarate and aspartate were determined by the decrease in absorbance of NADH at 340 nm. The reaction medium for the assay of oxoglutarate contained 50 mM phosphate buffer pH 7.3, 2 mM $MgSO_4$, 0.1 mM NH_4Cl and 0.4 mM NADH. The extract containing oxoglutarate was incubated in the reaction medium for 10 min at 25 ^oC. The reaction was initiated by the addition of 3.5 U glutamate dehydrogenase in $(NH_4)_2SO_4$ suspension. The extract containing aspartate was incubated for 10 min at 25 ^oC in the reaction medium containing 50 mM Tris-HCl pH 8.1, 2.5 mM $MgCl_2$, 0.2 mM oxoglutarate, 0.1 mM NADH and 18 U MDH. The reaction was initiated by the addition of 2 U, GOT (Bergmeyer, 1983).

The level of glutamate was determined by enzymatic assay coupled to NAD reduction at 340 nm (Bergmeyer, 1983). The assay medium (1 ml) contained 100 mM Tris-HCl, 630 mM Hydrazine sulfate, 1mM EDTA (pH 9.0), 0.5 mM ADP and 1.5 mM NAD. The extract was incubated for 10 min in the reaction medium at 25 ^oC. The reaction was initiated by the addition of 24 U glutamate dehydrogenase (solution in glycerine).

For the estimation of malate, the extract was incubated in the reaction medium containing 100 mM Tris-HCl, 630 mM Hydrazine sulfate, 1 mM EDTA, pH 9.0 and 1.5 mM NAD for 10 min at 25 ^oC. The reaction was initiated by the addition of 30 U MDH. The concentration of malate was proportional to the amount of NAD reduced at 340 nm (Heineke et al., 1991).

Estimation of Ascorbate

Ascorbate content was measured according to the method of Foyer et al. (1983). Protoplast samples equivalent to 25 μg of chlorophyll in total volume of 600 μl of reaction media during steady state photosynthesis with or without mitochondrial inhibitors after exposure to light, osmotic or temperature stress were withdrawn and mixed immediately with ice-cold $HClO_4$ (final concentration 0.5 M) and were frozen in liquid nitrogen. The samples were thawed and centrifuged at 4 ^{o}C for 10 min at 10,000 *g* and the supernatant was collected for analysis. The pH of the supernetant was increased approximately to 5.6 by stepwise addition of 1.25 M potassium carbonate (30-40 μl). The supernatant was collected by centrifugation (10,000 *g*, 6 min, 4 ^{o}C) and used for ascorbate estimation.

An aliquot of 100 μl of the extract (supernatant collected above) was added to 900 μl of 0.1 M sodium phosphate buffer pH 5.6. The absorbance (A) was read at 265 nm. Ascorbate oxidase (2.5 U, Roche Applied Science, Mannheim, Germany) was added to the above mixture (and also to the blank) and absorbance at 265 nm (B) was measured. A fresh aliquot containing extract was incubated with 10 mM reduced glutathione in 0.1 M Tricine–KOH buffer pH 8.5 for 15 min at room temperature. The volume was then made upto 1 ml with 0.1 M sodium phosphate buffer pH 5.6 and the absorbance (C) was read at 265 nm.

A standard curve was plotted for pure ascorbic acid (A_{265} vs concentration of ascorbate; standard plot 1). Aliquots of pure ascorbic acid were treated with 10 mM reduced glutathione and absorbance was measured at 265 nm for dehydroascorbic acid (standard plot 2). Ascorbate in protoplasts was measured as the amount of ascorbate

corresponding to an absorbance value of A-B, in the standard plot 1. Total ascorbate was measured as the amount corresponding to an absorbance value of C-B, in the standard plot 2. Dehydroascorbate was calculated as the difference between the total ascorbate and ascorbate in the reduced form.

Estimation of Glutathione

Protoplast samples equivalent to 25 μg of chlorophyll in total volume of 600 μl of reaction media during steady state photosynthesis with or without mitochondrial inhibitors after exposure to light, osmotic or temperature stress were withdrawn and mixed immediately with 7% sulfosalicylic acid and were frozen in liquid nitrogen. The samples were thawed and centrifuged at 8000 rpm for 10 min. 20 μl of 7.5 M triethanolamine was added to the supernatant to neutralize the sample. Total, oxidized, reduced glutathione was determined spectrophotometrically at 412 nm by the cycling method described by Griffith (1980). For total glutathione estimation the assay mixture contained 100 mM phosphate buffer (pH 7.5), 2 mM EDTA, 6.3 mM 5-5-dithiobis-2-nitrobenzoic acid (DTNB), 5 mM NADPH, 1 unit of GR (from yeast, Boehringer Mannheim) and the neutralized extract (100 μl) in a total volume of 2 ml and the change in absorbance at 412 nm was monitored. All values are expressed as GSH (reduced form of glutathione) equivalents, determined from a standard curve. For determination of GSSG (oxidized form of glutathione), 0.01 ml of 2-vinyl pyridine (2 V-P) was added to 0.5 ml of neutralized extract, so as to mask GSH and to allow the determination of GSSG alone. The standard plot for GSSG is also plotted. The solution was stirred for 1 min and incubated for 1 hr at 25 ^{o}C. Neutralized extraction medium serves as a blank. Total glutathione was determined by reference to a standard curve

and reduced glutathione was determined as the difference between the total glutathione and the oxidized form of glutathione.

SDS-PAGE

Reaction medium containing protoplasts equivalent to 10 μg Chl were withdrawn after exposure of protoplasts to stress treatments during steady state photosynthesis (with or without mitochondrial inhibitors) and centrifuged at 1000 rpm for 1 min and the pelleted protoplasts were snap frozen in liquid nitrogen. The frozen pelleted protoplasts were homogenized in 125 mM Tris-HCl (pH 6.8) containing 5% (w/v) SDS and 1mM PMSF. The homogenate was centrifuged at 10,000 *g* for 10 mins and supernatants were collected. To the supernatant, protein estimation was done following the method of Lowry et al. (1951). Polypeptides of the mesophyll protoplast extract were separated by denaturing SDS-PAGE according to Laemmli (1970) using mini gels (8 x 8 cm). The resolving gel was polymerized using 375 mM Tris-HCl buffer pH 8.8, 10% or 12.5% acrylamide, 0.1% SDS (w/v), 0.05% ammonium per sulphate (APS) and N.N.N^{1}.N^{1}.tetramethyl ethylenediamine (TEMED). The stacking gel (2 cm long and 8 cm wide) was made of 125 mM Tris-HCl (pH 6.7), 4% acrylamide, 0.1% SDS (w/v), 0.04% APS and TEMED. The crude extract was mixed with sample buffer containing 250 mM Tris-HCl pH 6.8, 8% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.04% (w/v) bromophenol blue and boiled at 100 ^{o}C for 3 min. The samples were stored at -20 ^{o}C until loading onto the gel, usually the next day. Electrophoresis was performed initially at 60V (until the dye front migrates into the resolving gel) and then at 120 V. Power was supplied through

Amersham Biosciences Electrophoresis Power Supply EPS-601 unit. The gels were visualized by coomassie staining or by silver staining.

Coomassie staining

Coomassie reagent was prepared by dissolving 0.5 gm of coomassie blue in 500 ml of fixative solution (50% methanol + 12.5% acetic acid). After electrophoresis the gel was placed in fixative solution for 1 hr and then the solution is discarded. Freshly prepared coomassie reagent was added on to the gel and the gel was kept under gentle shaking for 2 hr. After this the staining solution was removed and destaining solution (50% methanol + 12.5% acetic acid) was added to the gel and was kept under shaking until the blue colour of the gel disappears and clear bands are visualized. Molecular weight markers (Bangalore Genei, Bangalore) in the range of 14.3 kDa to 97.4 kDa (PMW-M) were added in the gels.

Silver staining

After electrophoresis, the gel was placed in a fixative solution containing 50% (v/v) methanol, 12.5% (v/v) glacial acetic acid and 0.5 ml of commercial formaldehyde [37% (v/v)] per litre, for 1 h. Later, the gel was washed thrice with 50% (v/v) ethanol (3 x 20). The gel was pre-treated with 0.02% (w/v) sodium thiosulfate solution for 1 min and rinsed with water for 1 min (3 x 20 s). The gel was impregnated with a mixture of 0.2% (w/v) silver nitrate and 0.75 ml formaldehyde/litre, for 20 mins. The gel was again washed with water for 1 min (3 x 20 s) and developed with a solution containing 6% (w/v) sodium carbonate and 0.5 ml formaldehyde/litre, until the bands appeared are clear and sharp. The reaction solution was discarded and the gel was washed with water quickly. Immediately the gel was treated with a solution containing

50% (v/v) methanol and 12.5% glacial acetic acid to prevent any further development of colour. The gel was kept in the above solution until scanned or photographed (2-3h). Molecular weight markers (from Bangalore Genei, Bangalore) in the range of 14.3 kDa to 97.4 kDa (PMW-M) were used in the gels.

Western blotting

The levels of D1 and AOX protein were examined using the western blot. Aliquot of mesophyll protoplasts containing 5 μg or 50 μg of total protein were loaded onto each well and electrophoresis was performed on a 12.5% SDS-PAGE for detection of D1 or AOX. The polyvinylidene difluoride (PVDF) membrane was cut equivalent to the size of the gel and placed in methanol for 10 min under shaking. The methanol was discarded and deionised water was added to the membrane and kept for shaking for 6 min (with 3 changes of deionised water). The membrane was transferred to transfer buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine and 20% (v/v) methanol. The gel was placed in transfer buffer until the treatment of membrane was done. The PVDF membrane was carefully laid on the gel without any air bubbles in between the gel and the membrane. The gel and the membrane were sandwiched between two layers of Whatman No. 3 chromatography papers. A single layer of sponge was placed on either sides of the chromatography papers. The entire sandwich was soaked in transfer buffer and placed in the cassette of the western blot unit (Amersham Biosciences, Hoefer miniVE, vertical electrophoresis system) and connected to a power pack (Amersham Biosciences Electrophoresis Power Supply EPS-601). A power supply of 30V was given for 6 hrs at 4 ^{o}C. The proteins from the gel were electrophoretically transferred to PVDF membranes (Towbin et al., 1979). The transfer of proteins was confirmed by Ponceau's staining (0.2% (w/v) Ponceau's stain in 3%

(w/v) trichloroacetic acid). Ponceau's stain was removed by repeated washing with distilled water.

The PVDF membrane was blocked, to saturate the non-specific binding sites, with 5% (w/v) non fat milk powder in Tris-Buffered Saline (TBS) containing 25 mM Tris-HCl pH 7.5 and 150 mM NaCl. The blocking was allowed for 1 hr at room temperature with constant shaking. The blocked membrane was washed for 45 min (3 x 15 min) with TBS-Tween (25 mM Tris-HCl pH 7.5, 150 mM NaCl, and freshly added 0.2% Tween 20). Now the PVDF membrane was treated for 1 h with 1:100 dilution of anti-AOX antibody or 1:2000 dilution of anti-D1 antibody. The PVDF membrane was washed 3 times with TBS-Tween (3 x 15 min) and then treated with goat anti-Rabbit IgG alkaline phosphatase conjugate (1:5000) for 1 h and finally washed with TBS-Tween. All the above treatments were done with the help of shaker. The washed blot was developed with 60 µl of *p*-nitro-blue-tetrazolium chloride (NBT, 50mg/ml stock) and 30 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 mg/ml stock) in 10 ml of 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 0.05 M $MgCl_2$. The protein bands would be developed after 10-20 min.

Detection of reactive oxygen species (ROS)

Intracellular production of ROS was measured by using a fluorescent dye, 2, 7, -dichlorofluorescein diacetate (H_2DCF-DA). This nonpolar compound was converted to membrane – impermeant polar derivative H_2DCF by cellular esterases and was rapidly oxidized to highly fluorescent DCF by intracellular H_2O_2 and other peroxides. Stocks of H_2DCF-DA (1 mM) were made in ethanol and stored in the dark at –80 ^oC. After 30 min pre-incubation in H_2DCF-DA (100 µM) in dark, mesophyll protoplasts were

collected after brief centrifugation step of 1000 rpm and resuspended in suspension medium so as to dilute the concentration of H_2DCF-DA to 5 μM. The mesophyll protoplasts with H_2DCF-DA (5 μM) were subjected to various stress treatments and illuminated for 10 min under saturating light intensities (1000-1200 μE m^{-2} s^{-1}) in the presence and absence of mitochondrial inhibitors. DCF fluorescence was measured by using a Hitachi F- 4010 fluorescence spectrophotometer with excitation and emission wavelengths set at 488 nm and 525 nm, respectively.

Enzyme Assays

Preparation of enzyme extract and assay of enzyme activity

Aliquots (600 μl) of reaction medium containing mesophyll protoplasts equivalent to 100 μg Chl were withdrawn during steady state photosynthesis (in the presence and absence of antimycin A or SHAM) after exposure of protoplasts to their respective stress treatments. The samples were centrifuged at 1000 rpm for 1 min and the pelleted protoplasts were snap frozen in liquid nitrogen. The pelleted protoplasts were homogenized in 50 mM phosphate buffer pH 7.0, with 1mM PMSF. The homogenate was centrifuged at 10,000 *g* for 10 min and the supernatants were collected and used for the enzyme assays of CAT, APX, MDAR and GR. For SOD assay the pelleted protoplasts were homogenized in 50 mM phosphate buffer pH 7.8, with 1mM PMSF. Protein concentrations in the enzyme extract were determined by the method of Lowry et al. (1951) using defatted BSA as a standard.

Superoxide dismutase (SOD, E.C. 1.15.1.1)

SOD activity was determined by the method of Beauchamp and Fridovich (1971). The required cocktail for SOD activity estimation was prepared by mixing

27 ml of sodium phosphate buffer (pH 7.8), 1.5 ml of methionine (300 mg ml^{-1}), 1 ml of NBT (14.4 mg/ 10 ml^{-1}), 0.75 ml of triton-X-100 and 1.5 ml of 2 mM EDTA. To 1 ml of this cocktail, 10 µl of riboflavin (4.4 mg 100 ml^{-1}) and 50 µg of protein (enzyme extract) were added. After mixing, the contents taken in a cuvette were illuminated for 8 min using three comptalux bulbs (100W, Philips India Ltd.). The temperature was maintained at 25 ^{o}C using a water bath. A tube with protein kept in dark served as blank, while the control tube was without enzyme and kept in light. The absorbance was measured at 560 nm. The reduction of NBT under illumination was measured without enzyme and also in the presence of the enzyme. The principle involved is the inhibition of NBT reduction by superoxide ion radicals generated photochemically. Activity of SOD is measured as the difference in NBT reduction without enzyme and with enzyme (enzyme extract). One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light.

Catalase (CAT, E.C. 1.11.1.6)

CAT activity was measured spectrophotometrically by measuring the rate of H_2O_2 disappearance at 240 nm ($\Delta\varepsilon$ = 43.6 mM^{-1} cm^{-1}) according to the method Patterson et al. (1984). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 19 mM H_2O_2 and 25 µg protein in a final volume of 3 ml. The activity was determined by the oxidation of H_2O_2 at 240 nm.

Ascorbate Peroxidase (APX, E.C. 1.11.1.11)

APX was assayed by the method of Nakano and Asada (1981). The reaction mixture for measuring APX activity contained 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 mM H_2O_2 and 50 µg of protein.

The activity of APX was recorded as decrease in absorbance at 290 nm for 1 min and the amount of ascorbate oxidized was calculated ($\Delta\varepsilon$= 2.8 mM^{-1} cm^{-1}).

Monodehydroascorbate reductase (MDAR, E.C. 1.6.5.4)

MDAR activity was assayed by monitoring the change in absorbance at 340 nm due to NADPH oxidation ($\Delta\varepsilon$ = 6.2 mM^{-1} cm^{-1}) for 1 min in a 3 ml reaction mixture containing 0.1 mM NADPH, 2.5 mM ascorbic acid, 50 mM sodium phosphate buffer, pH 6.0 and 50 μg of protein. The reaction was started by the addition of 4 units of ascorbate oxidase (Drazkiewicz et al., 2003).

Glutathione reductase (GR, E.C. 1.6.4.2)

The GR assay was performed according to Jiang and Zhang (2001) with little modifications. The reaction mixture contained 25 mM sodium phosphate buffer pH 7.5, 10 mM GSSG, 3 mM $MgCl_2$, and 1mM NADPH in a total volume of 2 ml. The reaction was started by addition of 50 μg protein and NADPH oxidation was recorded as the decrease in absorbance at 340 nm for 1 min.

Activity staining of SOD, CAT and APX

Native polyacrylamide gel electrophoresis (PAGE) was performed using the Laemmli (1970) buffer systems at 4 ^{o}C for SOD, CAT and APX. The resolving gel was polymerized using 375 mM Tris-HCl buffer pH 8.8, 10% acrylamide and 10% glycerol along with 0.05% APS and TEMED. The stacking gel (2 cm long and 8 cm wide) was made of 125 mM Tris-HCl (pH 6.7), 4% acrylamide, 10% glycerol, 0.04% APS and TEMED. The protein extracts were mixed with sample buffer (250 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, and 0.04% (w/v) bromophenol blue) before

loading onto the gels. In each lane 100 –150 μg of enzyme extract was loaded. The gels were run at constant current of 100V at 4 ^{o}C in Amersham Biosciences Electrophoresis Power Supply EPS-601 unit.

Isozymes of SOD were visualized according to Beauchamp and Fridovich (1971) as modified by Rucinska et al. (1999). After electrophoresis gels were soaked in 2.45 mM NBT for 20min, followed by incubation in a solution containing 50 mM potassium phosphate buffer (pH 7.8), 28 mM TEMED**,** 2.4 μM riboflavin and subsequently illuminated until the SOD bands appear on a dark back ground. Different isoforms of SOD were identified by selective inhibition with H_2O_2 and potassium cyanide following the method of Salin and Bridges (1981). To inhibit Cu/Zn SOD and Fe-SOD, gels were stained in buffer containing 5 mM H_2O_2. Selective inhibition of Cu/Zn SOD was achieved by incubation of the gels in buffer containing 5 mM KCN.

CAT isozymes were visualized in 7.5 % native gel according to Woodbury et al. (1971). After electrophoresis the gel was washed in three changes of double distilled water for 45 mins to remove the buffer from outer surface of the gel. The gel was then incubated in 20 mM H_2O_2 and kept in dark for 10 mins. The gel was then briefly washed with double distilled water and placed in a solution containing 1% ferric chloride and 1% potassium ferricyanide solution until catalase bands became visible.

In-gel APX activity staining was performed according to Lee and Lee (2000). Non-denaturing electrophoresis was performed in a buffer containing 2 mM ascorbate. Subsequent to electrophoresis the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM ascorbate for a total of 30 mins with the equilibration buffer changed every 10 min. The gel was then incubated with 50 mM sodium phosphate

buffer (pH 7.0) containing 4 mM ascorbate and 20 mM H_2O_2 for 20 mins. H_2O_2 was added to the solution just prior to the incubation of the gel, the gel was subsequently washed with sodium phosphate buffer (pH 7.8), 28 mM TEMED, and 2.4 mM NBT with gentle agitation for approximately 10 mins and stopped by brief wash with distilled water.

Replications

The data presented are the average values of results ($\pm$ SE) from three to four experiments conducted on different days.

Chemicals and Materials

Cellulase (Onozuka R-10) and Macerozyme R-10 (pectinase) were procured from Yakult Honsha Co. Ltd., Tokyo, Japan. Antimycin A, SHAM, secondary antibodies, DTNB, H_2DCF-DA, coomassie brilliant blue, NBT, BCIP and the enzymes used for the spectrophotometric assays were procured from Sigma-Aldrich Corporation, USA. Ascorbate oxidase was from Roche Applied Science, Mannheim, Germany. All other chemicals and materials were of analytical grade and were purchased from the following companies: Sisco Research Laboratories, Loba Chemie, Himedia Laboratories, Qualigens, all from Mumbai. Protein molecular weight markers for SDS-PAGE were purchased from Bangalore Genei, Bangalore.

Antibodies for AOX protein were a generous gift from Prof. Dr. Renate Scheibe, University of Osnabruck, Germany. Antibodies for D1 protein were purchased from Agrisera, Sweden.

Chapter 4

Role of ROS and Antioxidant System during the Beneficial Interactions of Mitochondrial Metabolism with Photosynthetic Carbon Assimilation

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Role of ROS and Antioxidant System during the Beneficial Interactions of Mitochondrial Metabolism with Photosynthetic Carbon Assimilation

Introduction

In photosynthetic tissues, mitochondria play an important role in benefiting chloroplastic photosynthesis by backing up carbon assimilation through cytochrome oxidase (COX) and alternative oxidase (AOX) pathways by different mechanisms (Padmasree and Raghavendra, 1999a,b,c; 2001a,b). The two terminal oxidases, COX and AOX associated with these two pathways are involved in reducing molecular oxygen to H_2O. While the energy released during the electron transport through COX pathway is coupled to the synthesis of ATP, the energy released during the electron transport through AOX pathway is liberated as heat. Although, the passage of electrons from ubiquinone to AOX pathway apparently appears to be wasteful in terms of ATP generation, it has a significant function in preventing over-reduction of not only respiratory complexes but also electron transport carriers of chloroplasts (Zhang et al., 2003; Yoshida et al., 2006, 2007; Noguchi and Yoshida, 2008).

The existence and operation of respiration in light was debated until past two decades (Graham, 1980) but in the recent past its importance in benefiting photosynthetic carbon assimilation through metabolic interactions between chloroplasts and mitochondria was revealed in several reviews (Raghavendra et al., 1994; Krömer, 1995; Hoefnagel et al., 1998; Gardeström et al., 2002, Padmasree et al., 2002; Raghavendra and Padmasree, 2003; Noctor et al., 2007; Noguchi and Yoshida, 2008; Nunes-Nesi et al., 2008). Usage of specific metabolic inhibitors or generation of

specific mutants / transgenic plants either anti-sense or over-expressing a specific key component related to respiratory process clearly established the importance of bioenergetic oxidative metabolism over TCA cycle in optimizing photosynthesis (Krömer et al., 1993; Padmasree et al., 1999a; Carrari et al., 2003; Dutilleul et al., 2003a; Fiorani et al., 2005; Nunes-Nesi et al., 2005; Yoshida et al., 2006, 2007). The different components of bioenergetic metabolism of mitochondria, which include the activities of oxidative phosphorylation, rotenone sensitive complex I, rotenone insensitive external and internal NAD(P)H dehydrogenases, antimycin A - sensitive complex of COX pathway, SHAM - sensitive AOX pathway and reactive oxygen species (ROS) activated uncoupling proteins (UCP) were found to be essential for efficient functioning of chloroplastic photosynthesis (Krömer et al., 1988; Igamberdiev et al., 1998; Møller, 2001; Dutilleul et al., 2003a; Sweetlove et al., 2006; Yoshida et al., 2006, 2007; Noguchi and Yoshida, 2008).

Generation of ROS from redox reactions of chloroplasts and mitochondria has been identified as an inevitable process of aerobic metabolism (Møller, 2001; Apel and Hirt, 2004; Noctor, 2006; Møller et al., 2007). Four major types of ROS: singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) are produced in mesophyll protoplasts during active photosynthesis. In chloroplasts, PSII and PSI are the major sites for the production of singlet oxygen and superoxide radicals (Apel and Hirt, 2004). In mitochondria, complex I, ubiquinone and complex III of electron transport chain are the major sites for the generation of superoxide radicals (Møller, 2001; Rhoads et al., 2006; Navrot et al., 2007). In both of these organelles O_2^- radicals are immediately dismutated to H_2O_2, a less toxic form of ROS by superoxide dismutase (SOD). H_2O_2 is also formed as a by-product of photorespiration during

oxidation of glycolate to glyoxylate in peroxisomes (del Rio et al., 2006). Both O_2^- and H_2O_2 participate in a fenton-type reaction with free Cu and Fe ions available in the cell and generate OH^- radicals.

Traditionally, ROS were considered to be toxic by-products of aerobic metabolism, which were disposed of using antioxidants (Asada, 1999; Blokhina et al., 2003, Apel and Hirt, 2004; Noctor et al., 2007; Shao et al., 2008). However, in recent years, it has become apparent that plants actively produce ROS as signaling molecules to control processes such as growth, cell cycle, programmed cell death, abiotic stress responses, pathogen defense and systemic signaling and development (Mittler et al., 2004; Møller et al., 2007). The intensity, duration and localization of different ROS signals are determined by the interplay between the ROS-production and ROS-scavenging pathways of the cell. AOX and uncoupling protein (UCP) of the mitochondrial electron transport were suggested to be proactive enzymes for ROS avoidance in plants. The suggested pathways for ROS scavenging along with catalase (CAT) were: (i) The water-water cycle, (ii) The ascorbate-glutathione (Asc-GSH) cycle and **(**iii) The glutathione peroxidase (GPX) cycle. In all these pathways, SOD acts as the first line of defense converting O_2^- into H_2O_2. H_2O_2 is subsequently detoxified by CAT, ascorbate peroxidase (APX) and GPX. In contrast to CAT, APX and GPX require an ascorbate (Asc) and/or a glutathione (GSH) regenerating cycle, which use electrons from NAD(P)H. The NAD(P)H-dependent pathways of ROS scavenging mechanisms act either by reducing ROS directly or by protecting or regenerating the oxidized proteins (Navrot et al., 2007). Using transmission electron microscopy, the localization of ROS scavenging pathways has been detected in all the following compartments of

the plant cells: chloroplasts (grana and stroma), peroxisomes, mitochondria and cytosol (Mittler et al., 2004).

Direct role of ROS in signal transduction is ensured only if ROS escape destruction by antioxidants or otherwise consumed in a ROS cascade. Thus, the major low molecular weight antioxidants Asc and GSH determine the specificity of the signal. Along with ROS, Asc and GSH also act as signal-transducing molecules that can either signal independently or further transmit ROS signals. The specific interplay between ROS and the Asc-GSH cycle constituents generate compartment (chloroplastic, mitochondrial, cytosolic and peroxisomal) specific changes in both the absolute concentrations of ROS and antioxidant compounds, and thereby changes in redox ratio of Asc (Asc/DHA) and GSH (GSH/GSSG) during *Botrytis cinerea* infection in tomato whole leaves (Kuzniak and Sklodowska, 2005).

The information on the role of redox related metabolites like malate (Mal), oxaloacetate (OAA), triose-P and PGA in the biochemical cross-talk between chloroplasts and mitochondria during active photosynthesis is well established (Padmasree and Raghavendra, 1999c; Baier and Dietz, 2005; Noguchi and Yoshida., 2008). Although, the role of ROS, ascorbate and NO as signals in the biochemical cross talk between these compartments has been suggested, so far no experimental evidence is reported on these aspects of research (Raghavendra and Padmasree, 2003). In this chapter we examined the importance of ROS and a few components of antioxidant system in fine tuning the beneficial interactions between chloroplasts and mitochondria to optimize photosynthetic carbon assimilation.

Results

The effect of mitochondrial inhibitors antimycin A (inhibitor of COX pathway) or SHAM (inhibitor of AOX pathway) on photosynthetic carbon assimilation were examined by monitoring the changes in the steady state rates of photosynthetic oxygen evolution in mesophyll protoplasts of pea at optimal conditions (1.0 mM $NaHCO_3$) CO_2 and saturating light intensity of 1000 μE m^{-2} s^{-1} (Fig. 4.1). The steady state rates (177 $\pm$ 7.7 μmoles mg^{-1} chl h^{-1}) of photosynthetic O_2 evolution attained after a brief lag period of 3 min illumination were stable over a time period of at least 20-30 min. However, so as to avoid errors due to perturbations in the stability of mesophyll protoplasts at room temperature, all treatments in the present study were restricted to a time period of 10 min illumination. Also, very low concentrations of antimycin A (0.1 μM) and SHAM (0.5 mM) were used during most of the experiments to minimize the general perturbation of the metabolic system caused due to their non-specific effects. At the choosen concentrations, both compounds had no effect on the $NaHCO_3$ dependent photosynthetic O_2 evolution rates in chloroplasts (Padmasree and Raghavendra, 1999a), but remarkably decreased the rates of photosynthetic oxygen evolution in mesophyll protoplasts by 51% and 44% respectively (Fig. 4.1; Padmasree and Raghavendra, 2001a).

Any restriction in mitochondrial electron transport is known to cause over-reduction of respiratory complexes and generation of ROS. Further, any over reduction in chloroplastic electron transport carriers also lead to the generation of ROS. Therefore, the changes in the intracellular ROS levels were monitored during steady state photosynthesis using a fluorescent dye 2, 7,- dichlorofluorescein diacetate

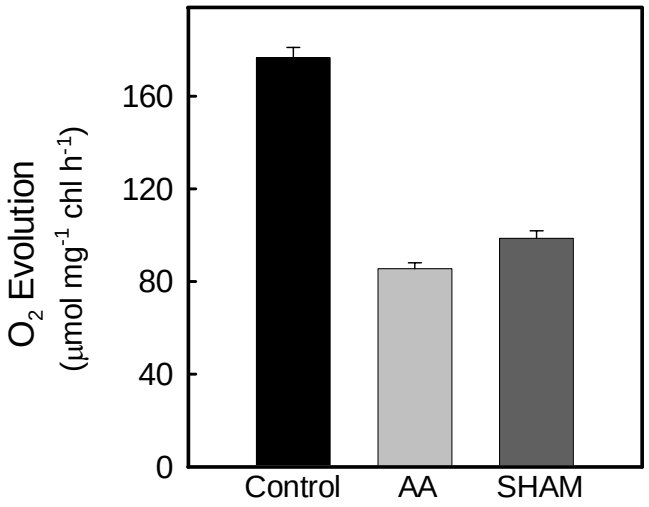

Figure 4.1. Effect of antimycin A (AA, 0.1 μM) or salicylhydroxamic acid (SHAM, 0.5 mM) on photosynthesis at optimal (1.0 mM $NaHCO_3$) CO_2 and saturating (1000 μE m^{-2} sec^{-1}) light intensity in mesophyll protoplasts of pea. Protoplasts were kept in darkness for 5 min before switching on the light. The reaction medium contained the test compounds at the indicated concentrations. The photosynthetic rates were measured at 10 min after illumination.

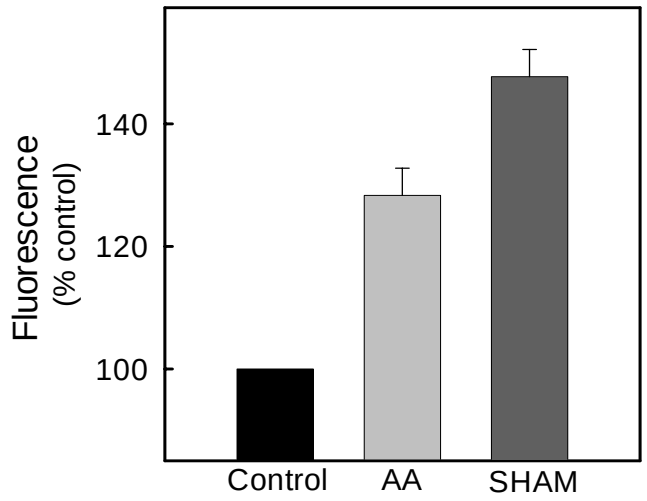

Figure 4.2. Effect of AA (0.1 μM) or SHAM (0.5 mM) on intracellular ROS in pea mesophyll protoplasts that were illuminated for 10 min at optimal (1.0 mM $NaHCO_3$) CO_2 and saturating light intensity (1000 μE m^{-2} sec^{-1}). Steady state ROS levels were assessed using the ROS-sensitive probe H_2DCF-DA as described in materials and methods. The DCF fluorescence in control was 1.6 ± 0.05 units min^{-1}.

(H_2DCF- DA) in the absence or presence of antimycin A and SHAM (Fig.4. 2). In contrast to the effects on photosynthesis, there was a remarkable rise in intracellular ROS as measured by DCF fluorescence when the electron transport through COX pathway or AOX pathway is restricted.

The relative units of DCF fluorescence increased by 28% and 48% over control (without inhibitor) in the presence of antimycin A and SHAM, respectively (Fig. 4.2). The relation between the rise in DCF fluorescence and the inhibition of photosynthesis was assessed in mesophyll protoplasts at a wider range of concentrations of antimycin A (0 to 1.0 μM) and SHAM (0 to 5.0 mM) to ensure that mitochondrial electron transport optimizes photosynthetic carbon assimilation through changes in intracellular ROS (Fig. 4.3, A and B). The DCF fluorescence increased from 1.6 (control, without inhibitor) to 10.08 and 13.47 respectively, while the photosynthesis decreased by 59% and 47% of control rates, respectively, in presence of antimycin A and SHAM (Fig. 4.3, A and B). A strong positive correlation between the rise in DCF fluorescence and inhibition of photosynthesis in presence of both antimycin A and SHAM demonstrates the importance of ROS in mediating or fine-tuning the beneficial interactions of mitochondrial electron transport with photosynthetic carbon assimilation (Fig. 4.3, A and B). Further, the changes in D1 protein, an important component of PS II was examined in presence of antimycin A or SHAM to understand if the decrease in photosynthetic oxygen evolution and increase in cellular ROS cause any damage to D1 protein. Inspite of the rise in intracellular ROS levels and decrease in photosynthetic O_2 evolution, D1 protein levels were very stable even at concentrations as high as 1.0 μM antimycin A or 5.0 mM SHAM (Fig. 4.3, C and D).

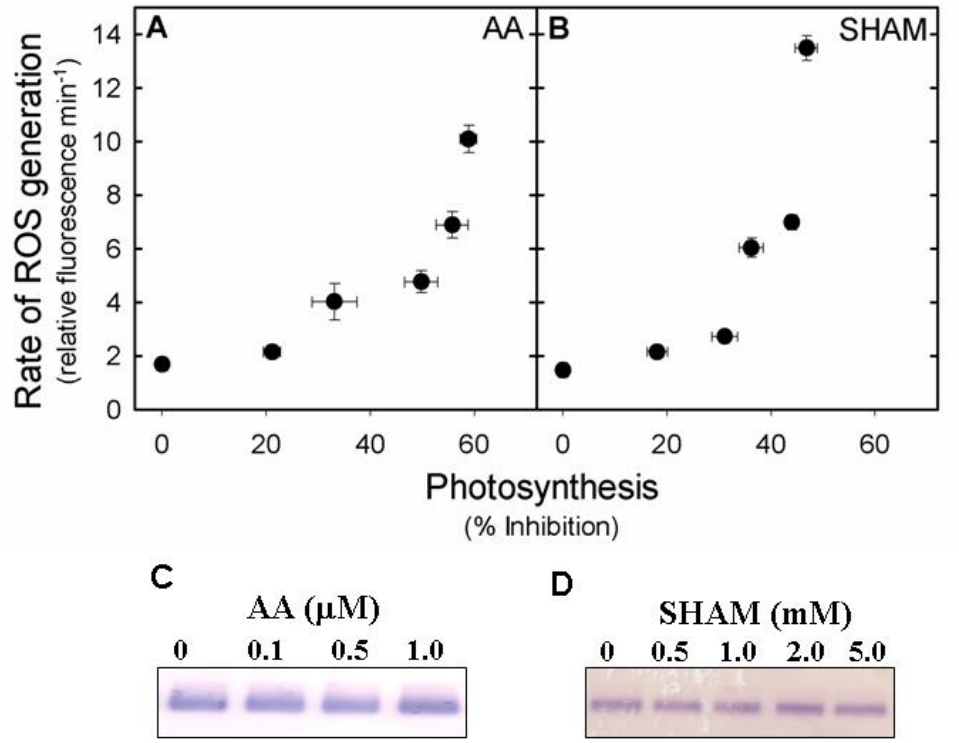

Figure 4.3. Correlation between the relative rates of photosynthesis (as % inhibition of control) and rate of ROS generation in mesophyll protoplasts during incubation with antimycin A (AA) or SHAM at optimal (1.0 mM $NaHCO_3$) CO_2 and saturating light intensity (1000 μE m^{-2} sec^{-1}) for 10 min. These data are obtained with different concentrations of mitochondrial inhibitors (A) 0 - 1 μM of AA (B) 0 - 5 mM of SHAM. Further details are as in Figs. 4.1 and 4.2 and as described in materials and methods. Western blot analysis of D1 protein in mesophyll protoplasts treated with AA (C) or SHAM (D). Mesophyll protoplasts illuminated for 10 min with AA and SHAM as described in (A) and (B) were homogenized in extraction buffer and the proteins (8 μg)were separated on SDS-PAGE. PVDF membranes with the transferred proteins were probed with polyclonal antibodies raised against D1. Representative results of three to four independent experiments are shown. Other details were as mentioned in materials and methods.

Any perturbation in the optimal level of cellular ROS in plant cells is likely to be detected through physiological, biochemical or molecular responses, for e.g., the molecular responses to changes in ROS levels include alteration in expression of nuclear genes encoding enzymes that function to regain cellular homeostasis by producing antioxidant compounds, protective and repair enzymes (Rhoads et al., 2006). However, in the present study the perturbations in cellular ROS are monitored through biochemical responses of the mesophyll cells. The effect of antimycin A or SHAM were examined on the following components of antioxidant system during steady state photosynthesis: (i) SOD, CAT and APX (antioxidant enzymes) (ii) Asc, DHA, GSH and GSSG (metabolites related to Asc-GSH cycle) and (iii) MDAR and GR (Asc-GSH cycle regenerating enzymes).

SOD is impermeable through membrane phospholipids. Therefore it is present in all cellular compartments where O_2^- is generated (Takahashi and Asada, 1983). Based on the metal cofactor used by the enzyme, SOD's are classified into three groups: (i) Fe-SOD, located in chloroplasts, (ii) Mn-SOD, located in mitochondria and (iii) Cu/Zn SOD, located in chloroplasts, peroxisomes and cytosol (Alscher et al., 2002). Activity staining of native PAGE revealed the presence of one isoform related to each of Mn-SOD, Cu/Zn-SOD and Fe-SOD in mesophyll protoplasts of pea (Fig. 4.4). Interestingly treatment of mesophyll protoplasts with antimycin A or SHAM showed remarkable increase in the activity of all the three SOD isoforms related to three groups in native gels during steady state photosynthesis (Fig. 4.5A). Further, the increase in the total enzymatic activities of SOD as detected by spectrophotometric method was more pronounced in presence of SHAM (33% of control rates) when compared with antimycin A (15% of control rates) (Fig. 4.5B).

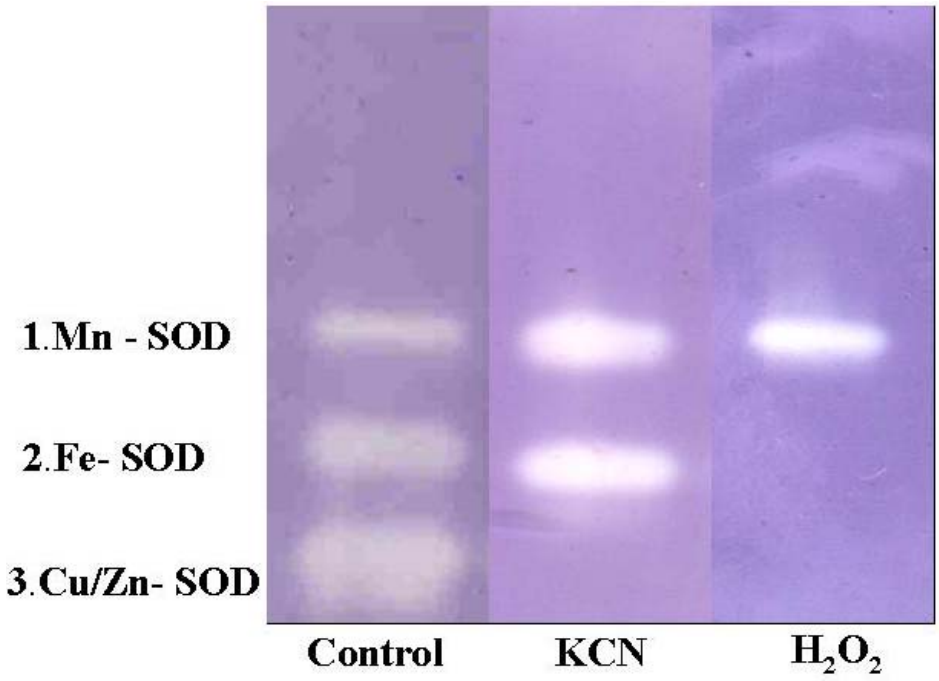

Figure 4.4. Identification of SOD isoforms in the mesophyll protoplasts of pea. Staining for activity was performed without any inhibitor (control), in the presence of 5 mM KCN that inhibits Cu/Zn-SOD, or in the presence of 5 mM H_2O_2, which inhibits both Cu/Zn- and Fe-SOD. Mesophyll protoplasts were illuminated for 10 min at optimal (1.0 mM $NaHCO_3$) CO_2 and saturating light intensity (1000 μE m^{-2} sec^{-1}). Proteins were extracted under non-denaturing conditions and separated on 10 % native PAGE (100 μg protein in each lane).

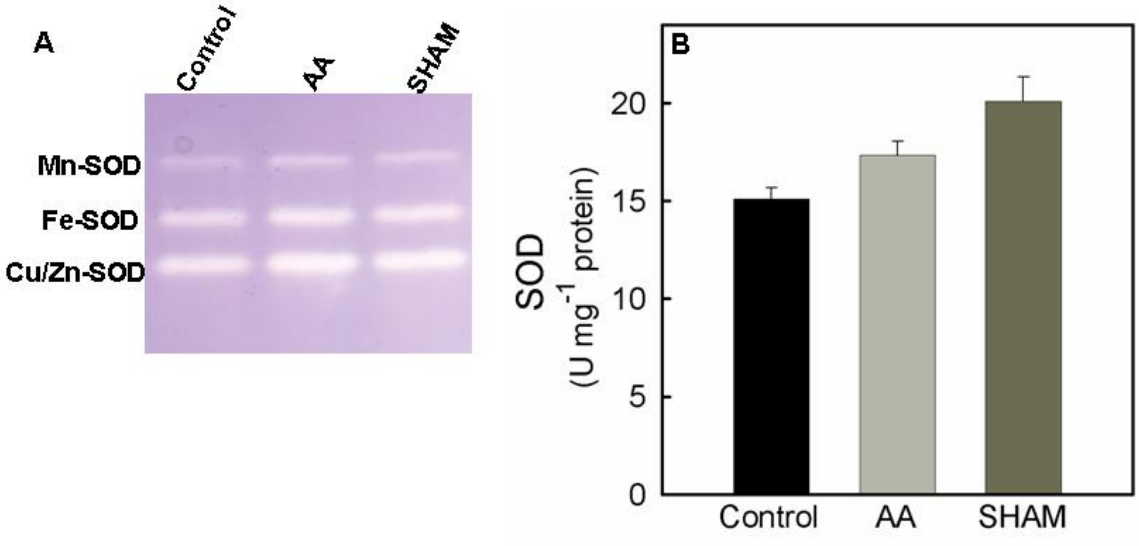

Figure 4.5. Effect of antimycin A (AA) or SHAM on the activity of SOD. (A) Activity staining of SOD as visualized on native PAGE showing changes in different isoforms in mesophyll protoplasts illuminated for 10 min with 0.1 μM AA or 0.5 mM SHAM. Further details are as indicated in Fig. 4.1 and 4.4 and as described in materials and methods. (B) Total cellular activity of SOD in mesophyll protoplasts illuminated for 10 min with AA or SHAM.

The total cellular activities of CAT and APX, which play an important role in scavenging H_2O_2 were shown in Figs. 4.6, A and B, respectively. Restriction of cytochrome pathway had no effect on the activities of either CAT or APX (<7%, increase or decrease in activity) during steady state photosynthesis. Restriction of alternative pathway caused marginal increase in the activity of CAT (17%), but not of APX (9%) which decreased when compared to control rates (Figs. 4.6, A and B). Activity staining for CAT and APX in native PAGE resulted in detection of single isoform for each of these enzymes in mesophyll protoplasts (Fig. 4.6, C and D). The changes in the activity staining of CAT and APX observed in native PAGE correlated with the results demonstrated by spectrophotometric method in presence of both antimycin A or SHAM (Fig. 4.6A-D).

In plant cells, the major soluble small molecular weight antioxidant metabolites, Asc and GSH with their corresponding oxidized forms DHA and GSSG, function as efficient redox couples in maintaining cellular redox homeostasis as well as secondary signals in ROS mediated signal transduction pathways. The cellular Asc redox state is indicated by the Asc/DHA ratio, while the GSH redox state is indicated by the GSH/GSSG ratio. Restriction of COX pathway or AOX pathway caused marked rise in total DHA levels (≥2.0 fold), while the levels of ascorbate (<10%) remained constant (Fig. 4.7, A and B). However the increase in cellular DHA was more pronounced in presence of SHAM when compared with antimycin A. The total ascorbate levels increased by ≥29% in presence of antimycin A or SHAM (Fig. 4.7C). Inspite of the constant levels of Asc, the Asc redox state decreased from 4.6 to 3.4 and 2.4, respectively, in presence of antimycin A and SHAM due to significant rise in cellular DHA levels (Fig. 4.7D). The changes in the metabolites related to the glutathione redox

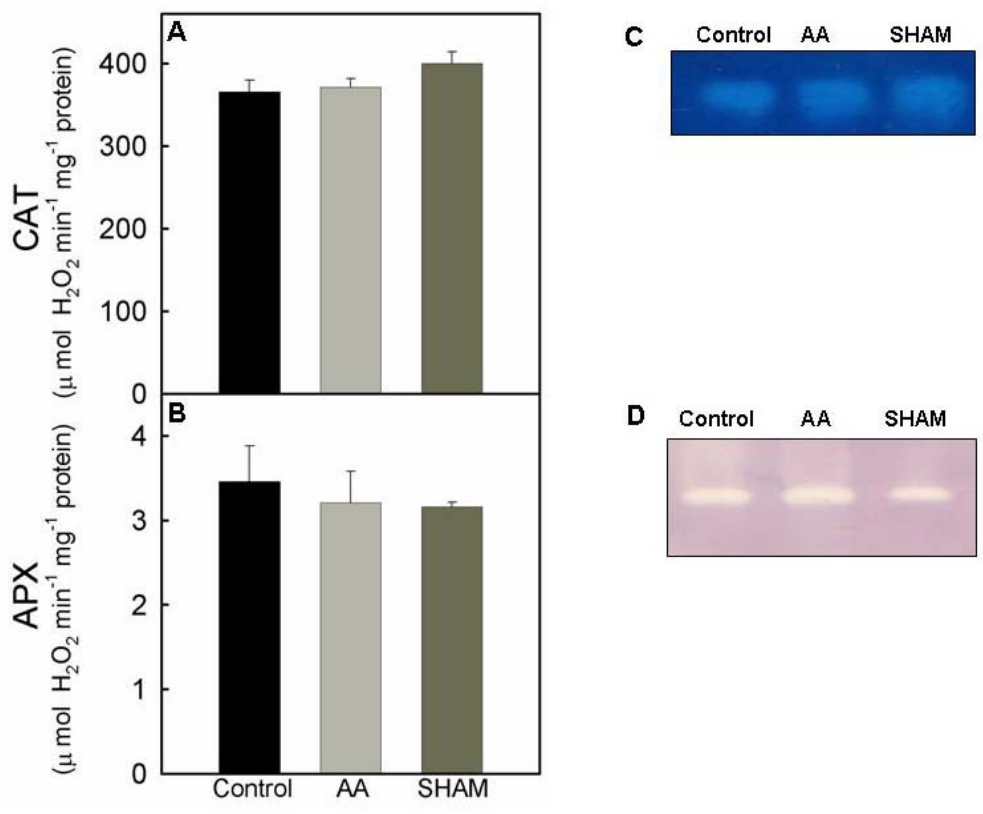

Figure 4.6. Effect of antimycin A (AA) or SHAM on activity of CAT and APX. (A) Total cellular activities of CAT and APX in mesophyll protoplasts illuminated for 10 min with 0.1 μM AA or 0.5 mM SHAM. (B) Activity staining of CAT and APX as visualized on 10 % native PAGE in mesophyll protoplasts treated with AA or SHAM. Further details are as described in Fig. 4.1.

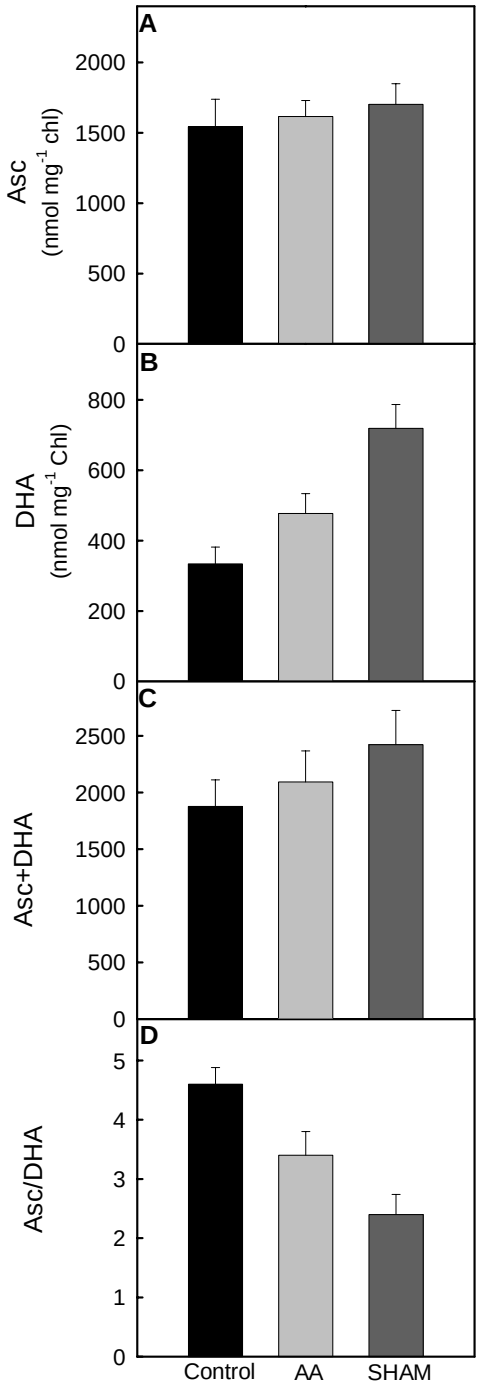

Figure 4.7. Effect of antimycin A (AA, 0.1 μM) and SHAM (0.5 mM) on the total cellular levels of Asc (A), DHA (B), Asc+DHA (C), and Asc/DHA (D), in mesophyll protoplasts that were illuminated for 10 min at optimal (1.0 mM $NaHCO_3$) CO_2 and saturating light intensities (1000 μE m^{-2} sec^{-1}). Further details are as described in Fig. 4.1.

couple, GSH and GSSG levels were ≤20% in presence of both antimycin A and SHAM (Fig. 4.8, A and B). Though the changes in GSH and GSSG were small they are highly significant. The total glutathione levels decreased marginally (≤ 11 %) in presence of both antimycin A or SHAM (Fig. 4.8C). Similar to Asc redox state, the GSH redox state decreased from 2.1 to 1.9, and 1.6 respectively in presence of antimycin A and SHAM (Fig. 4.8D). Restriction of COX pathway or AOX pathway caused remarkable increase in total cellular activities of Asc-GSH regenerating enzymes MDAR and GR (Fig. 4.9). However the increase in MDAR (≤ 24 %) and GR (≤19 %) was more in presence of antimycin A when compared to SHAM (Fig. 4.9, A and B).

Discussion

In chloroplasts, the coordination between different components of photosynthesis such as generation and use of assimilatory power (ATP and NADPH), the induction of photosynthesis, the activation of enzymes and the maintenance of metabolites is required to keep up the photosynthetic carbon assimilation at optimal rates. Mitochondrial oxidative metabolism through COX and AOX pathways benefit photosynthetic carbon assimilation by modulating any of the above components (Padmasree and Raghavendra, 1999a, b, c; 2001a, b; Yoshida et al., 2006, 2007). The interactions between chloroplasts and mitochondria were mediated by metabolites related to redox shuttles (Padmasree and Raghavendra, 2002; Raghavendra and Padmasree, 2003; Nunes-Nesi et al., 2007; Pesaresi et al., 2007; Sweetlove et al., 2007; Noguchi and Yoshida, 2008; Nunes-Nesi et al., 2008). In the present study we examined the importance of ROS and a few components of antioxidant system in fine-

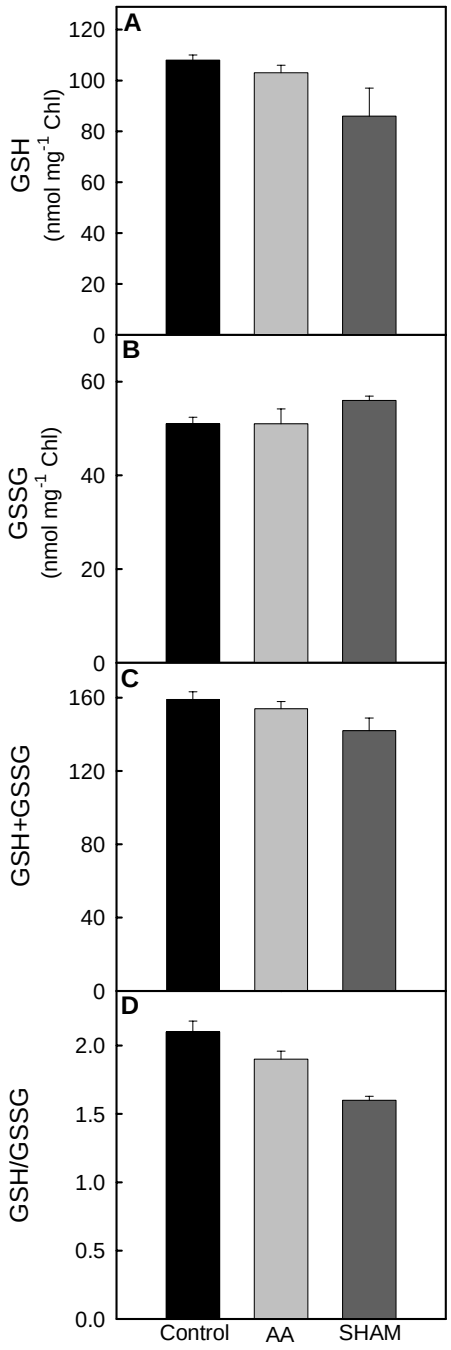

Figure 4.8. Effect of antimycin A (AA, 0.1 μM) and SHAM (0.5 mM) on the total cellular levels of GSH (A), GSSG (B), GSH+GSSG (C), and GSH/GSSG (D) in mesophyll protoplasts that were illuminated for 10 min at optimal (1.0 mM $NaHCO_3$) CO_2 and saturating light intensities (1000 μE m^{-2} sec^{-1}). Further details are as described in Fig. 4.1

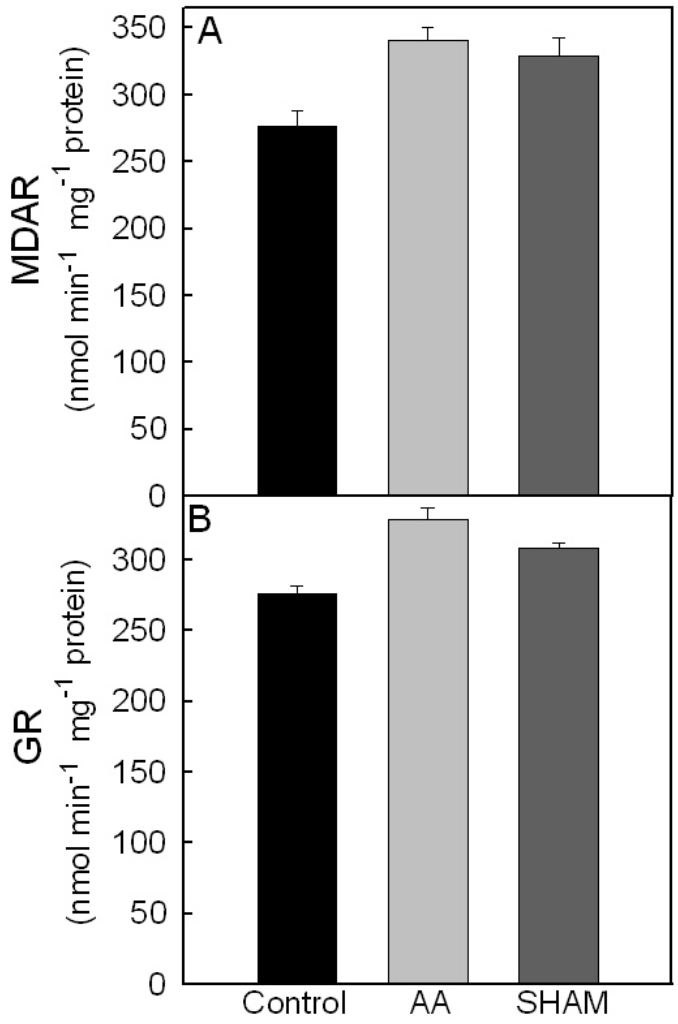

Figure 4.9. Effect of antimycin A (AA, 0.1μM) or SHAM (0.5 mM) on total cellular activities of MDAR (A) and GR (B) in mesophyll protoplasts illuminated for 10 min. Other details are as described in Fig. 4.1.

tuning the beneficial interactions of mitochondrial oxidative metabolism to optimize photosynthetic carbon assimilation.

The effects of mitochondrial inhibitors antimycin A (inhibitor of COX pathway) and SHAM (inhibitor of AOX pathway) on photosynthetic carbon assimilation were examined by monitoring the changes in the steady state rates of photosynthetic oxygen evolution in mesophyll protoplasts of pea under non-photorespiratory conditions (1.0 mM $NaHCO_3$) at a saturating light intensity of 1000 μE m^{-2} s^{-1}. We used very low concentrations of antimycin A (100 nM) and SHAM (0.5 mM) during most of the experiments to minimize the general perturbation of the metabolic system caused due to their non-specific effects. At the choosen concentrations, both compounds had no effect on the $NaHCO_3$ dependent photosynthetic O_2 evolution rates in chloroplasts (Padmasree and Raghavendra, 1999a), but remarkably decreased the rates of photosynthetic oxygen evolution in mesophyll protoplasts (Present study, Padmasree and Raghavendra, 1999a). Any restriction in mitochondrial electron transport is also known to cause over-reduction of respiratory complexes and generation of ROS in mitochondria (Møller, 2001; Foyer and Noctor, 2003; Navrot et al., 2007). Therefore, the changes in the intracellular ROS levels were monitored during steady state photosynthesis using a fluorescent dye H_2DCF-DA in the absence or presence of antimycin A and SHAM (Maxwell et al., 1999; Yip and Vanlerberghe, 2001; Hoffman et al., 2005). The strong positive correlation between DCF fluorescence and inhibition of $NaHCO_3$ dependent O_2 evolution, in presence of both antimycin A and SHAM demonstrated the importance of ROS in fine-tuning the beneficial interactions of mitochondrial electron transport with photosynthetic carbon assimilation.

Any perturbations in the steady-state level of ROS in plant cells are likely to be detected through physiological, biochemical or molecular responses (Foyer and Noctor, 2003; Baier and Dietz, 2005; Foyer and Noctor, 2005; Gechev et al., 2006; Noctor, 2006; Navrot et al., 2007). In the present study the perturbations in cellular ROS caused due to restriction in mitochondrial electron transport under steady state photosynthesis were monitored by examining the biochemical changes in the following components of antioxidant system: (i) antioxidant enzymes, SOD, CAT and APX (ii) metabolites related to Asc-GSH cycle (Asc, DHA, GSH and GSSG) and (iii) Activities of MDHAR and GR, which play a role in regeneration of metabolites related to Asc-GSH cycle (Noctor and Foyer, 1998; Mittler, 2002; Blokhina et al., 2003; Chew et al., 2003; Mittler et al., 2004; Shao et al., 2008). Antimycin A and SHAM on one hand, caused marked increase in the activities of SOD, CAT, MDAR and GR. On the other hand they caused marked decrease in the redox ratios of Asc (indicated by Asc/DHA) and GSH (indicated by GSH/GSSG). Interestingly, both these mitochondrial inhibitors did not cause any change in the activity of APX. However, except in MDAR and GR, the changes in all the components of the antioxidant system investigated in the present study were more pronounced in presence of SHAM when compared to antimycin A.

Taken together, all these results indicate that AOX pathway play a significant role over COX pathway in maintaining optimal levels of cellular ROS, which in-turn play an important role in fine-tuning the biochemical cross-talk between chloroplasts, mitochondria and cytosol directly or indirectly through the operation of redox shuttles associated with Asc-GSH cycle to optimize photosynthetic carbon assimilation. The present study is the first report indicating the importance of ROS and antioxidant

system during interactions between chloroplasts and mitochondria to optimize chloroplastic photosynthesis.

From the previous work done in our laboratory it is well known that both COX and AOX pathways play distinct roles to keep the chloroplastic carbon assimilation at maximal rates. While COX pathway play a significant role in supplying ATP for cytosolic sucrose synthesis, AOX pathway play a significant role in relieving the excess redox pressure on chloroplastic electron transport carriers created due to imbalances between the generation and utilization of reducing equivalents (Fig. 1.2; Padmasree and Raghavendra, 1999c, Yoshida et al., 2006). As energy in the form of NADPH cannot be directly transferred from chloroplasts, it is exported to cytosol as malate and triose-P in counter exchange with OAA and PGA through dicarboxylate translocator and Pi-translocator. The reducing equivalents carried through these redox shuttles are further oxidized in mitochondria through COX and AOX pathways (Padmasree and Raghavendra, 2003: Noguchi and Yoshida, 2008). The rise in the total cellular levels of malate and triose-P with concomitant decrease in OAA and PGA in presence of antimycin A and SHAM during steady state photosynthesis demonstrated the significant role of these metabolites in biochemical cross talk between chloroplasts and mitochondria to maintain cellular redox homeostasis (Padmasree and Raghavendra, 1999c).

The results from the present study and past work from our laboratory demonstrates that any perturbation in the operation of the mitochondrial oxidative electron transport leads to disturbances in the sustenence of photosynthesis. How does the mitochondria warn chloroplasts about the perturbations generated in its electron transport system to optimize photosynthetic carbon assimilation? One plausible

hypothesis that helps to explain such situation is any over-reduction in COX pathway or AOX pathway creates back pressure on chloroplastic redox carriers and ROS are generated in both chloroplasts and mitochondria. These ROS released from both mitochondrial electron transport chain as well as chloroplastic electron transport chain diffuse directly to different cellular compartments including cytosol, peroxisomes and nucleus and relay the information of over-reduction of mitochondrial and chloroplastic electron transport chain or indirectly relays this information through exchange of metabolite related to Asc-GSH cycle. Of all the ROS generated, H_2O_2 has more half-life and known to diffuse from its site of production to distant sites through special aquaporins. While doing so it may also alert the different components of Asc-GSH present in other cellular compartments. The Asc-GSH cycle while scavenging ROS, it also utilizes the reducing equivalents. The function of Asc-GSH cycle not only reduces the ROS levels but also keeps the different redox carriers (GSH or NADPH or thioredoxins etc) or whole cell redox levels in the oxidized state. Thus increase in ROS and corresponding changes in the intracellular redox levels of Asc/DHA and GSH/GSSG may in turn help to sustain photosynthetic carbon assimilation.

Although it is well known that biosynthesis of Asc occurs in mitochondria and GSH occurs partially in chloroplasts and partially in cytosol, the information on the occurrence of specific transporters for exchange of these antioxidant molecules across different compartments is not available. However, this type of shuttling of antioxidant molecules across the cellular compartments may help to bring the cellular redox homeostasis much faster (probably in few seconds) than through gene transcription / expression (which may take from several minutes to hours). For eg., shuttling of GSH or GSSG helps in glutathionylation of redox sensitive proteins which in turn lead to

temporary inactivation of proteins. Some of the light activated Calvin cycle enzymes are often subjected to such type of regulation. We observed marked reduction in light activation of chloroplastic enzymes NADP-GAPDH, PRK and FBPase when mesophyll protoplasts are illuminated in presence of antimycin A or SHAM (Padmasree and Raghavendra, 2001b). Such inactivation of these chloroplastic enzymes in presence of antimycin A or SHAM could be due to gluatathionylation. On the other hand AOX itself is known to be regulated by glutaredoxin.

A model was shown indicating the possible biochemical mechanism by which ROS and antioxidant system present in different compartments of the photosynthetically active mesophyll cells function in coordination with other cellular redox systems, malate/OAA and triose-P/PGA to mediate beneficial interactions between chloroplasts and mitochondria to keep up optimal rates of photosynthetic carbon assimilation (Fig.4. 9). Figure 4.10 indicates the modulation in the cellular antioxidant system in response to treatment with antimycin A and SHAM.

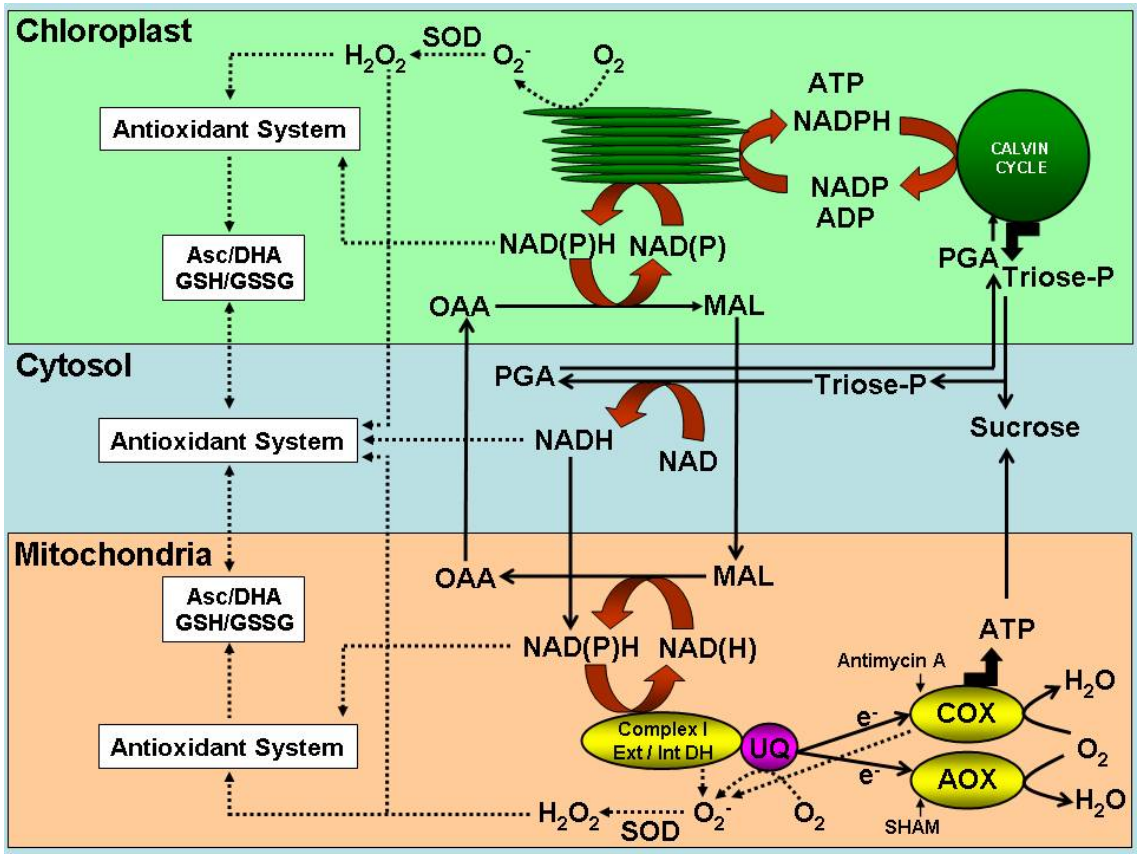

Figure 4.9. Biochemical cross talk between chloroplasts, mitochondria and cytosol mediated by metabolites. The excess reducing equivalents generated in chloroplasts are transported to mitochondria through Mal-OAA shuttle and Triose-P-PGA shuttle. Mal is either oxidized by complex I or Ext/Int DH while triose-P is utilized in sucrose synthesis. Mitochondria supply ATP for sucrose biosynthesis in cytosol. Any over-reduction in COX pathway or AOX pathway generates back pressure on chloroplastic redox carriers leading to generation of ROS in both chloroplasts and mitochondria. These ROS either diffuses directly from one cellular compartment to other or alternatively scavenged by the compartment specific antioxidant system (refer to Fig. 5.10). The antioxidant system operated in different compartments may cross talk through exchange of antioxidant metabolites [Asc/DHA] & [GSH/GSSG] related to Asc-GSH cycle. Solid lines indicate the known concept, where as the dashed lines indicate the proposed concept based on our results. Abbreviations: SOD, superoxide dismutase; MAL, malate; OAA, oxaloacetate; COX, cytochrome oxidase; AOX, alternative oxidase; SHAM, salicylhydroxamic acid; Asc/DHA, ascorbate/dehydroascorbate; GSH/GSSG, reduced glutathione/oxidized glutathione; UQ, ubiquinone; PGA, 3-phosphoglyceric acid or 3-phosphoglycerate.

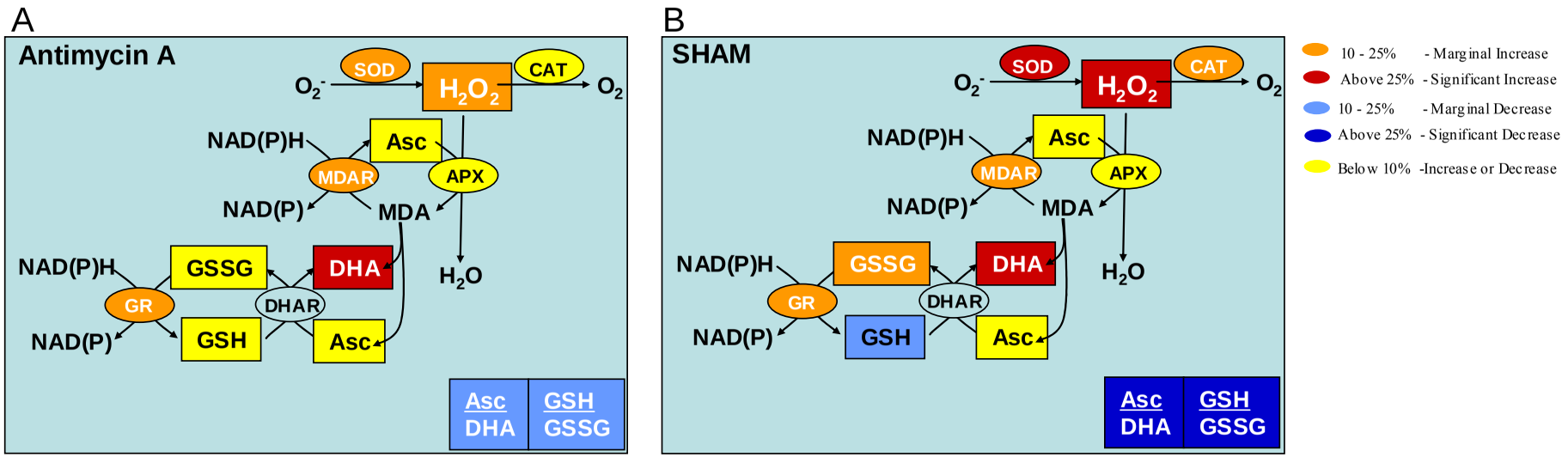

Figure 4.10. Antioxidant system represented in the Fig. 4.9 includes the antioxidant molecules and enzymes examined in the present study. Restriction of COX pathway (Fig. 4.10A) with antimycin A caused (i) marginal increase in ROS; (ii) remarkable increase in DHA; (iii) marginal decrease in Asc/DHA & GSH/GSSG & (iv) marginal increase in SOD, MDAR and GR. Restriction of AOX pathway (Fig. 4.10B) caused (i) significant increase in ROS; (ii) significant increase in DHA and marginal increase in GSSG and decrease in GSH; (iii) Significant decrease in Asc/DHA & GSH/GSSG & (iv) significant increase in SOD and marginal increase in CAT, MDAR and GR.

Chapter 5

Importance of Mitochondrial Oxidative Electron Transport in Optimizing Photosynthesis under Light, Osmotic or Temperature Stress

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Introduction

Redox, reactive oxygen species (ROS) and antioxidant levels are balanced by plant cells, including within mitochondria (Foyer and Noctor, 2003, 2005; Gechev et al., 2006; Noctor, 2006; Navrot et al., 2007). We observed using mesophyll protoplasts of pea that mitochondria optimize photosynthetic carbon assimilation by modulating all these parameters during steady state photosynthesis at saturating light intensities (Chapter 4; Padmasree and Raghavendra, 1999c). Plants, being immobile are known to adjust and acclimatize to the changing environmental conditions by altering one or all of these parameters and thereby maintain cellular redox homeostasis (Baier and Dietz, 2005; Scheibe et al., 2005; Noctor et al., 2006). It is intriguing to understand that the same parameters, redox status, ROS and antioxidant levels which were found to be crucial in mediating and/or fine-tuning the beneficial interactions between chloroplasts and mitochondria are also important in determining the capacity of plants to acclimatize and survive under frequently changing environmental conditions. However, we do not know whether mitochondria optimize chloroplastic photosynthesis under abiotic stress conditions as they do under saturating / sub-saturating light intensities and optimal / limiting CO_2 (Krömer and Heldt, 1991a, b; Padmasree and Raghavendra, 1999a; Yoshida et al., 2006).

1. If mitochondria do play a role, which component of the mitochondrial electron transport, cytochrome oxidase (COX) or alternative oxidase (AOX) pathway is important in optimizing photosynthesis?

2. What is the biochemical basis for such beneficial interactions between chloroplasts and mitochondria under stress conditions?

3. And, lastly does the priority of biochemical factors mediating the interactions between chloroplasts and mitochondria vary with abiotic stress to which plants are exposed?

The work done in the present chapter using mesophyll protoplasts of pea was aimed to address these questions under the following abiotic stress conditions: high light intensity (HL), hypo-osmotic stress (0.3 M, sorbitol), hyper-osmotic stress (1.0 M, sorbitol), sub- (10 ^{o}C) or supra-optimal (40 ^{o}C) temperatures.

The metabolism of a leaf cell is distributed between various compartments, e.g. chloroplast, mitochondrion, cytosol and peroxisome. Chloroplasts and mitochondria are the powerhouses of photosynthetic cells. As one of the main bioenergetic centres of the cell, the midochondrion is uniquely sensitive to ATP demands across the cell. For e.g., COX pathway of mitochondria back-up photosynthetic carbon assimilation by meeting the cytosolic demands for ATP during sucrose synthesis (Padmasree and Raghavendra, 1999c). The chloroplasts tend to produce lot of reduced compounds during dark reactions of photosynthesis utilizing the reducing equivalents generated through photochemical reactions. Both peroxisomes and mitochondria oxidize these reduced compounds during photorespiration and dark respiration, respectively. Peroxisomes require the cooperation of mitochondria in oxidizing glycine, an intermediate formed during photorespiration. The metabolic processes in these various compartments are coordinated through the exchange of metabolites. Cytosol provides a physical and metabolic link between these organelles (Padmasree et al., 2002).

The oxidation-reduction cascades of both the photosynthetic and respiratory electron transport chains not only provide the driving forces for metabolism in chloroplasts and mitochondria, but also generate redox signals which participate and regulate every aspect of plant biology from gene expression and translation to enzyme chemistry (Foyer and Noctor, 2003, 2005; Noctor, 2006). As the photosynthetic electron transport drives reductive metabolism, basically any metabolite synthesized in chloroplasts, depending on the availability of reducing energy, could act as a redox signal for maintaining cellular redox homeostasis. For example putative signaling metabolites like carbohydrates (sugar sensing) which are synthesized during photosynthesis are oxidized in mitochondria by respiration depending on the cellular energy status (Pasaresi et al., 2007). During light-enhanced dark respiration, malate is the preferred redox transmitter (Padmasree et al., 2002). Further the bioenergetic functions of chloroplasts and mitochondrion also result in the production of ROS, potent intracellular signaling molecules. Thus ROS provide a potential mechanism to link bioenergetic output to a signal transduction cascade ultimately leading to specific downstream responses (Neill et al., 2002; Mittler et al., 2004).

The production of ROS by mitochondria was suggested as the critical factor for the induction of AOX (Clifton et al., 2006, Rhoads et al., 2006). Accumulation of ROS is also a common feature of several types of abiotic stresses which ultimately leads to oxidative stress (Mittler, 2002; Mittler et al., 2004). While the amounts and activities of enzymes involved in ROS scavenging are known to be altered by environmental stresses such as chilling, drought and high salinity (Shao et al., 2008), reductive detoxification of ROS occurs through the cellular ascorbate and glutathione pools (Smirnoff, 2000; Blokhina et al., 2003; Noctor, 2006).

In the present work, we investigated the changes in the pattern of metabolites related to cellular energy and redox status along with changes in the ROS, antioxidant metabolites and antioxidant enzymes in the presence of antimycin A and SHAM to identify their relative importance in mediating the beneficial interactions between chloroplasts and mitochondria to optimize photosynthesis during light, osmotic or temperature stress.

Results

We examined the effects of anitmycin A (inhibitor of COX pathway) and SHAM (inhibitor of AOX pathway) on three different components of photosynthesis under light, osmotic or temperature stress: (i) Calvin cycle activity (measured as $NaHCO_3$ dependent O_2 evolution (ii) PSII activity (measured as BQ dependent O_2 evolution) and (iii) changes in D1 protein. The rates of $NaHCO_3$ dependent O_2 evolution (177 ± 7.7 μmoles mg^{-1} Chl h^{-1}) were maximum at light intensities between 500 – 1000 μE m^{-2} s^{-1}, 0.4 M sorbitol and 25 ^oC temperature (Fig. 5.1A-C). Pre-incubation of mesophyll protoplasts in step-wise with high light intensities (from 1000 to 3000 μE m^{-2} sec^{-1}), hypo-osmotic stress (from 0.4 M to 0.1 M sorbitol), hyper-osmotic stress (from 0.4 M to 1.0 M sorbitol), sub-optimal temperatures (from 25 ^oC to 10 ^oC) or supra-optimal temperatures (from 25 ^oC to 50 ^oC) caused remarkable decrease in the rates of $NaHCO_3$ dependent O_2 evolution (Fig. 5.1A-C). As shown in Fig. 5.1 (A-C), the observed decrease (40-67% of controls) in the rates of $NaHCO_3$ dependent O_2 evolution caused by the independent abiotic stress treatments were aggravated when the stress treatments of mesophyll protoplasts were over-lapped by treatments with antimycin A (67-79% of controls) or SHAM (60-75% of controls). The effects of abiotic stress treatments were also examined on the respiratory O_2 uptake in the

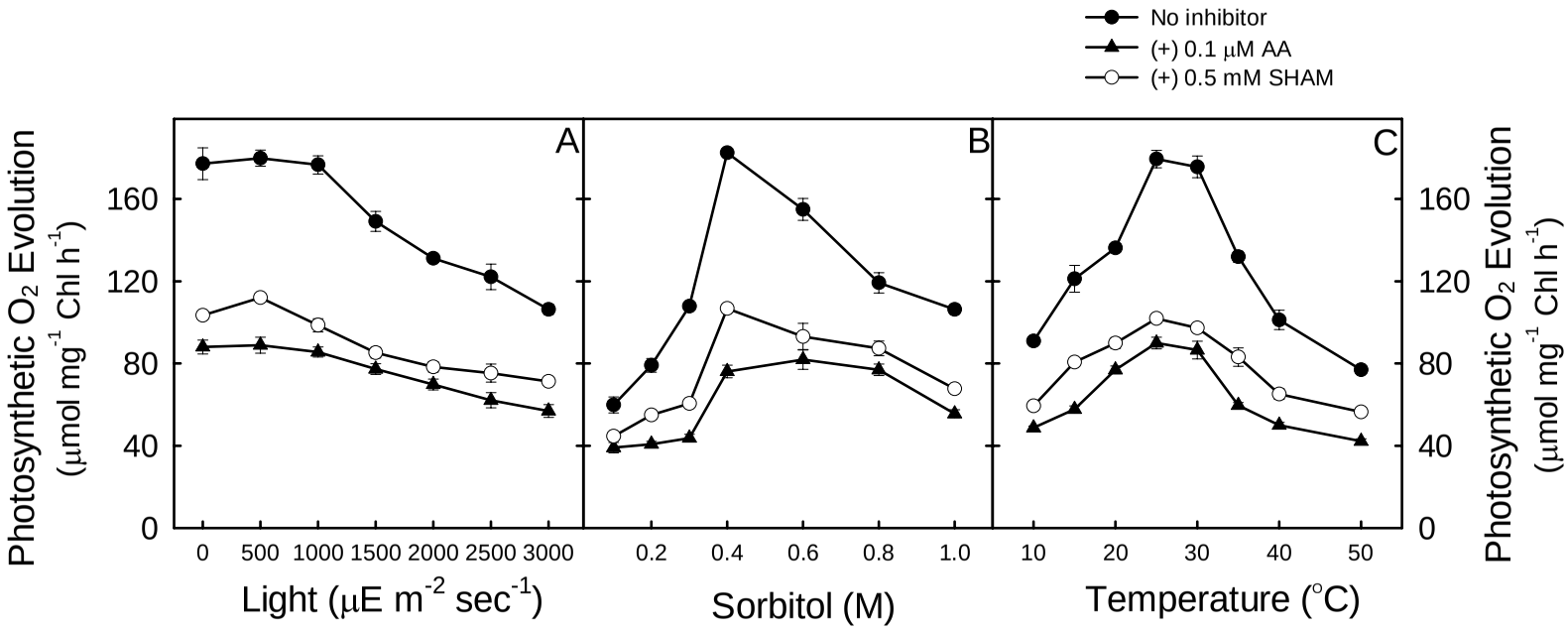

Figure 5.1. Effect of antimycin A (AA, ▲) or SHAM (○) on photosynthesis during light (A), osmotic (B) or temperature (C) stress in mesophyll protoplasts of pea. The rates of photosynthetic O_2 evolution were measured at optimal (1.0 mM, $NaHCO_3$) CO_2 and saturating light intensity (1000 μE m^{-2} s^{-1}) in the presence of AA (0.1 μM) and SHAM (0.5 mM) after pre-incubating mesophyll protoplasts under a wide range of stress treatments as described in materials and methods. The rates of O_2 evolution (μmol mg^{-1} Chl h^{-1}) in controls without inhibitor (●) were 177.19 ± 7.73 (darkness), 182.5 ± 2.06 (0.4 M sorbitol), 179.45 ± 4.22 (25 ^oC) respectively.

presence and absence of mitochondrial inhibitors (Fig. 5.2). In contrast to rates of photosynthesis, pre-incubation of mesophyll protoplasts with high light, hypo-/hyper-osmotic stress (or) sub-/supra-optimal temperatures caused marginal decrease (5-16% of control) in the rate of respiratory O_2 uptake (Fig. 5.2A-C). Also, over-lap of the stress treatments with antimycin A or SHAM enhanced further the decrease (6-20% of control rate) in the rates of respiratory O_2 uptake observed under independent abiotic stress treatments (Fig. 5.2A-C). The following conditions of high light of 3000 μE m^{-2} s^{-1} (HL), hypo-osmotic stress (0.3 M sorbitol), hyper-osmotic stress (1.0 M sorbitol), sub-optimal temperature (10 ^{o}C) and supra-optimal temperature (40 ^{o}C) were chosen in all further studies, where the decrease in steady-state photosynthesis did not extend beyond 50% of their respective controls (Fig. 5.3). However, the changes in respiratory O_2 uptake under these conditions did not extend beyond 5% of their respective controls (Fig. 5.4). The changes in the physical appearance of mesophyll protoplasts at 0.3 M sorbitol (hypo-osmoticum) and 1.0 M sorbitol (hyper-osmoticum) as observed under microscope were shown in Fig. 5.5.

Para-Benzoquinone (*p*-BQ) is an artificial electron acceptor and is often used to examine the PSII activity (Padmasree and Raghavendra, 2001b). The changes in PSII activity of intact mesophyll protoplasts as indicated by *p*-BQ dependent oxygen evolution under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C were shown in the inset of Fig. 5.6. Pre-incubation of mesophyll protoplasts under HL or 40 ^{o}C temperature caused remarkable changes in *p*-BQ dependent oxygen evolution in presence of both antimycin A and SHAM. The *p*-BQ dependent oxygen evolution was decreased by ≤42% and ≤33% of control rates in presence of antimycin A and SHAM, respectively (Fig. 5.6). However, both antimycin A and SHAM did not cause such

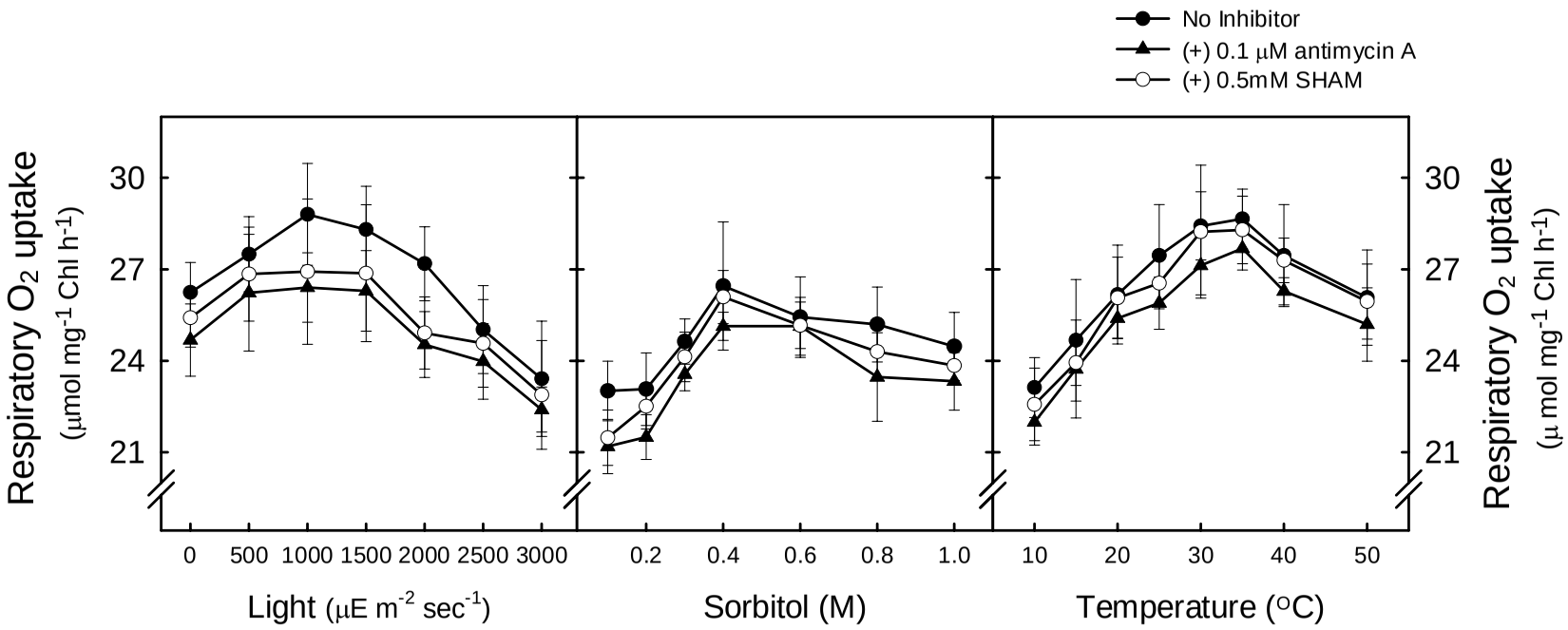

Figure 5.2. Effect of antimycin A (AA, ▲) or SHAM (○) on respiration during light (A), osmotic (B) or temperature (C) stress in mesophyll protoplasts of pea. The rates of respiratory O_2 uptake were measured in darkness in the presence of AA (0.1 μM) and SHAM (0.5 mM) after pre-incubating mesophyll protoplasts under a wide range of stress treatments as described in materials and methods. The rates of respiratory O_2 uptake (μmol mg^{-1} Chl h^{-1}) in controls without inhibitor (●) were 26.24 ± 0.98 (darkness), 26.45 ± 2.1 (0.4 M sorbitol), 27.45 ± 1.67 (25 ^{o}C) respectively.

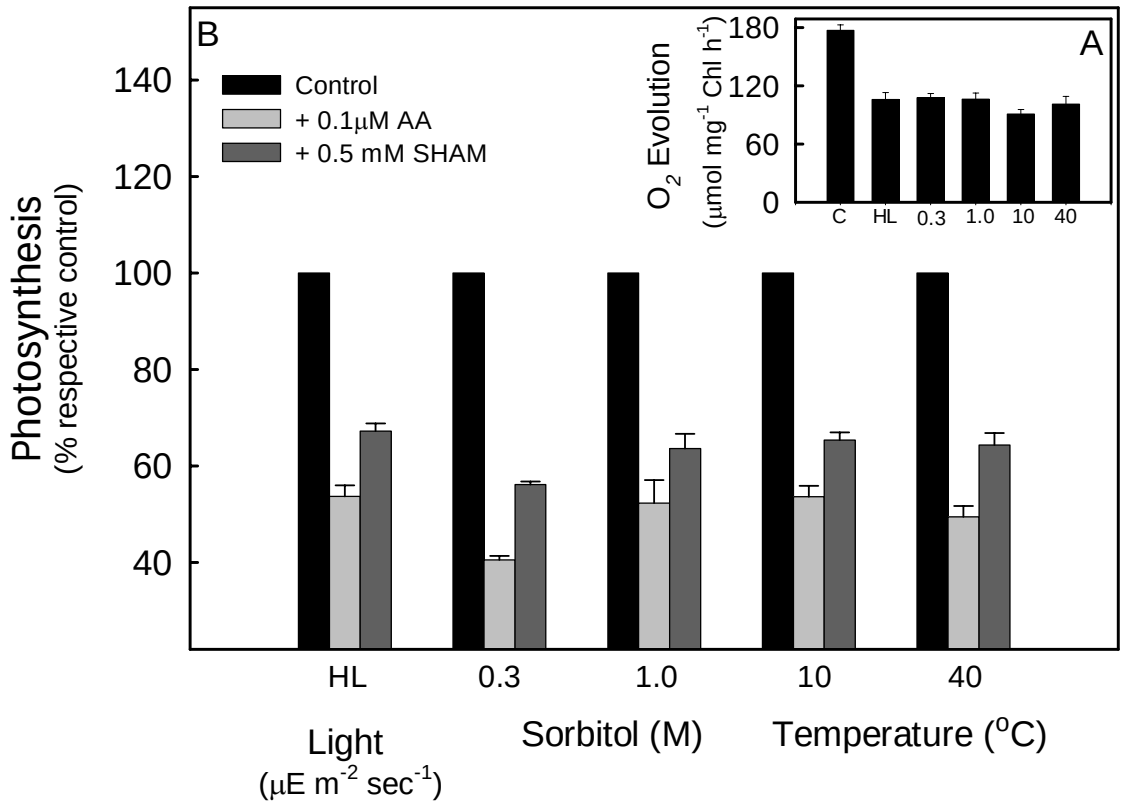

Figure 5.3. (A) Effect of high light (HL, 3000 μE m^{-2} s^{-1}), hypo-osmoticum (0.3 M), hyper-osmoticum (1.0 M), sub-optimal (10 ^{o}C) or supra-optimal (40 ^{o}C) temperature on $NaHCO_3$ (1.0 mM) dependent O_2 evolution in mesophyll protoplasts of pea. 'C' is control without stress treatment. (B) Effect of antimycin A (AA) and SHAM on $NaHCO_3$ dependent O_2 evolution in mesophyll protoplasts pre-incubated under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C (or) 40 ^{o}C temperature for 10 min. Controls (without inhibitor) were treated as 100% and the decrease in O_2 evolution in presence of inhibitors was shown as % respective control. The absolute values of O_2 evolution under different stress treatments were as indicated in the inset (A). Other details of stress treatments and measurement of photosynthesis were as described in materials and methods and Fig.5.1.

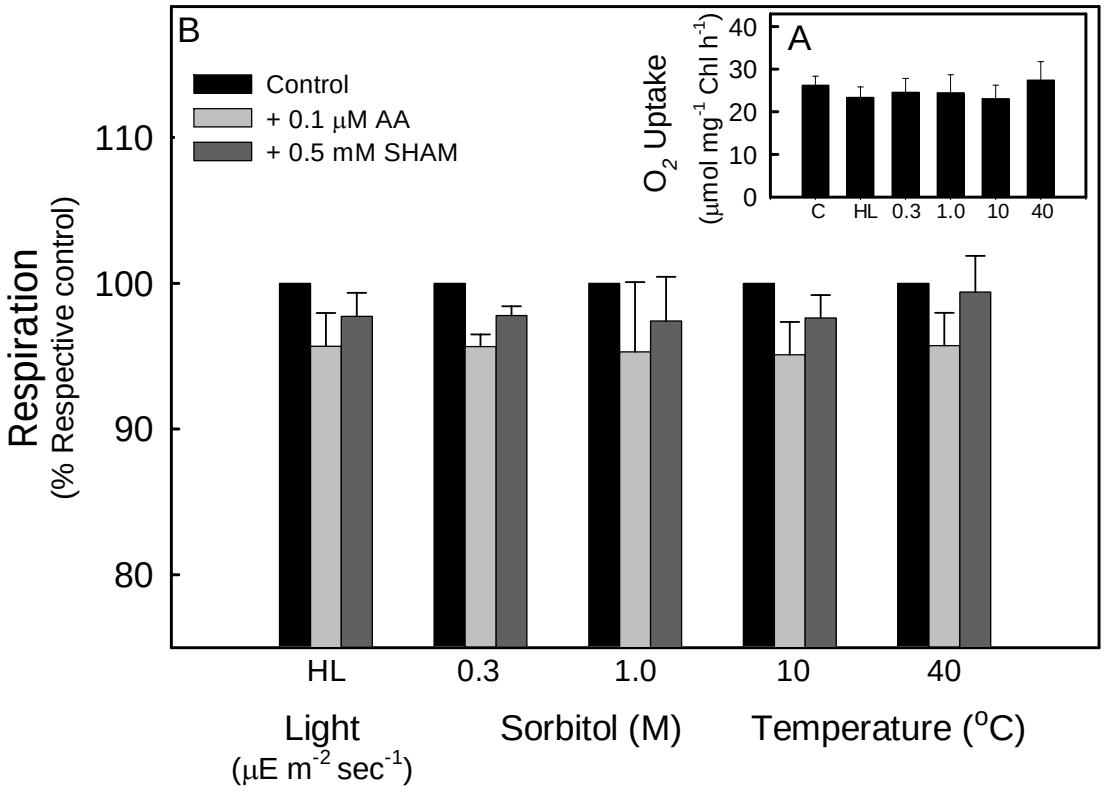

Figure 5.4. (A) The respiratory O_2 uptake in mesophyll protoplasts of pea pre-incubated under high light (HL), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min**.** (B) Effect of antimycin A (AA) or SHAM on respiratory O_2 uptake in mesophyll protoplasts of pea preincubated under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details were as described in Fig. 5.3 and materials and methods.

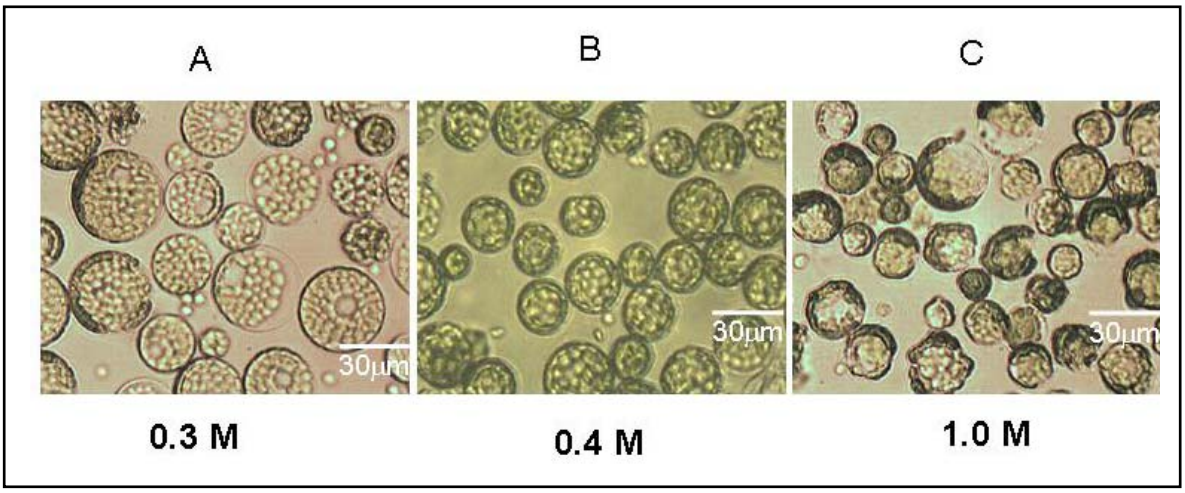

Figure 5.5. Microscopic examination of pea mesophyll protoplasts pre-incubated at (A) 0.3 M sorbitol (hypo-osmoticum); (B) 0.4 M sorbitol (iso-osmoticum) and (C) 1.0 M sorbitol (hyper-osmoticum) respectively for 10 min at 25 ^{o}C in reaction medium as described in materials and methods.

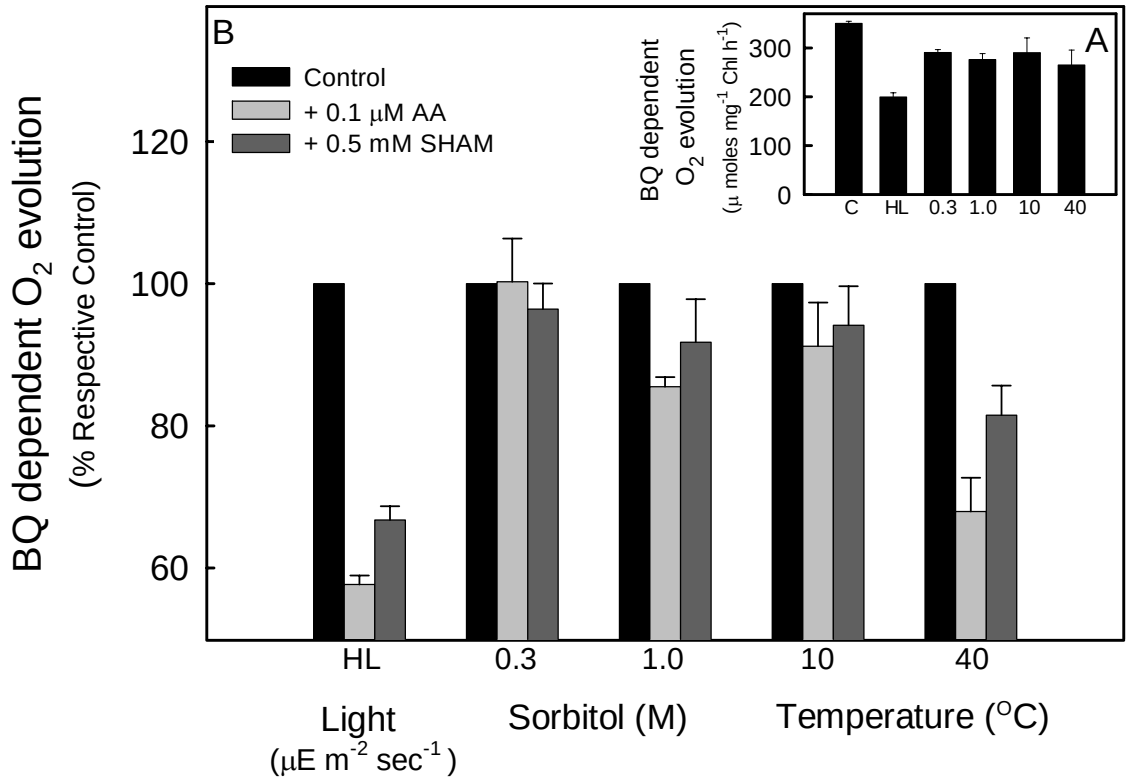

Figure 5.6. (A) The BQ dependent O_2 evolution in mesophyll protoplasts of pea pre-incubated under high light (HL), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min**.** (B) Effect of antimycin A (AA) or SHAM on BQ dependent O_2 evolution in mesophyll protoplasts of pea preincubated under HL, 0.3 M / 1.0 M (or) 10 ^{o}C / 40 ^{o}C temperature. Other details were as described in Fig. 5.3 and materials and methods.

remarkable changes in *p*-BQ dependent oxygen evolution under 0.3 M, 1.0 M sorbitol or 10 ^{o}C (Fig. 5.6). The changes in D1 protein caused due to HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C under steady state photosynthesis were represented in Fig. 5.7(A-F) and Fig. 5.8A. Pre-incubation of mesophyll protoplasts with HL and 10 ^{o}C caused significant decrease in D1 protein (Fig. 5.7, A and E; Fig. 5.8A). However, when the stress treatments were over-lapped by treatments with antimycin A or SHAM, the changes in D1 protein were remarkable only under 1.0 M sorbitol and 10 ^{o}C (Fig. 5.8B). Antimycin A treatment caused 17% decrease in D1 protein levels when compared to control under 1.0 M sorbitol. In contrast to hyper-osmotic stress treatment, both antimycin A and SHAM enhanced the D1 protein levels by ≤17% of their control at 10 ^{o}C (Fig. 5.8B).

Sucrose is the immediate end-product of carbon assimilation in mesophyll cells. The synthesis of sucrose occurs in cytosol and ATP required for its synthesis is mostly supplied by bioenergetic reactions of mitochondria (Fig.1.2; Padmasree and Raghavendra, 1999a,c; Raghavendra and Padmasree, 2003). While the earlier experiments described above demonstrated the essentiality of mitochondrial respiration in optimizing photosynthetic carbon assimilation during various abiotic stress conditions, the examination of adenylate levels (ATP and ADP) allows us to assess the importance of mitochondrial respiration in supplying ATP for sucrose synthesis under stress conditions. The relative changes in total cellular levels of ATP, ADP and ATP/ADP examined during steady state photosynthesis under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C and 40 ^{o}C were shown as inset figures (Fig. 5.9, A and C; Fig. 5.10A). All these abiotic stress factors caused a dramatic decrease in ATP levels (up to ≤48%) and increase in ADP levels (up to ≤23%), respectively, of their respective

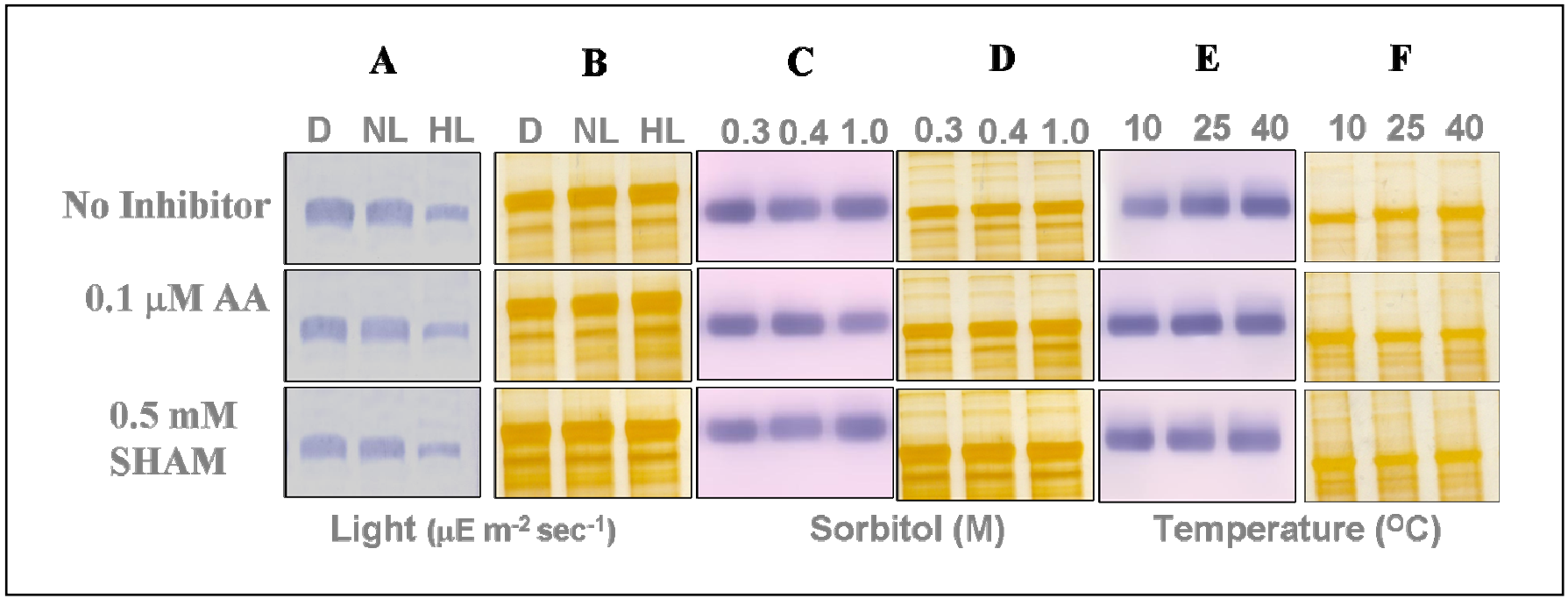

Figure 5.7. Western blot analysis of D1 protein from mesophyll protoplasts of pea preincubated with optimal (1.0 mM, $NaHCO_3$) CO_2 under (A) high light (HL, 3000 μE m^{-2} s^{-1}), (C) hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, (E) sub- (10 ^{o}C) supra- (40 ^{o}C) optimal temperature in the presence of AA or SHAM (A, C and E). 8 μg was loaded on to the gel. Equal loading of protein was confirmed by silver staining of a duplicate gel (B, D and E). Other details were as mentioned in materials and methods.

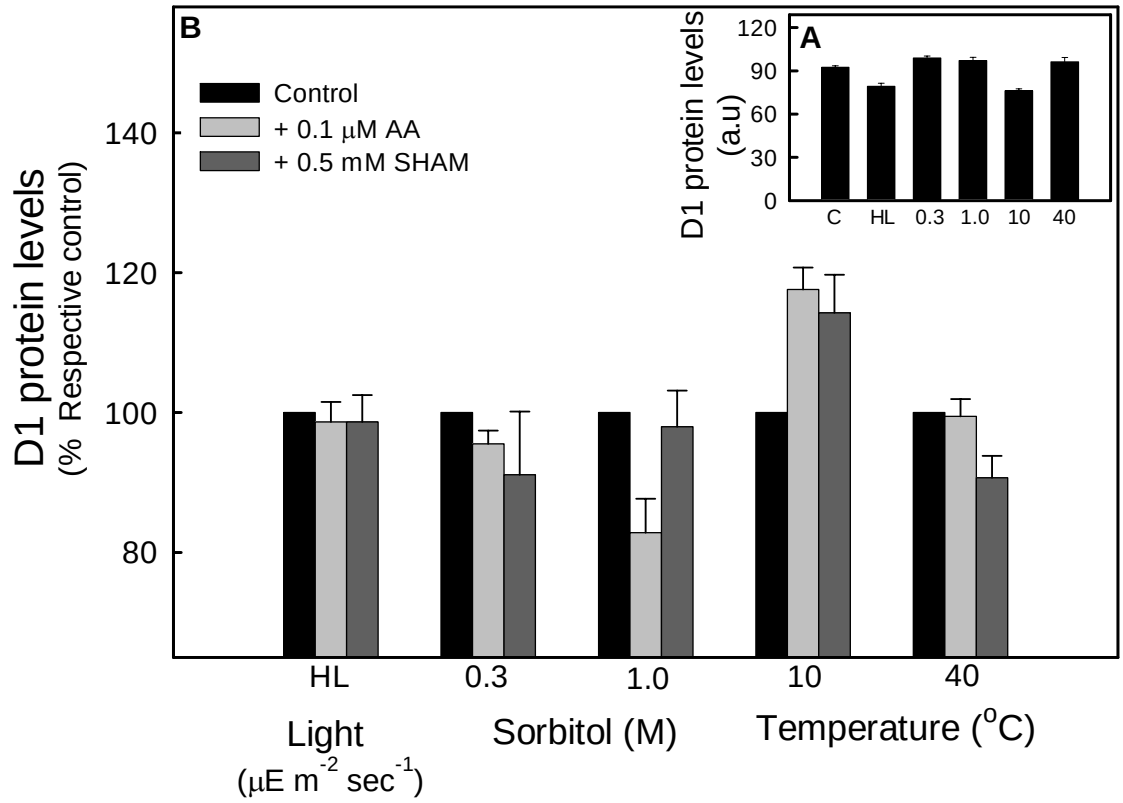

Figure 5.8. Densitometric analysis of D1 protein levels taken from the western blot of Figure 5.7 using a image J software 1.37 V, National Institute of Health, USA. (A) The D1 protein levels in mesophyll protoplasts of pea pre-incubated under high light (HL), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min. (B) Effect of AA and SHAM on D1 protein levels during steady state photosynthesis under HL, 0.3 M / 1.0 M (or) 10 ^{o}C / 40 ^{o}C temperature. Other details were as mentioned in Fig. 5.3 and materials and methods.

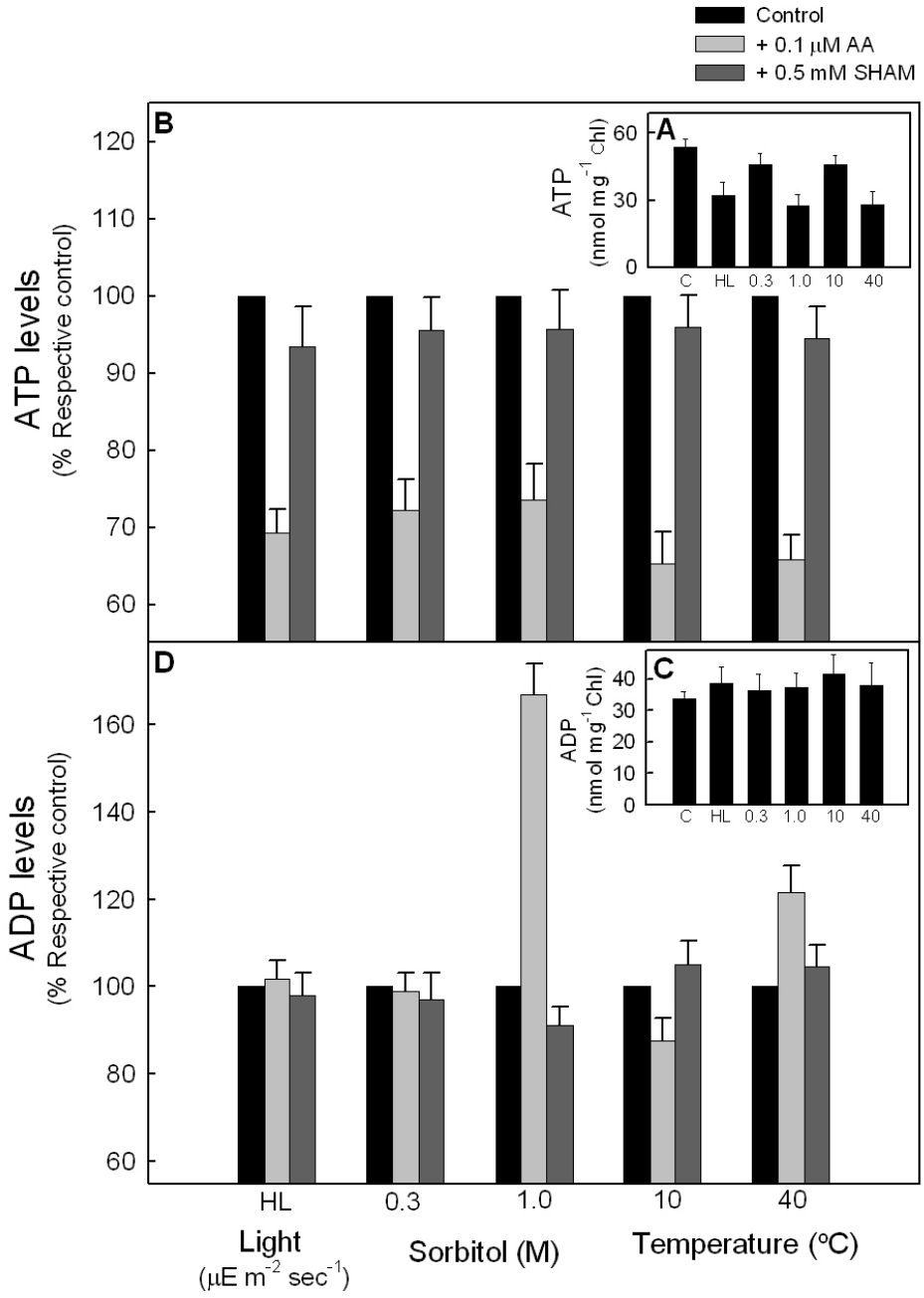

Figure 5.9. Intracellular levels of ATP (A) and ADP (C) in mesophyll protoplasts of pea pre-incubated under high light (HL, 3000 μE m^{-2} s^{-1}), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min. Effect of AA or SHAM on ATP (B) and ADP (D) levels during steady state photosynthesis under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details are as described in Fig. 5.3 and materials and methods.

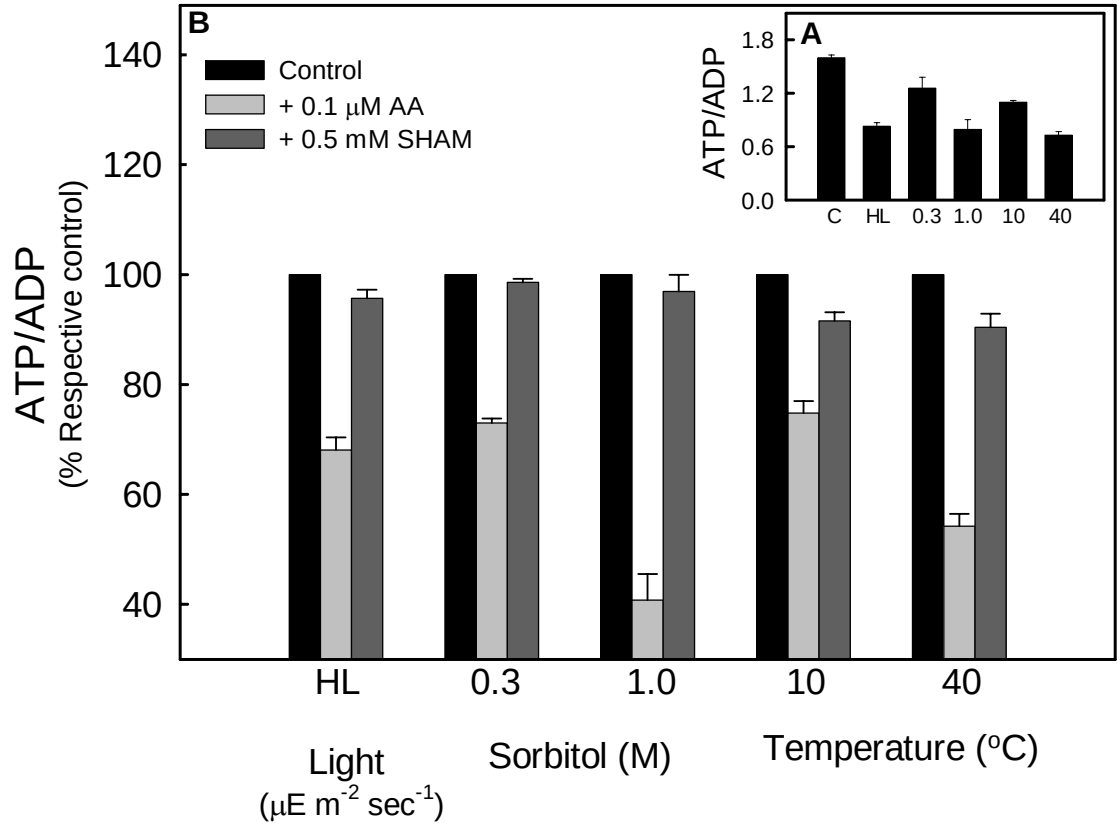

Figure 5.10. (A) The ratio of intracellular ATP/ADP in mesophyll protoplasts of pea pre-incubated under high light (HL, 3000 μE m^{-2} s^{-1}), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min. (B) Effect of AA or SHAM on the ratio of ATP/ADP during steady state photosynthesis under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details were as described in Fig. 5.3 and materials and methods.

controls (Fig. 5.9, A and C). Similar to ATP, the ATP/ADP ratios also decreased drastically (up to ≤54%) in presence of all abiotic stress factors examined in the present study (Fig. 5.10A). In presence of antimycin A, although increase in ADP was remarkable only under 1.0 M sorbitol (67%) and 40 ^{o}C (22%), the decrease in ATP (≤35%) and corresponding ATP/ADP ratios (≤46%) were remarkable under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C and 40 ^{o}C. In contrast, the effect of SHAM on the changes in the ATP, ADP and ATP/ADP were less than 10% under all abiotic stress conditions tested in the present study (Fig. 5.9, B and D; Fig. 5.10B). The results obtained in the present study not only reveal the importance of COX pathway in meeting the cytosolic ATP requirements for sucrose synthesis, but also suggests the usage of ATP/ADP as a biochemical marker or indicator of beneficial interactions between mitochondrial respiration and chloroplast photosynthesis under any environmental circumstances, being normal or abiotic stress situation (Fig. 5.9, B and D; Fig. 5.10B).

In view of the suggested functions of both chloroplasts to release reducing equivalents in excess of the requirements of Calvin cycle through malate valve and participation of mitochondria to use up the reducing equivalents generated in chloroplasts through COX and AOX pathways (Padmasree et al., 2002), we examined the changes in the different components of malate-OAA shuttle regulated by malate valve in presence of antimycin A and SHAM under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature, so as to assess the significance of malate-OAA shuttle during beneficial interactions between chloroplasts and mitochondria under stress conditions. Accordingly the changes in the total cellular composition of the following components of malate-OAA shuttle were determined: (i) malate, (ii) OAA, (iii) malate

+ OAA and (iv) Mal/OAA ratio (indicator of cellular malate redox state). The independent effects of HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature on the suggested components of malate-OAA shuttle were shown in the insets of the Figures 5.11 and 5.12. While treatment of protoplasts with HL, 0.3 M sorbitol or 1.0 M sorbitol caused remarkable rise in total cellular levels of Malate, Malate + OAA and Mal/OAA ratio (up to <89%) during steady state photosynthesis, treatment with either 10 ^{o}C or 40 ^{o}C temperature caused drastic decrease (<40%) in the cellular levels of all these components with respect to their controls (Figs. 5.11, A and E; Fig. 5.12A). In contrast to these three components of malate-OAA shuttle, OAA levels decreased by <25% in the controls under all conditions of stress examined in the present study (Fig. 5.11C).

Restriction of COX pathway or AOX pathway with antimycin A and SHAM showed differential effects on the different components of malate-OAA shuttle during stress (Figs. 5.11, B, D and F; Fig. 5.12B). In presence of antimycin A, the total cellular levels of malate and (malate+OAA) decreased drastically under 0.3 M sorbitol (36%), but increased under 40 ^{o}C (15%) when compared to their respective stress controls (Fig. 5.11, B and F). However such changes in malate and (malate + OAA) were marginal in presence of antimycin A under other abiotic stress treatments. In contrast to antimycin A, treatment of protoplasts with SHAM caused remarkable rise in malate and (malate + OAA) levels by <40% of their respective controls under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature (Figs. 5.11, B and F). On the other hand, in presence of antimycin A, the total cellular levels of OAA decreased by <40% of their respective stress controls under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C temperature. Interestingly, the effects of SHAM on intracellular levels of OAA was variable under

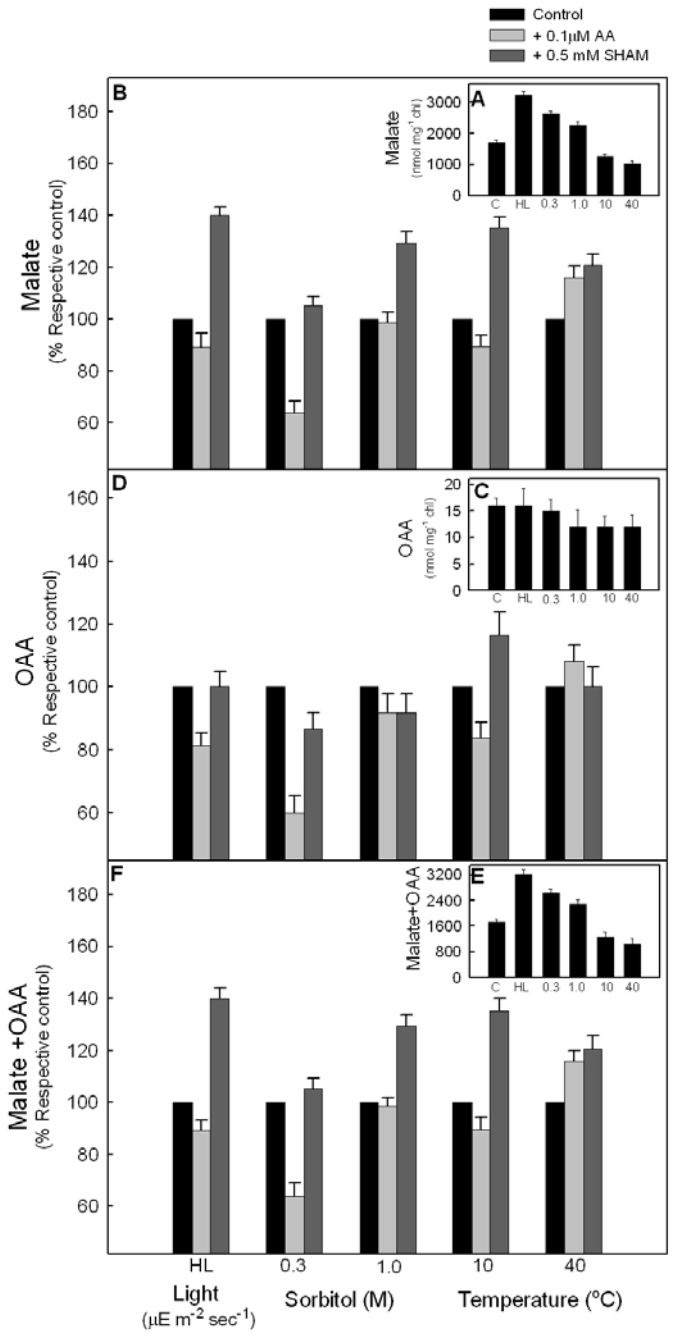

Figure 5.11. Intracellular levels of malate (A), OAA (C) and malate + OAA (E) in mesophyll protoplasts of pea pre-incubated under high light (HL, 3000 μE m^{-2} s^{-1}), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) and supra- (40 ^{o}C) optimal temperature for 10 min. Effect of antimycin A or SHAM on malate (B), OAA (D) and malate + OAA (F) levels during steady state photosynthesis under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details were as described in Fig. 5.3 and materials and methods.

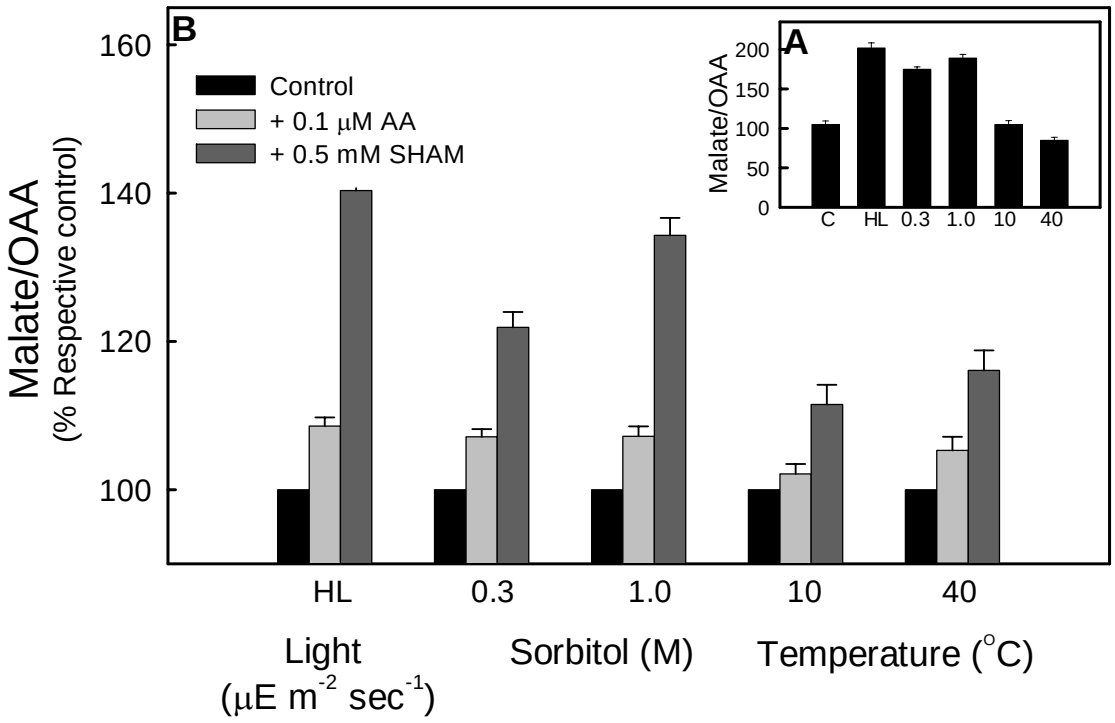

Figure 5.12. (A) The ratio of intracellular malate to OAA in mesophyll protoplasts of pea pre-incubated under high light (HL, 3000 μE m^{-2} s^{-1}), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min. These ratios were calculated from the data in fig. 5.11 (B) Effect of AA or SHAM on the ratio of malate to OAA during steady state photosynthesis under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details are as described in Fig. 5.3 and materials and methods.

different stress treatments. The OAA levels increased by 17% under 10 ^{o}C while decreased by 13% under 0.3 M sorbitol. However the effects of SHAM on OAA were insignificant under other abiotic stress treatments (Fig. 5.11D). The increase in the redox state of malate as determined by Mal/OAA ratio was more significant in presence of SHAM (≤40%) when compared to antimycin A (≤10%) under all abiotic stress treatments (Fig. 5.12B).

Results from the chapter 1 of the present study demonstrated the importance of ROS and antioxidant system in fine-tuning the beneficial interactions of mitochondrial respiration with chloroplastic photosynthesis. ROS and antioxidant molecules are also known to play an important role in cellular signaling during various abiotic stress conditions. Therefore examination of ROS and antioxidant molecules Asc and GSH along with their oxidized counter parts (DHA and GSSG) in presence of antimycin A and SHAM under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature reveal the importance of these molecules in fine-tuning the beneficial interactions between chloroplasts and mitochondria even during stress conditions (Figs. 5.13-5.16).

The changes in the total cellular levels of ROS as indicated by DCF fluorescence during HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature were represented as the inset figure (Fig. 5.13A). The total cellular levels of ROS increased significantly under HL (4.5 fold) and 40 ^{o}C temperature (2.6 fold) when compared to its increase under 0.3 M sorbitol (20%). However such a rise in ROS levels was not observed under both 1.0 M sorbitol and 10 ^{o}C temperature (Fig. 5.13A). Surprisingly when mesophyll protoplasts were treated with antimycin A or SHAM under stress conditions, the changes in the total cellular ROS levels observed under each of the independent abiotic stress treatments were modulated (Fig. 5.13B). Though the

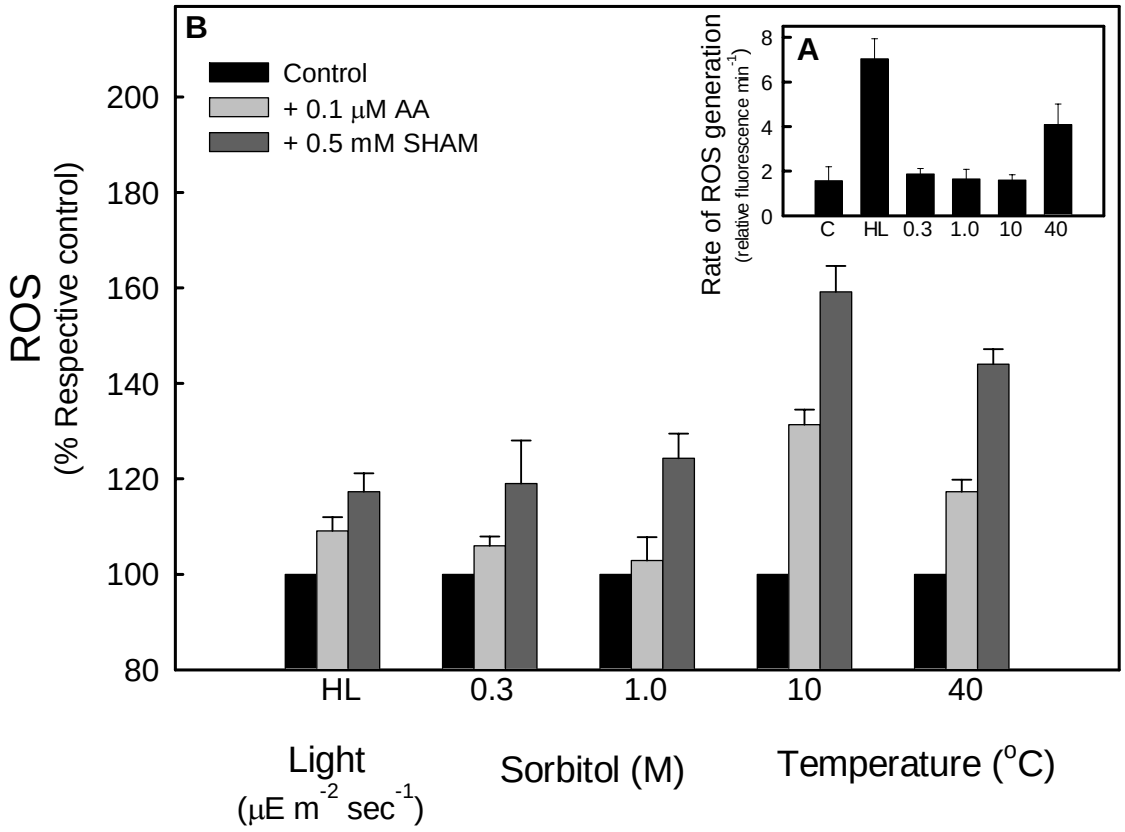

Figure 5.13. (A) Intracellular ROS levels in mesophyll protoplasts of pea pre-incubated under high light (HL, 3000 μE m^{-2} s^{-1}), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min. (B) Effect of antimycin A or SHAM on intracellular ROS levels during steady state photosynthesis under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details are as described in Fig. 5.3 and materials and methods.

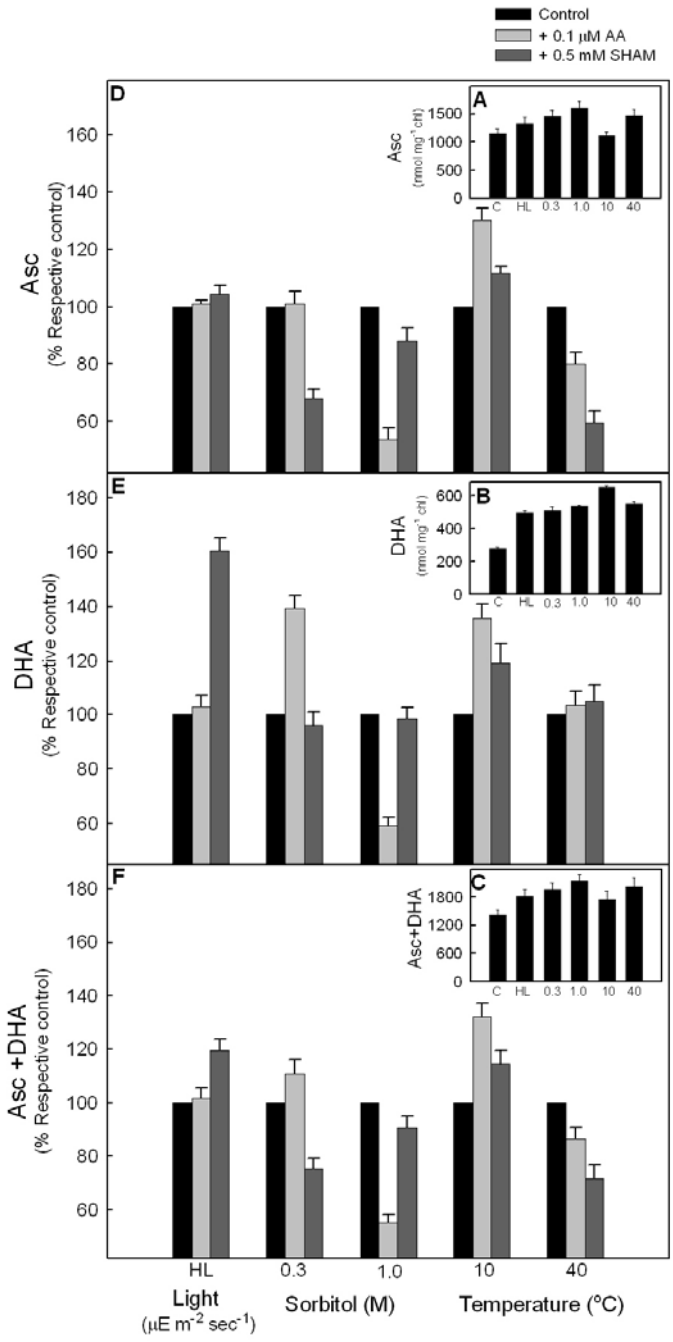

Figure 5.14. Intracellular levels of Asc (A), DHA (B) and Asc + DHA (C) in mesophyll protoplasts of pea pre-incubated under high light (HL, 3000 µE m^{-2} s^{-1}), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) and supra- (40 ^{o}C) optimal temperature for 10 min. Effect of AA or SHAM on Asc (D), DHA (E) and Asc + DHA (F) levels during steady state photosynthesis under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details are as described in Fig. 5.3 and materials and methods.

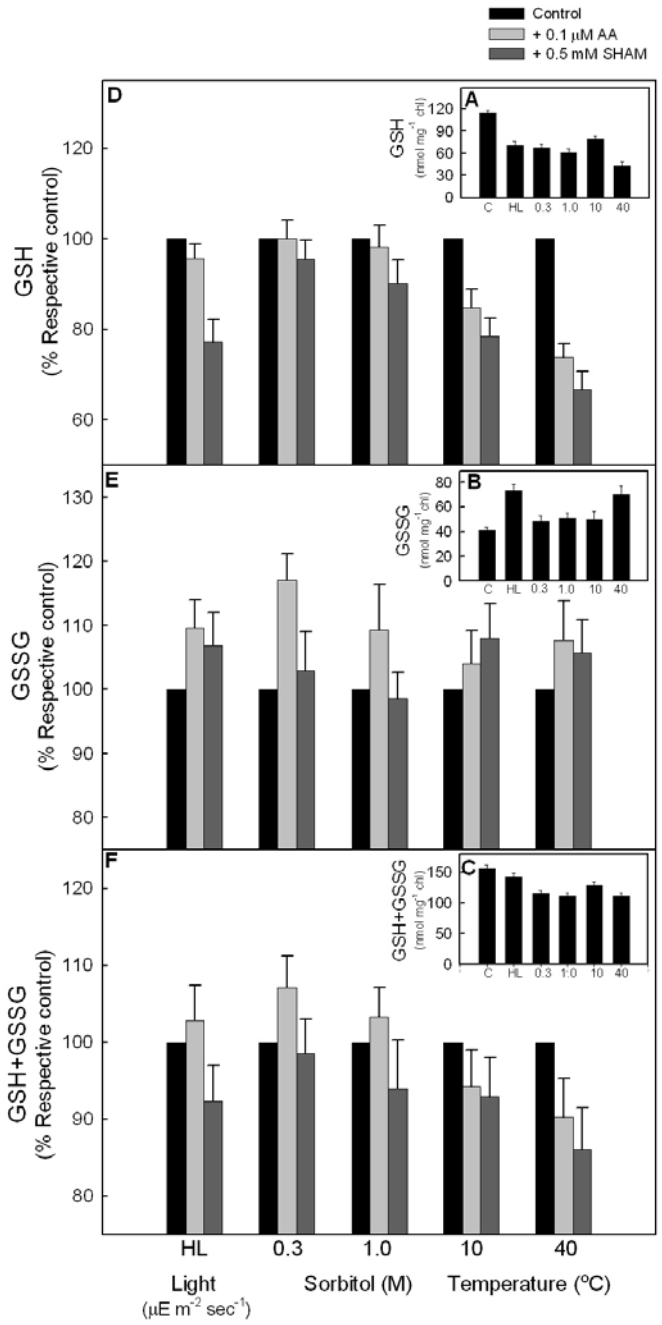

Figure 5.15. Intracellular levels of GSH (A), GSSG (B) and GSH + GSSG (C) in mesophyll protoplasts of pea pre-incubated under high light (HL, 3000 μE m^{-2} s^{-1}), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) A or supra- (40 ^{o}C) optimal temperature for 10 min. Effect of AA or SHAM on GSH (D), GSSG (E) and GSH + GSSG (F) levels during steady state photosynthesis under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details are as described in Fig. 5.3 and materials and methods.

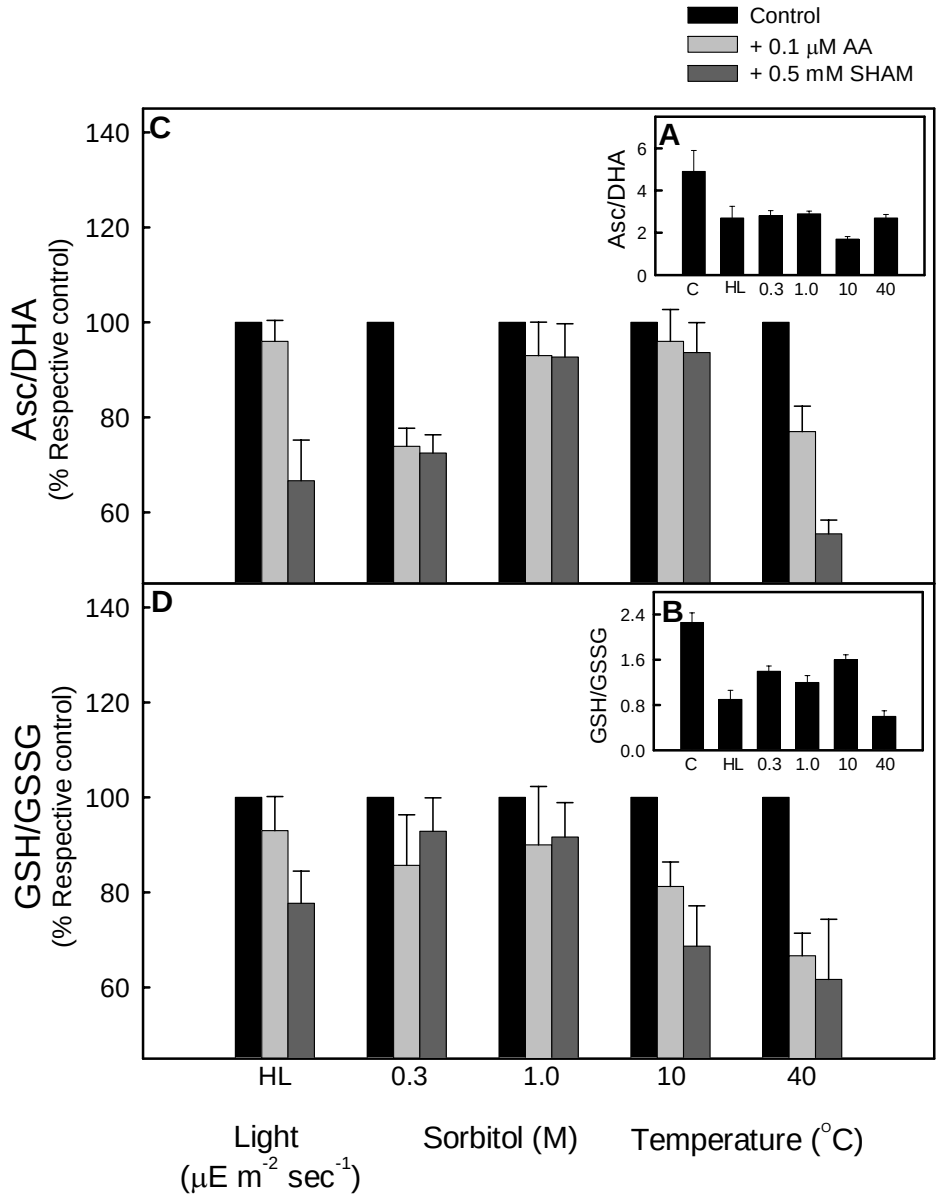

Figure 5.16. Asc/DHA (A) and GSH/GSSG (B) ratio in mesophyll protoplasts of pea pre-incubated under high light (HL, 3000 μE m^{-2} s^{-1}), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmoticum, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min. These ratios were calculated from the data in Fig. 5.14 and 5.15 (B) Effect of antimycin A or SHAM on the ratios of Asc/DHA (C) and GSH/GSSG (D) during steady state photosynthesis under HL, 0.3 M / 1.0 M sorbitol (or) 10 ^{o}C / 40 ^{o}C temperature. Other details are as described in Fig. 5.3 and materials and methods.

changes were small, they were consistent and significant. Restriction of COX pathway with antimycin A caused remarkable rise in cellular ROS levels only under 10 ^{o}C (31%) and 40 ^{o}C (17%) temperature, but not under HL, 0.3 M or 1.0 M sorbitol. In contrast to COX pathway, restriction of AOX pathway raised total cellular levels of ROS by 17-59% of their respective controls under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature (Fig. 5.13B).

The changes in the antioxidant molecules (i) Asc and GSH (Fig. 5.14A; Fig. 5.15A); (ii) their oxidized forms DHA and GSSG (Fig. 5.14B; Fig. 5.15B): (iii) total ascorbate (Asc + DHA) or glutathione (GSH + GSSG) levels (Fig. 5.14C; Fig. 5.15C) and (iv) Asc or GSH redox state indicated by Asc/DHA and GSH/GSSG ratios (Fig. 5.16, A and B), respectively, were assessed under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature and control values corresponding to each of these tested parameters were represented as the inset figures.

During steady state photosynthesis, the total cellular levels of Asc and GSH decreased by ≤28% and ≤64% of their corresponding controls under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature (Fig. 5.14A; Fig. 5.15A). In contrast to Asc and GSH, the total cellular levels of their oxidized forms DHA and GSSG increased by ≤2.3 fold and ≤78%, respectively, of their controls (Fig. 5.14B; Fig. 5.15B). Inspite of the remarkable increase in total ascorbate (Asc + DHA) by ≤1.5 fold, the total glutathione (GSH + GSSG) levels decreased by ≤28% of their controls (Figs. 5.14C; Fig. 5.15C). But the redox states of both Asc (Asc/DHA) and GSH (GSH/GSSG) decreased by ≤65% and ≤73% respectively, of their control values (Fig. 5.16, A and B).

Restriction of COX pathway or AOX pathway of mesophyll protoplasts under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C caused different effects on total

cellular levels of Asc and DHA during steady state photosynthesis (Fig. 5.14, D and E). In presence of antimycin A, the Asc levels drastically increased by 30% under 10 ^{o}C and decreased by ≤46% under 1.0 M sorbitol or 40 ^{o}C temperature, respectively, when compared to their respective stress controls. On the other hand, in presence of SHAM, Asc levels drastically decreased by ≤41% of their respective stress controls under 0.3 M sorbitol or 40 ^{o}C temperature (Fig. 5.14D). While the changes in DHA and total ascorbate (Asc + DHA), modulated by antimycin A were more remarkable under 0.3 M, 1.0 M sorbitol and 10 ^{o}C temperature, the changes in these metabolites modulated by SHAM were remarkable under HL and 10 ^{o}C temperature (Fig. 5.14, E and F). However, the presence of antimycin A caused remarkable decrease (≤27%) in the redox state of Asc only under 0.3 M sorbitol and 40 ^{o}C, but not under other abiotic stress conditions (Fig. 5.16C). On the other hand, the presence of SHAM caused significant decrease (≤44%) in Asc redox state only under HL, 0.3 M sorbitol and 40 ^{o}C (Fig. 5.16C).

Restriction of COX pathway or AOX pathway in mesophyll protoplasts by antimycin A or SHAM caused decrease in the total cellular levels of GSH and increase in the GSSG under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C (Fig. 5.15,D and E). However, the decrease (<33%) in GSH was more remarkable than increase in GSSG (<10%) in presence of both antimycin A and SHAM under most of the abiotic stress conditions examined (Fig. 5.15, D and E). While the changes in the total GSH were marginal, the changes in redox state of GSH were remarkable in presence of both antimycin A and SHAM under stress (Fig. 5.15F; Fig. 5.16D). The redox state of GSH levels decreased by ≤33% in presence of antimycin A and ≤38% in presence of SHAM when compared to their respective stress controls (Fig. 5.16D). To further assess the

significance of ROS and redox metabolites related to Asc-GSH cycle during the beneficial interactions of mitochondrial respiration with chloroplast photosynthesis under stress conditions, the changes in the activities of antioxidant enzymes: SOD, CAT, APX, MDAR and GR were examined in the presence of antimycin A and SHAM under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature (Fig. 5.17)

Table 5.1 shows the effects of HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature, independently on the total cellular activities of antioxidant enzymes. These values were treated as controls for each of the antioxidant enzymes examined in presence of antimycin A and SHAM under stress conditions (Fig. 5.17). Restriction of COX pathway or AOX pathway by antimycin A and SHAM also caused differential changes in the activities of antioxidant enzymes. The activities of SOD increased by ≤21% and ≤71%, respectively, when compared to their stress controls in presence of antimycin A and SHAM under HL and 10 ^{o}C (Fig. 5.17A). The activities of CAT increased by ≤29% and ≤159%, respectively, of their stress controls in presence of antimycin A and SHAM under HL (Fig. 5.17B). In contrast to SOD and CAT, the activities of APX decreased by ≤54% with respect to their stress control in presence of both antimycin A and SHAM under 10 ^{o}C and 40 ^{o}C temperature (Fig. 5.17C). On the other hand, the changes in the activities of MDAR and GR were more pronounced in presence of antimycin A when compared to SHAM under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature (Fig. 5.17, D and E). The changes in the activity staining of three different isoenzymes related to SOD: Mn-SOD, Fe-SOD and Cu/Zn-SOD on Native PAGE in presence of antimycin A and SHAM under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature were represented in Fig. 5.18A-C.

Table 5.1. Effect of highlight (HL), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol) osmotic, sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature on the total cellular activities of antioxidant enzymes. Mesophyll protoplasts were preincubated under stress conditions for 10 min and the antioxidant enzyme activities were examined. The increase or decrease in enzyme activities upon stress treatments compared to their controls (without stress) were show n in brackets.

Parameters	**Control**	**High light** (HL)	**Hypo-osmotic stress** (0.3 M sorbitol)	**Hyper-osmotic stress** (1.0 M sorbitol)	**Sub-optimal temperature** (10 ^{o}C)	**Supra-optimal temperature** (40 ^{o}C)
SOD (U mg^{-1} protein)	14.6 ± 0.2 (1.0)	16.6 ± 0.2 (1.14)	14.6 ± 0.4 (1.0)	15.4 ± 0.12 (1.05)	14.1 ± 0.9 (0.97)	14.8 ± 1.0 (1.01)
CAT (μmol min^{-1} mg^{-1} protein)	279 ± 10.6 (1.0)	153 ± 2.7 (0.55)	267 ± 5.9 (0.95)	446.7 ± 8.21 (1.6)	268.5 ± 7.1 (0.96)	455 ± 7.5 (1.63)
APX (μmol min^{-1} mg^{-1} protein)	3.7 ± 0.1 (1.0)	2.12 ± 0.1 (0.57)	3.3 ± 0.3 (0.89)	3.4 ± 0.12 (0.91)	4.67 ± 0.1 (1.26)	1.89 ± 0.1 (0.51)
MDAR (nmoles mg^{-1} protein min^{-1})	249 ± 22.5 (1.0)	138 ± 4.6 (0.55)	342 ± 7.6 (1.37)	189.6 ± 4.35 (0.76)	329 ± 1.83 (1.31)	170.4 ± 12.7 (0.68)
GR (nmoles mg^{-1} protein min^{-1})	212 ± 5.0 (1.0)	112 ± 2.7 (0.52)	242 ± 4.7 (1.14)	174.3 ± 9.93 (0.82)	222 ± 4.4 (1.04)	69.4 ± 2.3 (0.33)

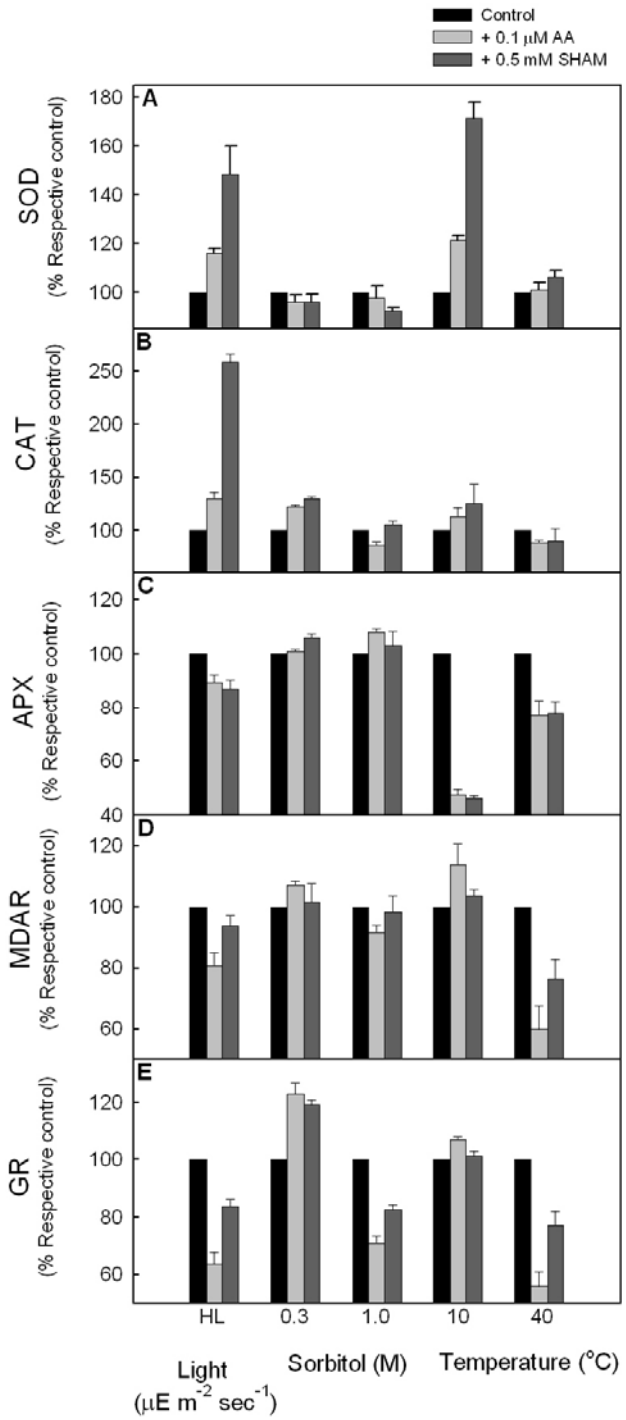

Figure 5.17. Effect of antimycin A (AA) or SHAM on SOD (A), CAT (B), APX (C), MDAR (D) and GR (E) activities during steady state photosynthesis. The mesophyll protoplasts are preincubated under highlight (HL), hypo- (0.3 M sorbitol), hyper- (1.0 M sorbitol), sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min and enzyme activities were determined as described in materials and methods. The absolute values of controls corresponding to each stress treatment are shown in Table 5.1.

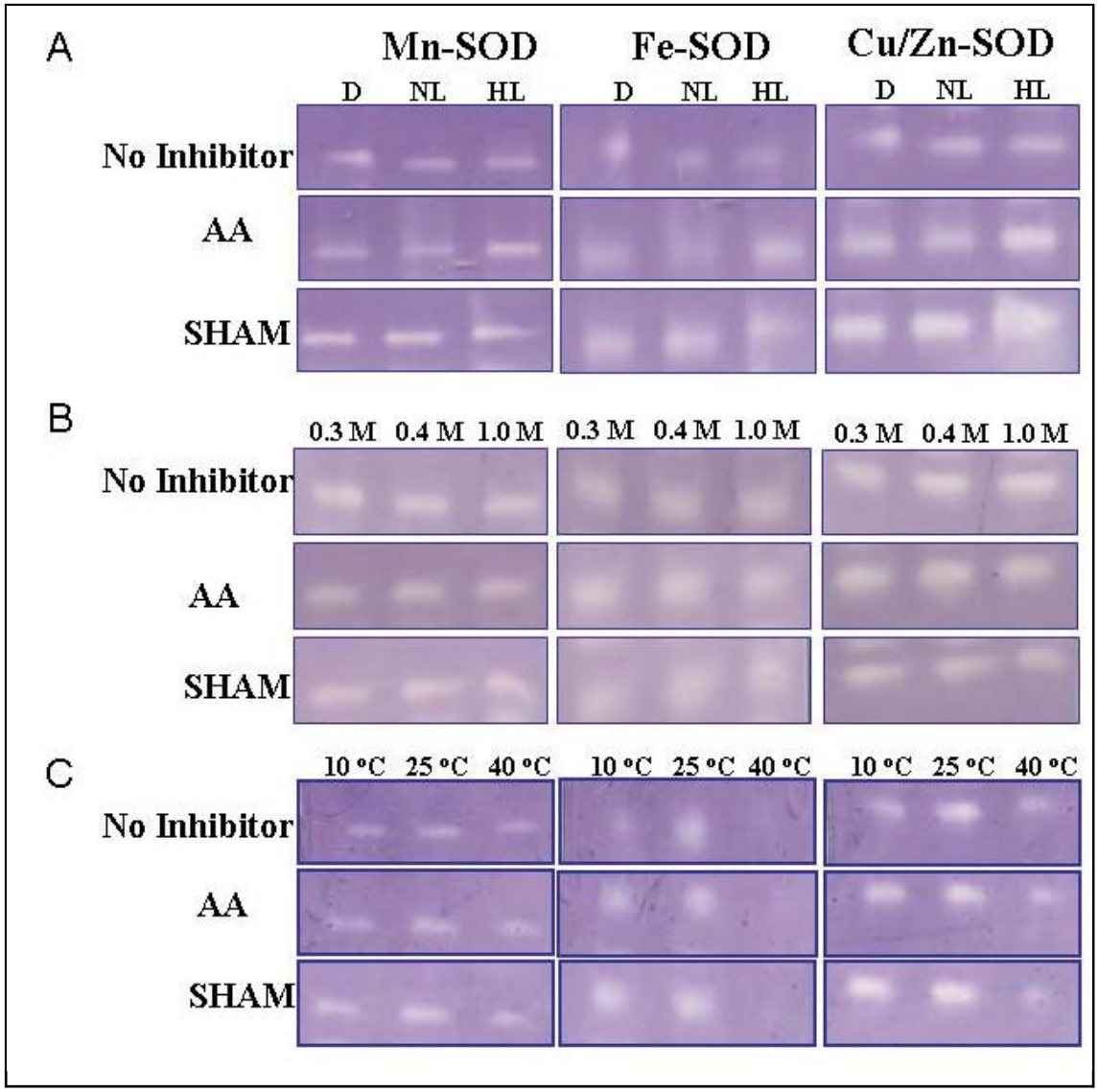

Figure 5.18. Activity staining of Mn-SOD, Fe-SOD and Cu/Zn-SOD in non-denaturing gels from mesophyll protoplasts of pea preincubated under (A) HL, (B) hypo- (0.3 M sorbitol) hyper- (1.0 M sorbitol) osmotic, (C) sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min in the presence of antimycin A and SHAM. Normal light (NL, 1000 μE m^{-2} s^{-1}) treated mesophyll protoplasts were considered as additional positive control along with dark treated ones. (D: darkness; NL: normal light; HL: high light treatment). Other details were as described in materials and methods.

Similar to SOD, the changes in activity staining of CAT and APX detected on native-PAGE in presence of antimycin A and SHAM under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature were shown in the Figures 5.19 and 5.20.

Figures 5.21 and 5.22 represents the flow chart indicating the importance of each of the components examined in the present study in mediating the beneficial interactions of mitochondrial respiration with chloroplast photosynthesis through COX or AOX pathway under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 40 ^{o}C temperature.

Discussion

It has been reported from the previous studies in our laboratory and literature that even a marginal interference in electron transport through COX or AOX pathway of mitochondrial respiratory chain caused a significant drop in photosynthetic carbon assimilation at optimal/limiting CO_2 or saturating/sub-saturating light intensities (Krömer et al., 1993; Padmasree and Raghavendra, 1999a; Dutilleul et al., 2003a; Yoshida et al., 2006). Though the importance of COX pathway in protecting photosynthesis from photoinhibition was examined earlier in mesophyll protoplasts of pea (Saradadevi and Raghavendra, 1992), this is the first report emphasizing the significance of AOX pathway in optimizing photosynthesis during abiotic stress.

We examined the relative importance of both COX and AOX pathways in benefiting different components of photosynthesis under high light, hypo- / hyper-osmoticum and sub- / supra-optimal temperatures. The optimal conditions to attain maximum efficiency of photosynthetic and respiratory processes in mesophyll protoplasts were found to be (i) light intensity of 500 to 1000 μE m^{-2} sec^{-1}; (ii) osmoticum of 0.4 M sorbitol and (iii) a temperature of 25 ^{o}C. Any deviation from these

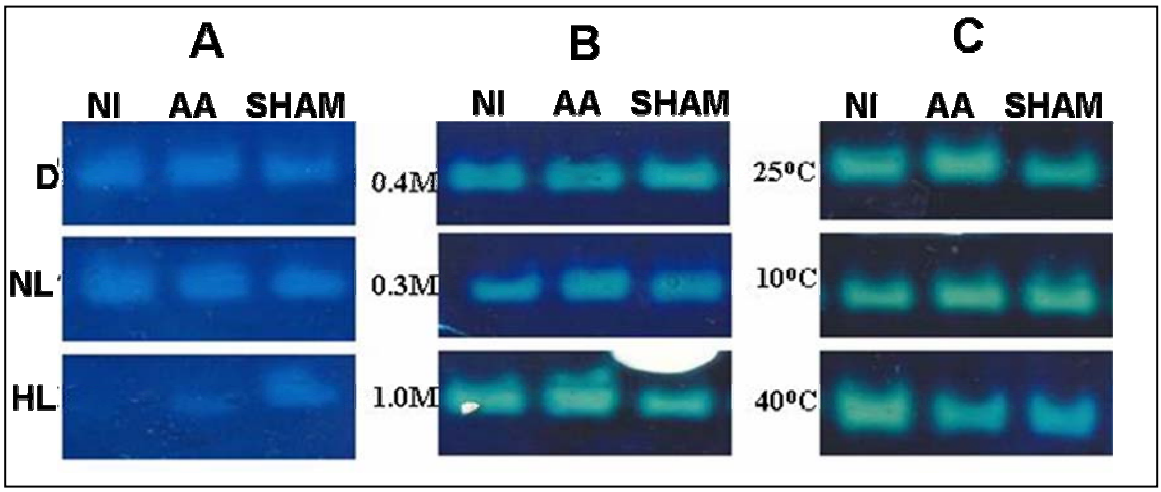

Figure 5.19. Activity staining of catalase in non-denaturing gels from mesophyll protoplasts of pea preincubated under (A) high light (HL, 3000 μE m^{-2} s^{-1}), (B) hypo- (0.3 M sorbitol) hyper- (1.0 M sorbitol) osmotic, (C) sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min in the presence of antimycin A and SHAM. Normal light (NL, 1000 μE m^{-2} s^{-1}) treated mesophyll protoplasts were considered as additional positive control along with dark treated ones. (D: darkness; NL: normal light; HL: high light treatment, NI: no inhibitor). Other details were as described in materials and methods.

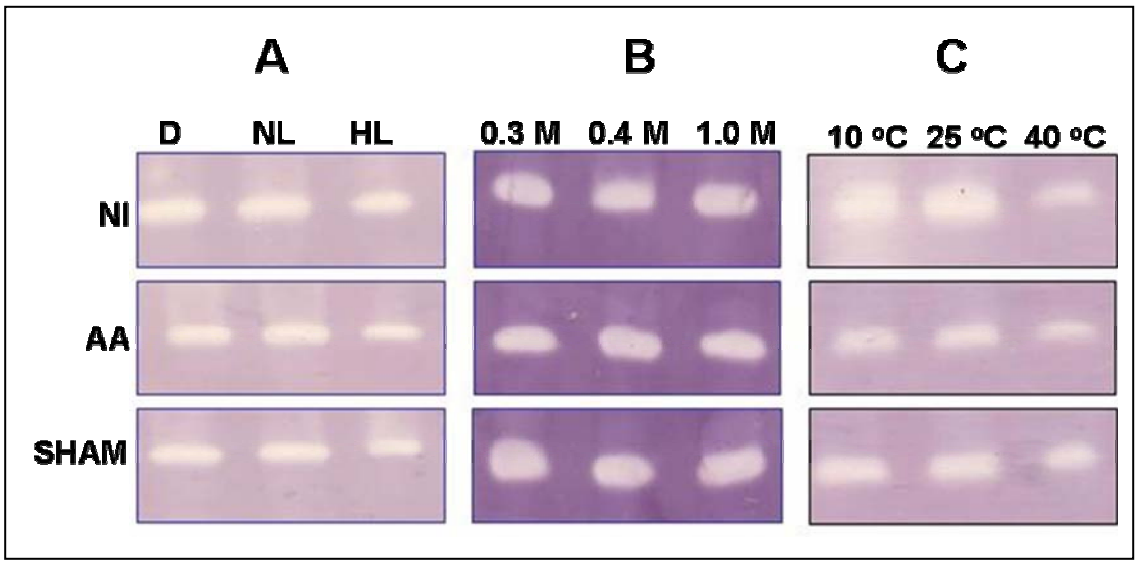

Figure 5.20. Activity staining of ascorbate peroxidase in non-denaturing gels from mesophyll protoplasts of pea preincubated under (A) high light (HL, 3000 μE m^{-2} s^{-1}), (B) hypo- (0.3 M sorbitol) hyper- (1.0 M sorbitol) osmotic, (C) sub- (10 ^{o}C) or supra- (40 ^{o}C) optimal temperature for 10 min in the presence of antimycin A and SHAM. Normal light (NL, 1000 μE m^{-2} s^{-1}) treated mesophyll protoplasts were considered as additional positive control along with dark treated ones. Other details were as described in Fig. 5.19 and materials and methods.

optimal conditions resulted in drastic reduction in the rates of both photosynthetic O_2 evolution and respiratory O_2 uptake (Fig. 5.1 A-C). Significant decrease in photosynthesis, above and below the optimum temperature was observed by Berry and Björkman (1980) in tree species. Saradadevi and Raghavendra (1994) observed a significant decrease in photosynthetic rates in mesophyll protoplasts on exposure to solutions of increasing osmolarity. In the present study, upon restriction of COX pathway or AOX pathway of mesophyll protoplasts using low concentrations of antimycin A or SHAM, respectively under light, osmotic or temperature stress conditions, the inhibition of photosynthesis ($NaHCO_3$ dependent O_2 evolution or Calvin cycle activity) observed under abiotic stress was aggravated (Fig. 5.1). Taken together these results suggest that both COX and AOX pathways play a significant role in optimizing chloroplastic photosynthesis not only under normal conditions but also under abiotic stress conditions.

Respiration in mesophyll protoplasts was either stimulated slightly or remained unchanged upto 1000 $\mu E\ m^{-2}\ sec^{-1}$. But, was inhibited only marginally under all given abiotic stresses (Fig. 5.2A-C). Thus, once again it was evident that a marginal interference in respiratory rates under abiotic stress conditions caused remarkable decrease in rates of photosynthetic O_2 evolution. Treatment with antimycin A or SHAM further decreased the respiratory O_2 uptake (Fig. 5.2 A-C). However, this decrease in respiration was not as pronounced as observed with photosynthesis (Figs. 5.3 & 5.4). The results from Saradadevi and Raghavendra (1992) showed decrease in both the photosynthesis and dark respiration in mesophyll protoplasts on exposure to supra-optimal light. In a cyanobacterium *Anacystis nidulans,* dark respiration was suppressed immediately after exposure to photoinhibitory light (Shyam et al., 1993),

while in *Chlamydomonas reinhardtii*, dark respiration declined after an initial rapid increase at supra-optimal light (Singh et al., 1996).

The protoplasts did not lose the integrity of plasma membrane under 0.3 M sorbitol, which was lost marginally at 0.1 M and 0.2 M sorbitol due to breakage and resealing of plasma membrane. On the other hand, treatment of protoplasts with osmoticum greater than 0.4 M sorbitol (isotonic) caused marginal shrinkage in plasma membrane up to 1.0 M sorbitol (Fig. 5.5). Therefore, we restricted our studies to 0.3 M sorbitol and 1.0 M sorbitol for examining the effects of hypo- and hyper-osmotic stress on photosynthesis in presence of antimycin A and SHAM.

In pea, restriction of COX or AOX pathway caused marked reduction in Calvin cycle activity but not of photochemical reactions at saturating light intensities (Padmasree and Raghavendra, 2001a). But in broad bean inhibition of COX or AOX pathway at saturating PPFD caused remarkable decrease in both steady state photosynthetic O_2 evolution rates and operating efficiency of PSII (ϕ II) (Yoshida et al., 2006). In the present study, restriction of COX or AOX pathways caused marked reduction in PSII activity under high light and supra-optimal temperatures but not under osmotic and sub-optimal temperatures (Fig.5.6). Our results are in agreement with those of Saradadevi and Raghavendra (1994) which suggested that the benzoquinone dependent O_2 evolution was not sensitive to osmotic stress. Bartoli et al. (2005) reported that AOX inhibition in leaves of drought-exposed wheat caused over-reduction of PSII. Taken together these results suggest AOX plays an important role in maintaining the photosynthetic electron transport chain in a more oxidized state, especially under stressful conditions.

Previous reports suggested that respiration provides ATP to repair proteins degraded during photoinhibition, particularly D1 protein of the PS II in chloroplasts (Atkin et al., 2000a). Therefore in order to evaluate this suggested function of mitochondria in meeting ATP demands for D1 protein turnover, we monitored the effects of mitochondrial inhibitors on D1 protein levels during steady state photosynthesis under abiotic stress conditions. Hyper-osmotic stress caused marginal decrease (17% of control) in D1 protein which was in correlation to reduction in ATP in presence of antimycin A. On the other hand, under sub-optimal temperatures inspite of decrease in ATP, antimycin A caused increase ($\leq$17% of control) in D1 proteins (Figs. 5.7 – 5.9).

Excess light intensities can generate harmful singlet oxygen ($^1O_2$). Due to the short life time of the $^1O_2$, it is possible that the oxidative damage is confined to PSII and targets the D1 protein for degradation (Takahashi et al., 2002; Nishiyama et al., 2006). In the present study though we observed remarkable decrease in PSII activity and increase in ROS levels under HL in presence of mitochondrial inhibitors, the changes in D1 protein were negligible (Figs. 5.6-5.8, 5.13). These results suggest that both COX and AOX pathways benefit photosynthesis by preventing over-reduction of PSII and degradation of D1 protein under HL. The increased ROS under HL in presence of antimycin A and SHAM might have different role in fine-tuning the beneficial interactions between chloroplasts and mitochondria as suggested in chapter 1 (Fig. 5.13). The general loss of PSII and D1 protein under high light observed in the present study in the absence of inhibitors (Figs. 5.6B and. 5.7A) were in agreement with studies of Bertamini and Nedunchezlan (2004) on leaves of *Vitis berlandieri* and

Vitis rupestris, whose results also emphasized the loss in PSII activity and D1 protein on illumination under high light.

Our results showed a substantial decrease in cellular ATP and ATP/ADP ratios under all abiotic stress conditions (Figs. 5.9 A & 5.10A). When electron transport through cytochrome pathway was restricted the decrease in cellular ATP and ATP/ADP was further promoted indicating the importance of cytochrome pathway in meeting cellular energy requirements even under stress (Fig 5.10). COX pathway is coupled to oxidative phosphorylation via proton translocation. Therefore, the inhibition of COX pathway results in the decrease of mitochondrial ATP synthesis. SHAM had marginal effect on cellular ATP or ATP/ADP ratio (Figs. 5.9B & Fig.5.10B). As electrons flow from ubiquinol to AOX, the rate of ATP synthesis is no more than one-third of the maximum rate (Noguchi et al., 2001). Therefore, it is logical that inhibition of AOX by SHAM has marginal effect on Cellular ATP/ADP ratio. Further it is interesting that despite marginal effects of SHAM on the ATP/ADP ratio the decrease in photosynthesis and increase in ROS was always marked (Figs. 5.3 & 5.13). Using the mitochondrial electron transport inhibitor oligomycin, Krömer et al. (1993) showed that inhibition of mitochondrial ATP synthesis caused a decline in cytosolic ATP/ADP ratios and a substantial decrease in SPS activity a cytosolic enzyme associated with sucrose biosynthesis in mesophyll cells.

Sucrose biosynthesis in the cytosol requires a continuous supply of carbon skeletons and energy. Although it is obvious that chloroplasts play a significant role in supplying the carbon compounds for the synthesis of sucrose, the relative importance of mitochondria in meeting the cytosolic demands for ATP, particularly during sucrose formation is emphasized only during the last decade (Raghavendra et al., 1994;

Gardeström and Lernmark, 1995; Krömer, 1995; Hoefnagel et al., 1998; Padmasree and Raghavendra, 1998; Atkin et al., 2000b). Studies with a starchless mutant NS 458 of *Nicotiana tabacum* (defective in plastid phosphoglucomutase) in the presence of oligomycin also suggested that the mitochondrial supply of ATP could affect assimilate partitioning into sucrose and thereby modulate photosynthesis (Hanson, 1992). The transfer of redox equivalents generated during the oxidation of TCA cycle intermediates along the mitochondrial electron transport chain accumulate significant amounts of ATP in the mitochondrial matrix. Mitochondria have a very high capacity for ATP synthesis, in fact higher than that of chloroplasts, producing up to 3 ATP per NAD(P)H compared with 1.5 to 2.0 ATP per NAD(P)H in the chloroplast (Hoefnagel et al., 1998; Siedow and Day, 2000). It is possible that the ATP pools generated in the mitochondrial matrix are translocated to cytosol (through adenylate translocator) to be used in sucrose synthesis (Fig. 1.2, Chapter 1; Hoefnagel et al., 1998). It is possible in light that mitochondrial respiration is subjected to adenylate control (Hoefnagel et al., 1998).

However, the degree to which mitochondrial ATP supply in the light required for optimal photosynthesis depends on the balance of ATP production and consumption in chloroplasts. Two key observations indicate the primary role of mitochondria in assisting chloroplasts in meeting the cytosolic demands of ATP for sucrose synthesis: (1) An increase in cytosolic or cellular levels of glucose-6-P and other phosphates (e.g., fructose-6-P and fructose-2,6-bisphosphate) in the presence of oligomycin or antimycin A (Krömer and Heldt, 1991a; Krömer et al., 1993; Padmasree and Raghavendra, 1999c). Subcellular analysis of protoplasts revealed that the increase in hexose monophosphates was mostly in the cytosol, demonstrating the restriction of sucrose

biosynthesis (Krömer et al., 1992); (2) Restriction of mitochondrial ATP synthesis by oligomycin or antimycin A or photorespiratory glycine oxidation using AAN in isolated protoplasts caused a marked reduction in ATP/ADP ratios in the cytosolic and mitochondrial compartments than that of chloroplasts (Gardeström et al., 1987; Gardeström and Wigge, 1988; Krömer and Heldt, 1991a; Krömer et al., 1993; Igamberdiev et al., 1998).

The Glc-6-P level increased by about 19 to 30% in the presence of both oligomycin and antimycin A at optimal CO_2 conditions. The marked increase in Glc-6-P in mesophyll protoplasts in the presence of only oligomycin or antimycin A but not SHAM suggested that the cytochrome pathway of electron transport (and oxidative phosphorylation) modulates sucrose biosynthesis, while the alternative pathway may not have a significant role (Padmasree and Raghavendra, 1999a). The restriction of mitochondrial ATP synthesis by oligomycin and antimycin A would not only limit sucrose synthesis but also suggested to cause feedback inhibition of photosynthetic activity because the phosphate translocator in the inner chloroplast membrane is regulated by the equilibrium of the triose-P concentration in the stroma and the cytosol. However, an elevated cytosolic level of DHAP or reduced flux of DHAP from chloroplast can also lead to decreased stromal PGA level and thereby decreased Calvin cycle activity (Krömer et al., 1993, Padmasree and Raghavendra, 1999a; Flügge and Heldt, 1991). Thus, mitochondrial oxidative electron transport through COX pathway was suggested to play a significant role in optimizing photosynthetic carbon assimilation by sustaining sucrose biosynthesis even during abiotic stress (Figs. 5.9 & 5.10).

Under abiotic stress conditions over reduction of chloroplastic electron transport chain occurs. Excess reducing equivalents generated in the chloroplasts through photosynthesis is exported as malate to the cytosol (Atkin et al., 2000b), and the malate/OAA shuttle is a well known valve for the transport of reducing equivalents from chloroplasts to mitochondria (Heineke et al., 1991, Scheibe, 2004) for redox regulation in chloroplasts. It is known from the previous literature that respiration helps the plant to cope with excess photosynthetic redox equivalents (Raghavendra and Padmasree, 2003). In order to examine the importance of COX and AOX pathways of mitochondrial electron transport chain in dissipating the redox equivalents from chloroplasts and to protect the photosynthetic machinery, we examined the malate/OAA ratios in mesophyll protoplasts of pea under different abiotic stress conditions in the presence of antimycin A and SHAM. Malate/OAA ratios remarkably increased under high light and osmotic stress (Fig.5.12A) suggesting the accumulation of reducing equivalents in the chloroplasts. Upon restriction of ETC by antimycin A and SHAM the promotion in the increase of the ratios was observed (Fig.5.12B) indicating the importance of oxidative electron transport pathway for dissipation of accumulated reducing equivalents from chloroplasts even during stress. The promotion in the increase in Mal/OAA was more when the AOX pathway was restricted suggesting the relative importance of AOX pathway over COX pathway in dissipation of reducing equivalents. Up-regulation of mitochondrial alternative oxidase (AOX), accompanied by the accumulation of reducing equivalents in the chloroplast stroma and mal/OAA activity was observed by Yoshida et al. (2007) in *Arabidopsis thaliana* under high light.

According to Taylor et al. (2004) the major factors for ROS generation in plants during abiotic stress conditions are either the disruption or inhibition of mitochondrial electron transport chain or photosynthetic apparatus, although ROS are generated at many other sites in the cell. There are many reports suggesting the involvement of ROS to cause the photodamage to PS II and finally cleave the D1 protein invitro (Ananyev et al., 1992; Chen et al., 1992; Miyao et al., 1995; Okada et al., 1996; Nishiyama et al., 2006). Our results showed a significant increase in ROS levels when mitochondrial electron transport is restricted using antimycin A or SHAM (Fig. 5.13). The increased ROS levels due to the inhibition of either the COX pathway or AOX pathway did not affect the D1 protein levels (Fig.5.13; Fig. 5.8) suggesting that, the ROS is acting as a signaling molecule rather than playing a role in cellular destruction. Discoveries made over the past few decades have demonstrated that ROS are not only destructive, but can also be important signals (Ledford and Niyogi, 2005). The marked promotion in the intracellular ROS parallel to the decrease in photosynthetic O_2 evolution in presence of antimycin A and SHAM (Fig. 5.3; Fig. 5.13), under various stress treatments indicates the possible role for ROS as signaling molecule to coordinate the beneficial interactions between chloroplasts and mitochondria to optimize photosynthesis even under stress conditions.

Our results suggest that mitochondrial electron transport generates more ROS when AOX pathway is restricted when compared to COX pathway (Fig. 5.13). These results indicate that ROS acts as a positive modulator to activate AOX pathway and thereby optimize photosynthetic carbon assimilation by dissipating excess reducing equivalents generated under stress conditions. Restriction of AOX pathway may also perturb extramitochondrial metabolism in such a way that other sources of ROS are

generated. Therefore the ROS that is generated upon AOX restriction by SHAM may not be emanating totally from the mitochondria but also could be extramitochondrial e.g., chloroplastic as described in chapter 4. The biological importance of this particular observation is the fact that ROS are potentially very toxic to plant cells and are produced when plants are subjected to stress. But in our case the generation of ROS is observed under stress conditions is further enhanced remarkably when the mitochondrial electron transport is restricted, thereby explaining the importance of mitochondrial electron transport chain in diminishing the ROS.

In mitochondria the first step in ROS avoidance is considered to be played by AOX activity, which may minimize over reduction of ETC and thus electron leakage (Møller, 2001). AOX could act as a means to dispose the reductant that is generated by photosynthetic electron transport. Exacerbation of mitochondrial ROS by antimycin A in transgenic cultured tobacco cells with altered levels of AOX was observed by Maxwell et al., 1999. The data presented in our study show that ETC inhibition (particularly AOX) enhances total intracellular ROS levels. This ROS might be able to intensify the signaling pathway(s) that control the expression of antioxidant defense genes from different compartments of the cell which is evident by the significant modulation in both the antioxidant molecules and antioxidant enzymes in the present study.

Accumulation of ROS is antagonized by enzymatic and non enzymatic antioxidants (Dietz et al., 2006). Under favourable conditions the biosynthesis and the activity of these antioxidants increase (Horling et al., 2003, Mittler et al., 2004) to stabilize the chloroplast redox poise (Asada, 2000). In transgenic tobacco (*Nicotiana tabacum*) which lack AOX, an increase in antioxidant defenses was observed

(Amirsadeghi et al., 2006). Therefore, in the present study we examined the pattern of the levels and activities of non-enzymatic and enzymatic antioxidants upon restriction of mitochondrial electron transport chain by antimycin A and SHAM under light, osmotic or temperature stress conditions since ROS levels are increased remarkably under this situation.

The decrease in Asc/DHA ratio was observed under light, osmotic or temperature stress conditions (Fig. 5.16C). Recently an increase in H_2O_2 content associated with the reduction in Asc level was reported in chloroplasts from pea plants subjected to salt stress (Gomez et al., 2004). Therefore we suggest that the decrease in Asc levels observed under different abiotic stress conditions in the present study could be due to the increased ROS levels. Bartoli et al. (2006) showed the over-accumulation of Asc in the leaves of AOX overexpressing plants than wild type or antisense leaves. In support of this hypothesis the Asc/DHA levels significantly decreased upon restriction of AOX pathway. Upon restriction of COX pathway and AOX pathway a remarkable decrease GSH/GSSG ratio was also observed under the tested abiotic stress conditions (Fig. 5.16D). Therefore we suggest that the cellular concentration of the Asc might decrease or DHA might increase due to excess ROS generated under stress conditions. In some stressed samples no significant changes in Asc/DHA ratio and APX were observed where other antioxidant enzymes may function. Inspite of remarkable decrease in the APX activity in protoplasts treated with antimycin A and SHAM under sub-optimal temperature no significant changes in the Asc/DHA ratio was observed (Fig. 5.17C; Fig. 5.16C). In catalase (*Cat-1*) deficient tobacco, cAPX protein and APX activity were increased by accumulated H_2O_2 under high light conditions (Willekens et al., 1997). This represents the flexibility of the plant system to vary the response of

antioxidant enzymes to particular stress. The dramatic increase in ROS observed under high light and high temperatures correlated well with the decrease in the activities of APX, MDAR and GR under high light and supra-optimal temperature (Fig. 5.3A; Table 5.1). The activity of SOD increased in presence of antimycin A and SHAM under high light and sub-optimal temperature (Table.5.17). The significant rise in SOD activity under high light intensities and sub-optimal temperatures, only in the presence of mitochondrial inhibitors, but not in their absence indicates the primary role of mitochondrial oxidative metabolism in preventing ROS formation to protect photosynthesis (Table 5.1; Fig. 5.17A). The activity of CAT decreased under high light which is possibly due to enhancement of H_2O_2 levels (Fig. 5.13A; Table 5.1; Lee et al., 2001). The significant increase in CAT activity under high light in the presence of SHAM indicates the antagonistic function of AOX pathway and CAT under highlight conditions (Fig. 5.17). Mizuno et al. (2005) suggested that CAT and AOX cooperatively regulate to reduce the accumulation of ROS in potato cell suspensions. Maxwell et al. (1999) observed increase in the expression of CAT mRNA in transgenic tobacco cells suppressing AOX activity. Maxwell et al. (1999) observed increase in the expression of CAT mRNA in transgenic tobacco cells suppressing AOX activity. The relative activities of all the antioxidant enzymes were modulated differently under different abiotic stress treatments in presence of antimycin A and SHAM (Figs. 5.17-5.20).

Our results suggest that under all abiotic stress conditions examined in the present study, mitochondrial oxidative metabolism through COX and AOX optimizes chloroplastic carbon assimilation primarily, while protection against PSII and D1 degradation was secondary (Fig. 5.21; Fig. 5.22). Further, figures 5.21 and 5.22 clearly

visualize the changes in all the biochemical factors examined in the present study in presence of antimycin A and SHAM under HL, 0.3 M sorbitol, 1.0 M sorbitol, 10 ^{o}C or 25 ^{o}C temperature. COX pathway of mitochondrial electron transport optimizes different components of photosynthesis primarily through changes in ATP/ADP ratios under all abiotic stresses, while the role of ROS and redox was secondary (Fig. 5.21). AOX pathway of mitochondrial electron transport optimizes different components of photosynthesis primarily through ROS and redox, while the role of ATP/ADP was secondary. The significance of adenylates, ROS and redox in mediating the beneficial interactions between chloroplasts and mitochondria during abiotic stress conditions is highly appreciated when the changes observed in each of these biochemical factors are applied in the model proposed in the Chapter 4. ROS and antioxidant molecules Asc and GSH fine tune the metabolic interactions between chloroplasts and mitochondria under different abiotic stress conditions by regulating the activities of antioxidant enzymes, which is evident by the increase in the Fe-SOD and Cu/Zn-SOD (chloroplastic, cytosolic, peroxisomal isoforms) along with increase in the Mn-SOD (mitochondrial isoform) (Fig. 5.18).

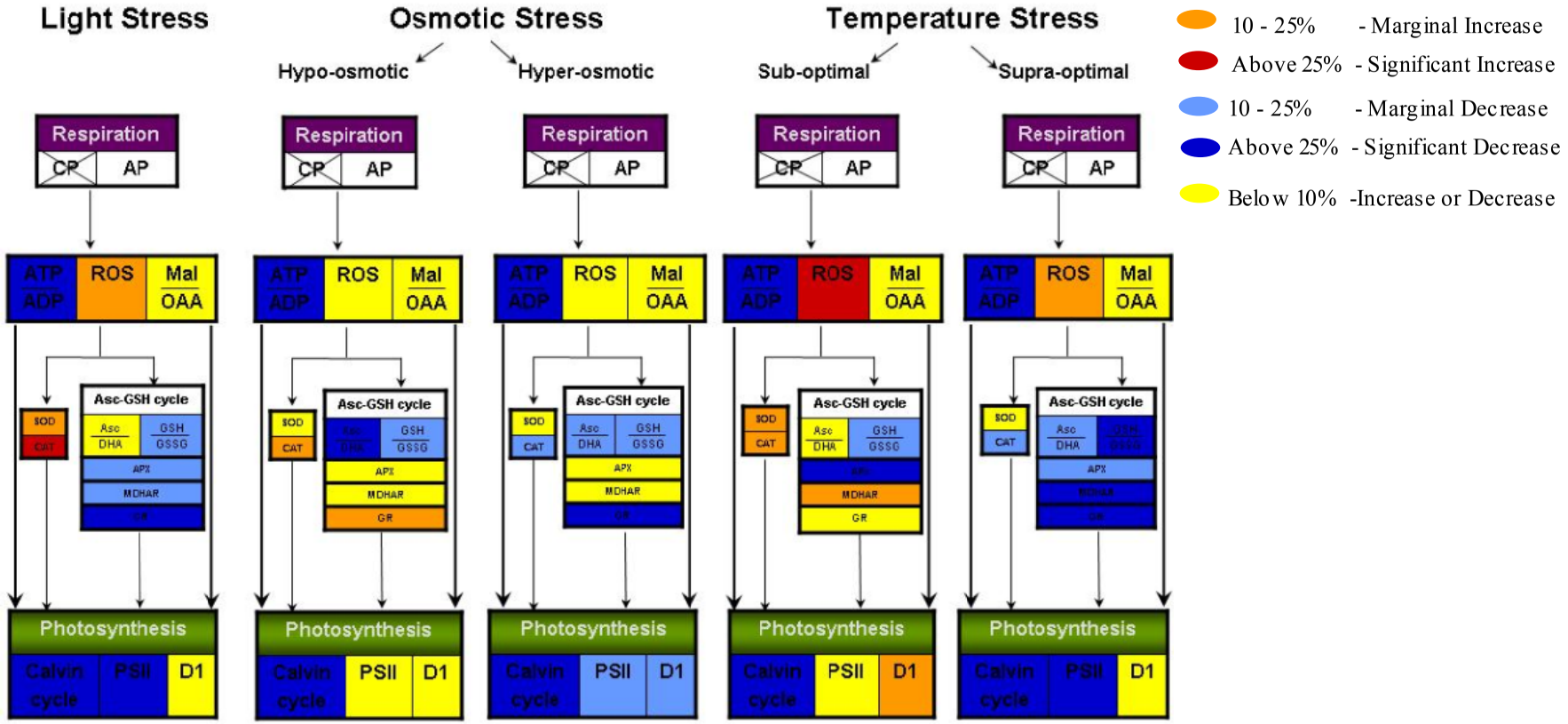

Figure 5.21. Effect of restriction of COX pathway (CP) of mitochondrial electron transport chain on different components of photosynthesis (calvin cycle, PS II, D1) under HL, hypo- (0.3 M sorbitol) / hyper- (1.0 M sorbitol) osmoticum (or) sub- (25 ^{o}C) / supra- (40 ^{o}C) optimal temperature. The changes in different biochemical components related to cellular adenylates (ATP/ADP), redox (Mal/OAA), ROS, antioxidant metabolites and enzymes in the presence of antimycin A (AA) under different abiotic stresses were represented. The colours indicated on the right hand side of the figure indicate corresponding increase or decrease in the levels of the different biochemical factors examined in the present study.

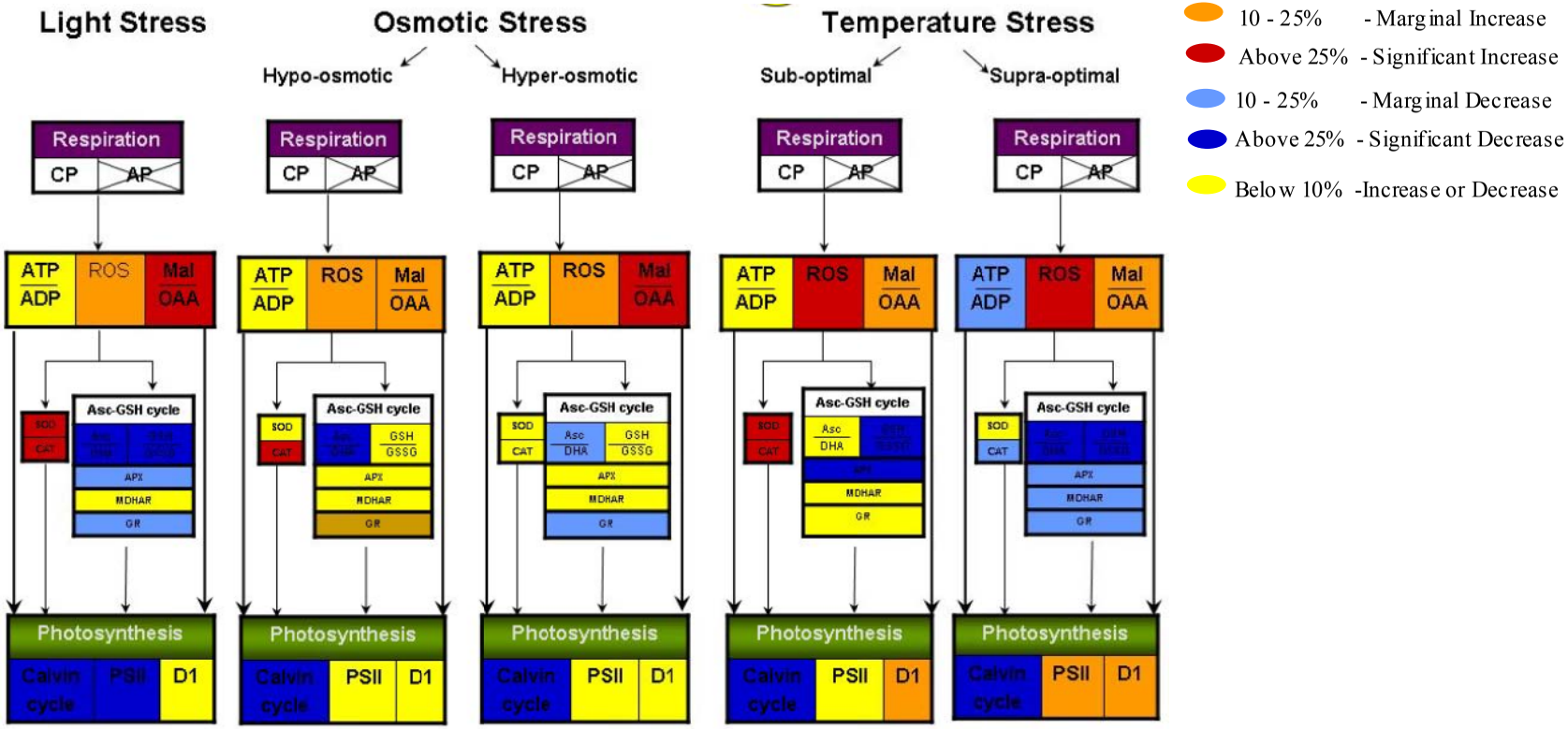

Figure 5.22. Effect of restriction of AOX pathway (AP) of mitochondrial electron transport chain on different components of photosynthesis (calvin cycle, PS II, D1) under HL, hypo- (0.3 M sorbitol) / hyper- (1.0 M sorbitol) osmoticum (or) sub- (25 ^{o}C) / supra- (40 ^{o}C) optimal temperature. The changes in different biochemical components related to cellular adenylates (ATP/ADP), redox (Mal/OAA), ROS, antioxidant metabolites and enzymes in the presence of salicylhydroxamic acid (SHAM) under different abiotic stresses were represented. The colours indicated on the right hand side of the figure indicate corresponding increase or decrease in the levels of the different biochemical factors examined in the present study.

Chapter 6

Relative Contribution of Cytochrome Pathway and Alternative Pathway to Total Respiration under Light, Osmotic or Temperature Stress

Chapter 6

Relative Contribution of Cytochrome pathway and Alternative pathway to Total Respiration under Light, Osmotic or Temperature stress

Introduction

The abiotic stresses light, osmoticum or temperature caused significant reduction in both photosynthesis and respiration. In presence of these abiotic stresses, even a marginal interference in respiration due to restriction of COX pathway or AOX pathway caused remarkable decrease in photosynthetic carbon assimilation in mesophyll protoplasts of pea (Chapter 2). Thus the strong positive correlation between the responses of photosynthesis and respiration provoked to examine the relative activities of COX and AOX pathways to total respiration under each of the abiotic stress condition choosen in the present study: light, osmoticum (hypo- / hyper-) and temperature (sub- / supra-) stress.

The responses of the plant respiration to abiotic stress are known to vary with the intensity or magnitude of the stress and also with the duration of the treatment or exposure to such stress factors (Poorter et al., 1992, Collier and Cummins 1996, Lambers et al., 1998). The relative contribution of the COX pathway and the AOX pathway to total respiration is known to be flexible and depends on the environmental conditions (Gonzalez-Meler et al., 1999). However, several reports have suggested that changes in electron partitioning between the two respiratory pathways under a given stress were mostly due to the decrease in the activity of the COX pathway rather than the increase in the activity of the AOX pathway (Peñuelas et al., 1996; Lambers et al., 2005). While Gonzalez-Meler et al. (1997) showed in bean and pepper leaves that water stress decreased SHAM-resistant respiration, with no effect on KCN- resistant

respiration, Ribas-Carbo et al. (2005) showed that severe water stress caused a significant shift of electrons from the COX to the AOX pathway. Electron distribution / partitioning between the AOX and COX pathways during steady-state respiration has been suggested as a crucial measurement to quantitatively analyze the effects of the various levels of regulation of the alternative oxidase (Sluse and Jarmuszkiewicz, 1998).

The partitioning of electrons to AOX is regulated in a dynamic manner, which is dependent upon both the carbon and redox status of the mitochondrion. Both matrix pyruvate level and the redox state of matrix NAD(P) alter the kinetic properties of AOX, modulating its ability to compete with the COX pathway for electrons. Metabolic conditions that lead to accumulation of reducing equivalents and pyruvate in the mitochondrial matrix will favour partitioning of electrons towards AOX. Such conditions arise when there is an imbalance between upstream carbon metabolism and downstream electron transport, for example during shifts in metabolism, developmental changes, nutrient availability, biotic and abiotic stress conditions (Vanlerberghe and Ordog, 2002). Accumulation of cellular reducing equivalents in the form of malate during treatment of mesophyll protoplasts with HL, 0.3M sorbitol (hypo-osmotic) and 1.0 M sorbitol (hyper-osmotic stress) was evident from our reported results in the chapter 2 (refer, Fig. 5.11A).

The usage of KCN was highly essential to determine the contribution of COX / AOX pathways to total respiration by using the method of metabolic inhibitors (Vani and Raghavendra, 1994). Since KCN is highly hazardous to the mankind, we evaluated the usage of antimycin A in place of KCN during titration experiments to assess the relative contribution of COX and AOX pathways to total respiration.

Results

The respiratory rates of mesophyll protoplasts of pea were assessed in the presence of wide range of concentrations of KCN (inhibitor of complex IV) and antimycin A (complex III) in reaction medium containing 0.4 M sorbitol (iso-osmoticum) at 25 ^{o}C (Fig. 6.1). As the concentration of KCN (0 to 0.4 mM) or antimycin A (0 to 2.0 mM) was raised, the respiratory rates decreased by ≤50% of their control rates (27.45 ± 1.67 μmoles mg^{-1} Chl h^{-1}). At 0.1 mM of KCN and 1 mM of antimycin A saturation kinetics in respiratory O_2 uptake was observed (Fig. 6.1). However, the respiratory rates of mesophyll protoplasts were not completely suppressed by these inhibitors even at high concentrations of 0.4 mM KCN or 2.0 mM antimycin A, suggesting the presence of a cyanide / antimycin A resistant AOX pathway in mesophyll protoplasts (Fig. 6.1, A and B). Subsequently, the operation of AOX pathway and its contribution to total respiration in mesophyll protoplasts was ascertained by monitoring the respiratory rates in the presence of a wide range of concentrations of SHAM (0 to 10 mM) with and without COX pathway inhibitors: KCN (0.1 mM) or antimycin A (1 mM) (Fig. 6.2). The respiratory O_2 uptake was more sensitive to SHAM in the presence of KCN or antimcyin A (decreased by <65% of control rates) when compared to treatment with SHAM alone (decreased by <29% of control rates) (Fig. 6.2, A and B).

Table 6.1 represents the relative proportions of the COX pathway, AOX pathway (along with its engagement) and residual respiration derived in mesophyll protoplasts using KCN or antimycin A as COX pathway inhibitors. The replacement of KCN with antimycin A did not show any deviation in the corresponding activities of COX or AOX pathway, along with engagement of AOX pathway. Therefore, antimycin A was successfully used in all further experiments to determine the relative

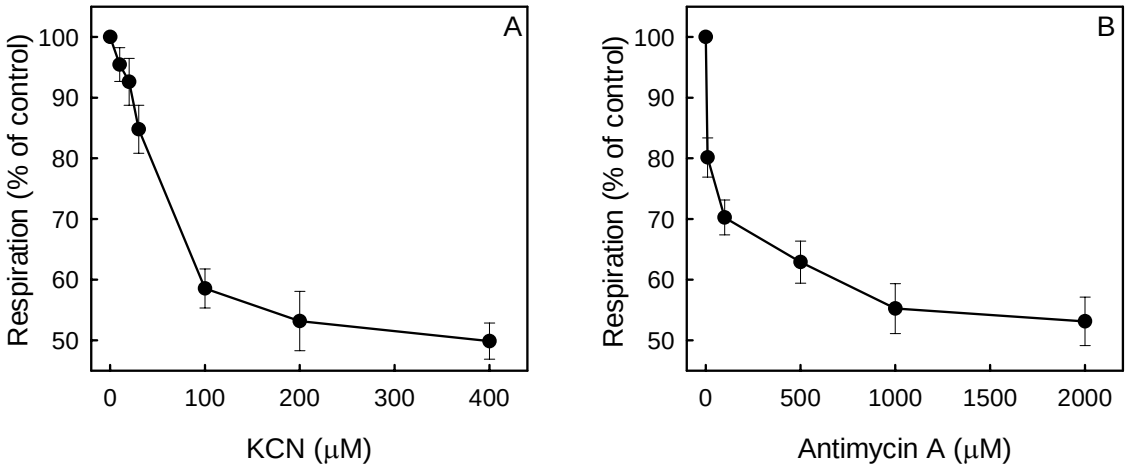

Figure 6.1. The sensitivity of respiration to increasing concentrations of KCN (A) and antimycin A (B) in mesophyll protoplasts of pea. The respiratory rates in control in the absence of KCN and antimycin A was 27.45 ± 1.67 μmol mg^{-1} Chl h^{-1}.

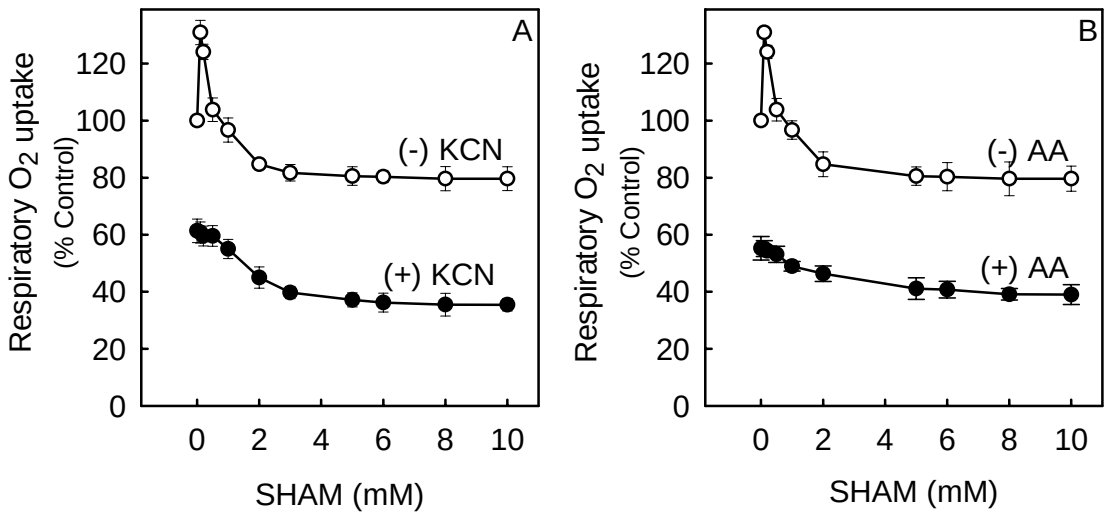

Figure 6.2. The sensitivity of respiration in pea mesophyll protoplasts to SHAM in the absence and presence of 0.1 mM KCN (A) and 1.0 mM antimycin A [AA] (B). The respiratory O_2 uptake rates (μmol O_2 mg^{-1} Chl h^{-1}) in the control sets were 27.45 ± 1.67 (no inhibitor), 16.83 ± 1.87 (0.1 mM KCN) and 15.16 ± 1.32 (1.0 mM AA).

Table 6.1. Comparison of the relative proportion of the COX and AOX respiration in mesophyll protoplasts of pea derived using KCN or antimycin A (AA) to inhibit COX pathway. The percent of COX and AOX pathways from total respiration, residual respiration and fraction of the capacity of the alternative pathway engaged (ρ) were calculated using Fig. 6.2 as described in materials and methods.

Parameter (Activity)	**KCN (0.1 mM)**	**AA (1.0 mM)**
COX pathway (%)	41.99 ± 3.12	39.43 ± 3.76
AOX pathway (%)	18.28 ± 2.13	19.45 ± 2.64
Residual respiration (%)	39.73 ± 2.97	41.12 ± 4.13
Engagement of AOX pathway (ρ)	0.788	0.792

contribution of COX and AOX pathways to total respiration under increasing light intensities, hypo- /hyper-osmotic stress or sub- / supra-optimal temperatures.

The sensitivity of respiratory O_2 uptake to SHAM (0 to 10 mM) both in the presence and absence of antimycin A under increasing light intensities was represented in Figure 6.3 (A-G). Under each choosen light intensity, treatment of mesophyll protoplasts by SHAM was over-lapped with antimycin A at a concentration where saturation kinetics in COX- linked respiratory O_2 uptake was initiated (data not shown). As the light intensity was increased from darkness to 3000 $\mu E\ m^{-2}\ s^{-1}$, the respiratory O_2 uptake decreased upto <73% in presence of SHAM (without antimycin A) and <84% in presence of SHAM (with antimycin A) (Fig. 6.3, A-G). The relative contribution of COX pathway or AOX pathway (and AOX capacity) along with the residual respiration under different light intensities was represented in Table. 6.2. Data presented in Table 6.2 suggests that the magnitude of the AOX pathway increased from 26% in darkness to <73% with increasing light intensity (3000 $\mu E\ m^{-2}\ sec^{-1}$). On the other hand, COX pathway decreased from <33% (in darkness) to <11% at high light intensities (up to 3000 $\mu E\ m^{-2}\ sec^{-1}$). Similar to COX pathway, the residual respiration decreased from 41% (in darkness) to 16% (at 3000 $\mu E\ m^{-2}\ sec^{-1}$). However, the fraction of the total capacity of AOX pathway that is engaged (ρ) also changed with increasing light intensity, i.e., it increased from 0.808 (in darkness) to 0.990 (at 3000 $\mu E\ m^{-2}\ sec^{-1}$) (Table 6.2).

Similar to high light intensities, the sensitivity of respiratory O_2 uptake to SHAM (without antimycin A) and SHAM (with antimycin A) were monitored both under hypo- and hyper-osmotic stress (Fig. 6.4, A-G). As the concentration of sorbitol decreased from 0.4 M to 0.1 M or increased from 0.4 M to 1.0 M in the reaction

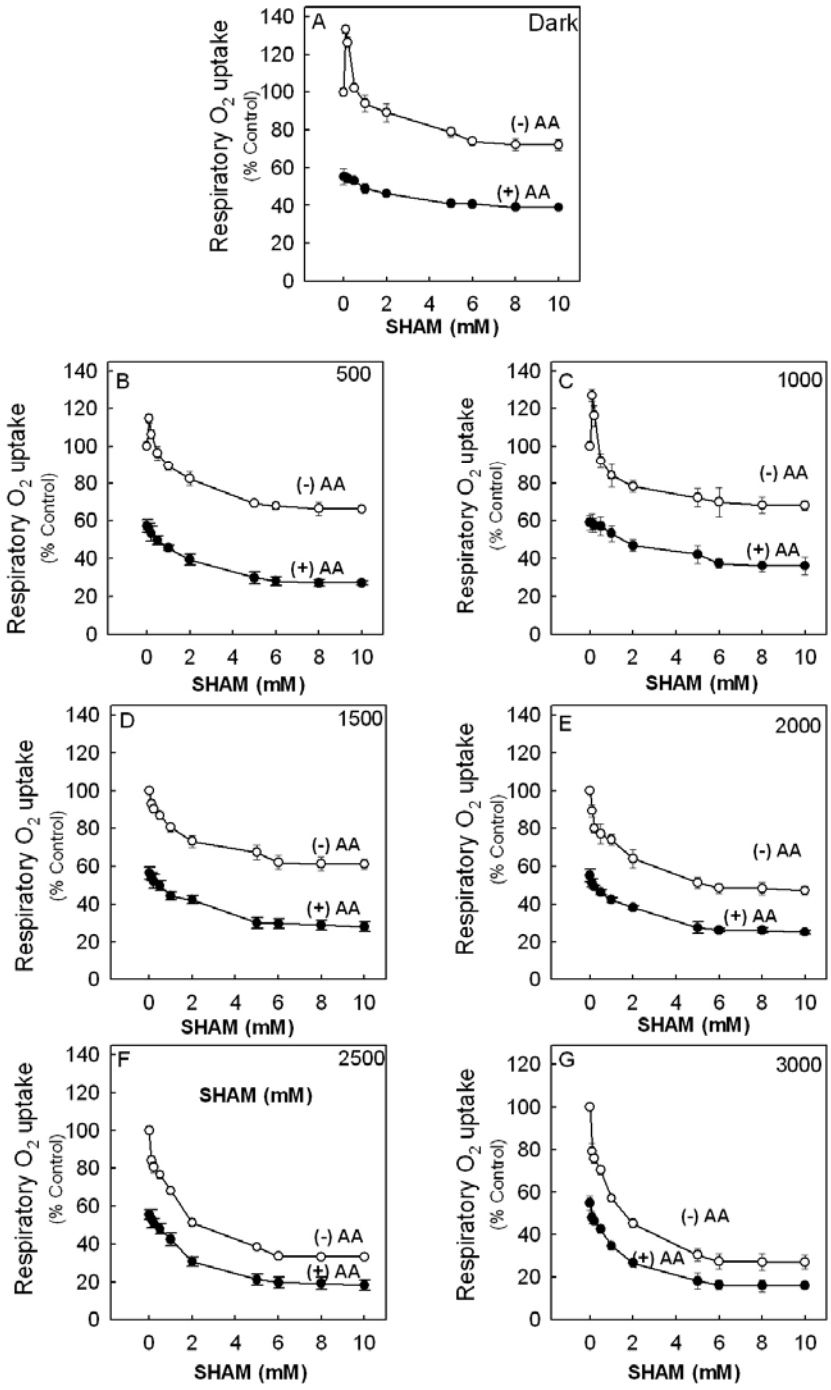

Figure 6.3. The sensitivity of respiration in pea mesophyll protoplasts to SHAM, with or without antimycin A (AA) to increasing light intensities. The control rates of respiration (μmol O_2 mg^{-1} Chl h^{-1}) were 26.24 ± 0.98 under darkness (A), 27.49 ± 1.23 at 500 (B), 28.8 ± 1.67 at 1000 (C), 28.3 ± 1.45 at 1500 (D), 27.18 ± 1.45 at 2000 (E), 25.02 ± 1.78 at 2500 (F) and 23.41 ± 1.89 at 3000 (G) μE m^{-2} sec^{-1} respectively.

Table 6.2. Relative proportion of the activities of COX pathway, AOX pathway, and residual respiration to total respiration in mesophyll protoplasts of pea at different light intensities. The percent of COX and AOX pathways to total respiration; residual respiration and engagement of AOX pathway (ρ) were calculated from Fig. 6.3 as described in materials and methods.

Light stress ($\mu E\ m^{-2}\ sec^{-1}$)	**COX Pathway (%)**	**AOX Pathway (%)**	**Residual Respiration (%)**	**Engagement of AOX pathway (ρ)**
Darkness	32.77 ± 2.34	26.11 ± 2.11	41.12 ± 3.98	0.808
500	38.00 ± 3.62	32.13 ± 2.67	29.87 ± 3.12	0.947
1000	32.39 ± 3.47	30.11 ± 2.64	37.5 ± 3.87	0.949
1500	31.94 ± 4.02	37.92 ± 2.89	30.14 ± 3.12	0.967
2000	21.08 ± 2.14	51.43 ± 4.89	27.49 ± 3.76	0.974
2500	13.71 ± 2.09	65.71 ± 4.67	19.68 ± 2.54	0.977
3000	11.01 ± 2.36	72.61 ± 6.34	16.38 ± 2.78	0.990

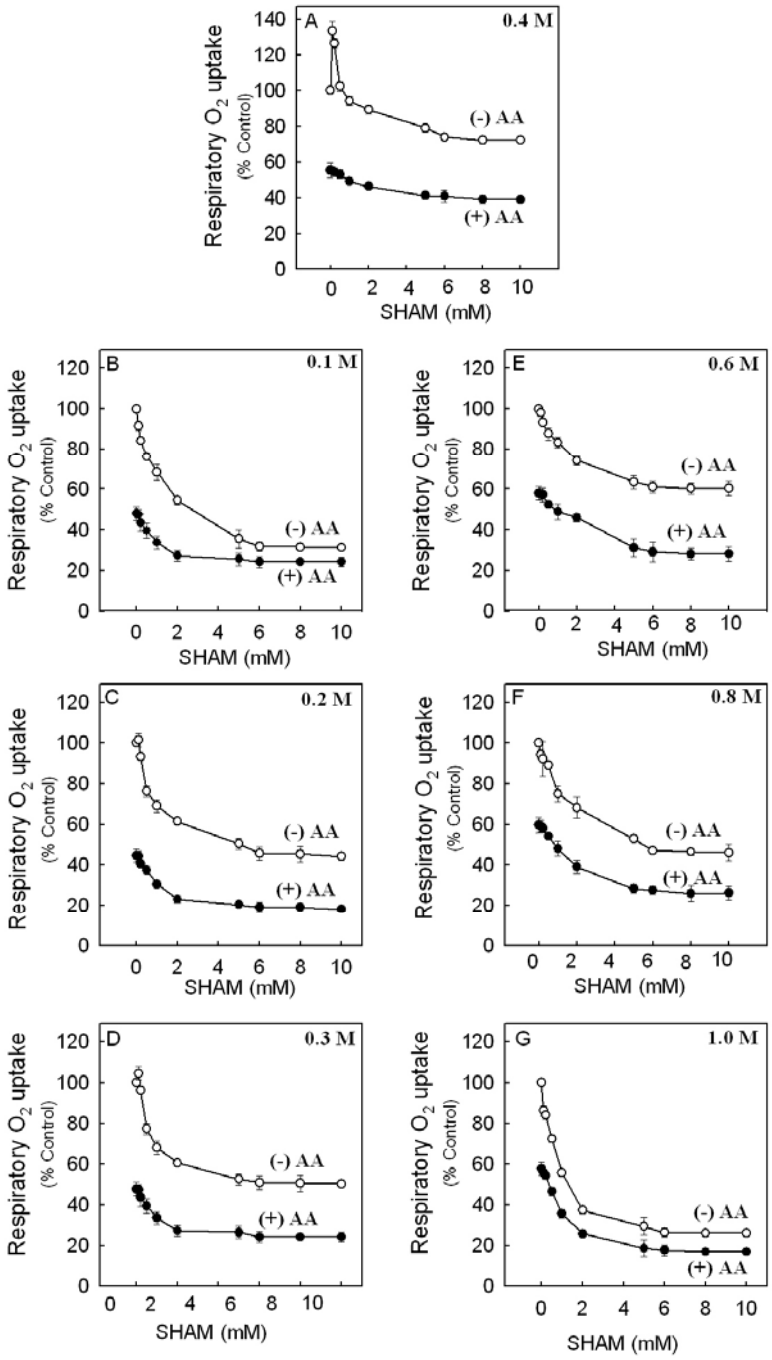

Figure 6.4. The sensitivity of respiration in pea mesophyll protoplasts to SHAM (with or without antimycin A) at hypo- (0.4 M to 0.1 M sorbitol) and hyper- (0.4 M to 1.0 M sorbitol) osmotic stress. The control rates of respiration (μmol O_2 mg^{-1} Chl h^{-1}) were 26.45 ± 2.1 at 0.4 M (A), 23.01 ± 0.98 at 0.1 M (B), 23.07 ± 1.19 at 0.2 M (C), 24.63 ± 0.74 at 0.3 M (D), 25.43 ± 1.32 at 0.6 M (E), 25.19 ± 1.23 at 0.8 M (F), and 24.47 ± 1.12 at 1.0 M (G) sorbitol respectively.

medium, the respiratory O_2 uptake of mesophyll protoplasts decreased by ≤74% and ≤83%, respectively, in presence of SHAM (with and without antimycin A). Table 6.3 represents the relative contribution of COX and AOX pathways to total respiration both under hypo- and hyper-osmotic stress. While the contribution of COX pathway and residual respiration decreased upto <9 % and <18% respectively under hypo- / hyper-osmotic stress, the contribution of AOX pathway increased significantly upto ≤73%. The total capacity of AOX pathway that is engaged (ρ) also increased from 0.808 (0.4 M sorbitol) to 0.958 (0.1 M sorbitol) and 0.987 (1.0 M sorbitol), respectively.

Figure 6.5 (A-G) shows the SHAM sensitive respiration (with or without antimycin A) at sub-optimal (below 25 ^{o}C) and supra-optimal (above 25 ^{o}C) temperatures in mesophyll protoplasts. Treatment of mesophyll protoplasts with either sub- (25 ^{o}C to 10 ^{o}C) or supra-optimal (25 ^{o}C to 50 ^{o}C) temperatures decreased the SHAM sensitive respiratory O_2 uptake by ≤62% (without antimycin A) and ≤77% (with antimycin A), respectively (Fig. 6.5 A-G). The relative proportion of alternative pathway increased from 26% (25 ^{o}C) to >53% (10 ^{o}C) and >60% (50 ^{o}C), where as the cytochrome pathway decreased from 33% (25 ^{o}C) to 27% (10 ^{o}C) and 15% (50 ^{o}C). Similar to COX pathway, the contribution of residual respiration decreased from 41% (25 ^{o}C) to <19% (10 ^{o}C) and 24% (50 ^{o}C). The total capacity of alternative pathway that is engaged (ρ) also increased from 0.808 (25 ^{o}C) to 0.901 (10 ^{o}C) and 0.920 (50 ^{o}C) (Table 6.4).

Mitochondrial electron transport chain is actively engaged in the synthesis and supply of ATP for cellular needs. Any modulation in COX pathway is therefore expected to be reflected on the total cellular levels of ATP. Therefore, in the present study, the changes in the total cellular levels of ATP and ADP were monitored under

Table 6.3. Relative proportion of the activities of COX pathway, AOX pathway, and residual respiration to total respiration in mesophyll protoplasts of pea at different osmoticum. The percent of COX and AOX pathways to total respiration; residual respiration and engagement of AOX pathway (ρ) were calculated from Fig. 6.4 as described in materials and methods.

Osmotic stress	Sorbitol (M)	COX Pathway (%)	AOX Pathway (%)	Residual Respiration (%)	Engagement of AOX pathway (ρ)
Control	**0.4**	32.77 ± 2.34	26.11 ± 2.11	41.12 ± 3.98	0.808
Hypo-osmotic stress	**0.1**	7.75 ± 1.02	68.13 ± 4.12	24.12 ± 3.87	0.958
	0.2	25.37 ± 2.89	54.13 ± 3.87	20.5 ± 2.98	0.972
	0.3	26.75 ± 2.12	49.13 ± 3.98	24.12 ± 2.13	0.976
Hyper-osmotic stress	**0.6**	32.08 ± 2.76	38.92 ± 3.98	29.00 ± 3.12	0.961
	0.8	19.42 ±1.89	52.88 ± 4.87	27.7 ± 3.56	0.981
	1.0	8.71 ± 1.43	73.61 ± 4.98	17.68 ± 2.84	0.987

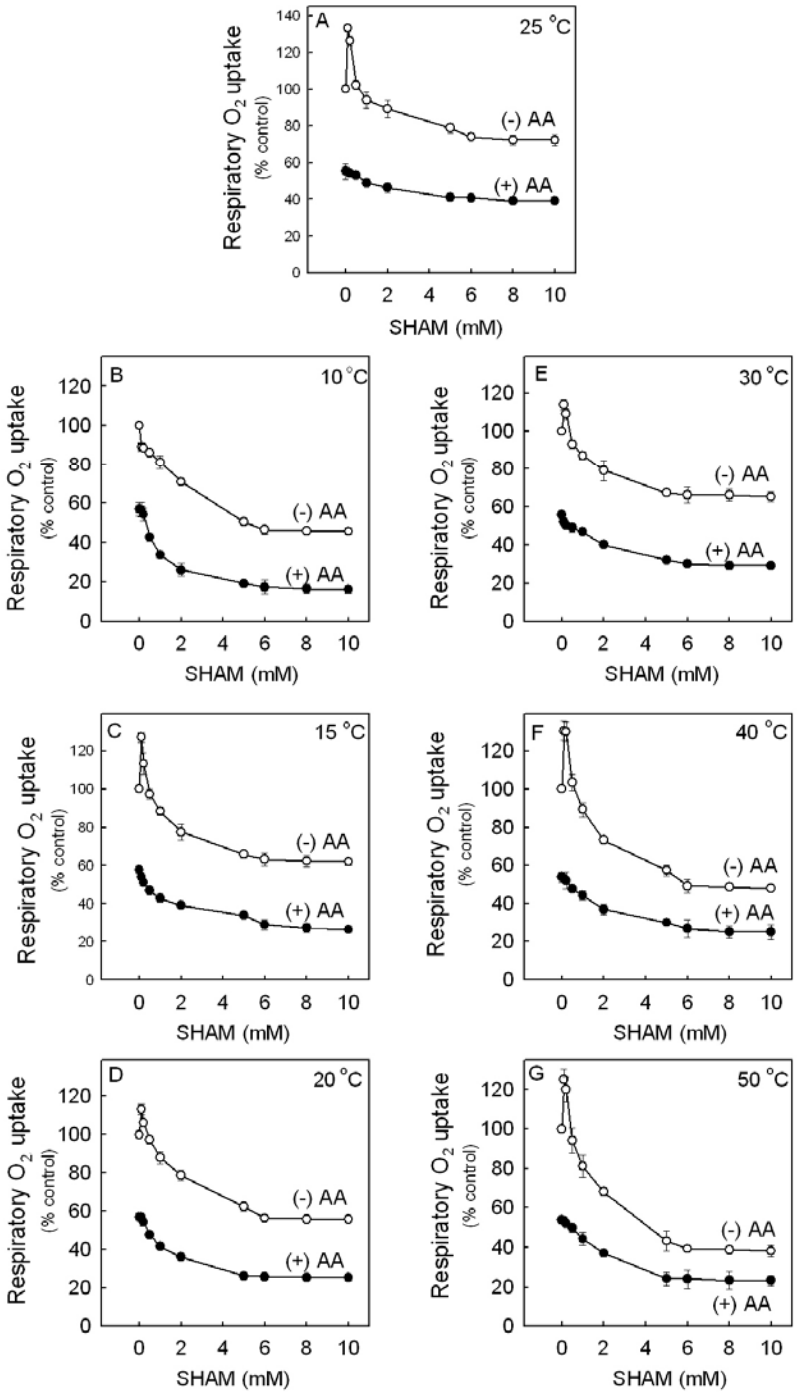

Figure 6.5. The sensitivity of respiration in pea mesophyll protoplasts to SHAM with or without antimycin A at sub- (25 ^{o}C to 10 ^{o}C) and supra- (25 ^{o}C to 50 ^{o}C) optimal temperatures. The control rates of respiration (μmol O_2 mg^{-1} Chl h^{-1}) 27.45 ± 1.67 at 25 ^{o}C (A), 23.12 ± 1.93 at 10 ^{o}C (B), 24.67 ± 1.98 at 15 ^{o}C (C), 26.17 ± 1.62 at 20 ^{o}C (D), 28.42 ± 1.12 at 30 ^{o}C (E), 27.45 ± 1.67 at 40 ^{o}C (F), and 26.07 ± 1.56 at 50 ^{o}C (G) temperature respectively.

Table 6.4. Relative proportion of the activities of COX pathway, AOX pathway, and residual respiration to total respiration in mesophyll protoplasts of pea at different temperatures. The percent of COX and AOX pathways to total respiration; residual respiration and engagement of AOX pathway (ρ) were calculated from Fig. 6.5 as described in materials and methods.

Temperature stress	(^{o}C)	COX Pathway (%)	AOX Pathway (%)	Residual Respiration (%)	Engagement of AOX pathway (ρ)
Control	**25**	32.77 ± 2.34	26.11 ± 2.11	41.12 ± 3.98	0.808
Sub-optimal temperature	**10**	27.1 ± 2.13	53.61 ± 4.98	19.29 ± 2.01	0.901
	15	34.19 ± 2.14	36.83 ± 3.62	28.98 ± 3.29	0.904
	20	30.1 ± 2.56	43.61 ± 3.87	26.29 ± 2.48	0.905
Supra-optimal temperature	**30**	37.0 ± 3.23	32.87 ± 3.63	30.13 ± 2.87	0.881
	40	22.32 ± 4.12	50.7 ± 4.12	26.98 ± 1.92	0.917
	50	15.18 ± 1.12	60.17 ± 5.98	24.12 ± 1.12	0.920

increasing light intensities, hypo-/hyper-osmoticum or sub-/supra-optimal temperatures (Fig. 6.6) and correlated the total cellular ATP levels with the COX pathway activity observed under the corresponding abiotic stresses (Fig. 6.7). As the light intensity increased from darkness to 3000 μE m^{-2} s^{-1}, there was a remarkable decrease in total cellular levels of ATP by ≤41%. Correspondingly, the ADP levels increased by ≤15% of control (darkness) on exposure of mesophyll protoplasts to high light intensities (Fig. 6.6A).

The effect of hypo-osmotic stress on the intracellular levels of ATP were marginal (decreased by >16%) while the effect of hyper-osmotic stress was significant (decreased by 51%). When compared to ATP, the changes in ADP were very marginal, which increased by only <15% both under hypo- / hyper-osmotic stress (Fig. 6.6B). Also, the changes in intracellular levels of ATP were marginal (decreased by 24%) at sub-optimal temperature (10 ^{o}C), while significant (decreased by >47%) under supra-optimal temperatures (40 ^{o}C). In contrast to ATP, the changes in ADP were more significant under sub-optimal temperature (increased by 28%), while marginal under supra-optimal temperatures (increased by 7%) (Fig. 6.6C).

A strong positive relationship was observed between changes in intracellular levels of ATP and COX pathway activity under light, hypo-/hyper-osmotic or sub-/supra-optimal temperatures (Fig. 6.7A-E). The relation between intracellular ATP and COX pathway activity in mesophyll protoplasts was linear under increasing light intensities, hypo-osmoticum, sub-optimal temperatures, while the relation was biphasic under hyper-osmoticum and sigmoidal type under supra-optimal temperatures (Fig. 6.7 A-E).

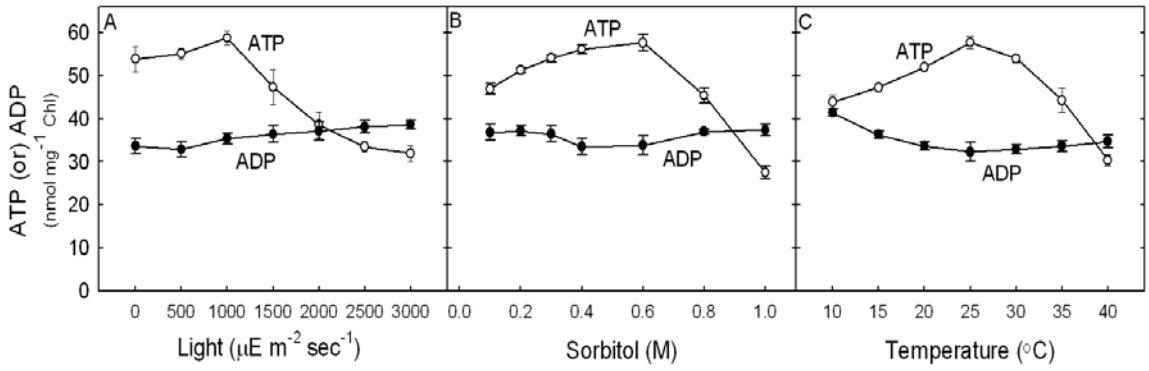

Figure 6.6. Effect of light (A), osmotic (B) or temperature (C) stress on intracellular levels of ATP (open circle) and ADP (closed circle) in pea mesophyll protoplasts. At the end of stress treatments (10 min) HCIO4 was added to the reaction medium and the samples were frozen dry in liquid nitrogen for analysis of ATP and ADP as described in materials and methods.

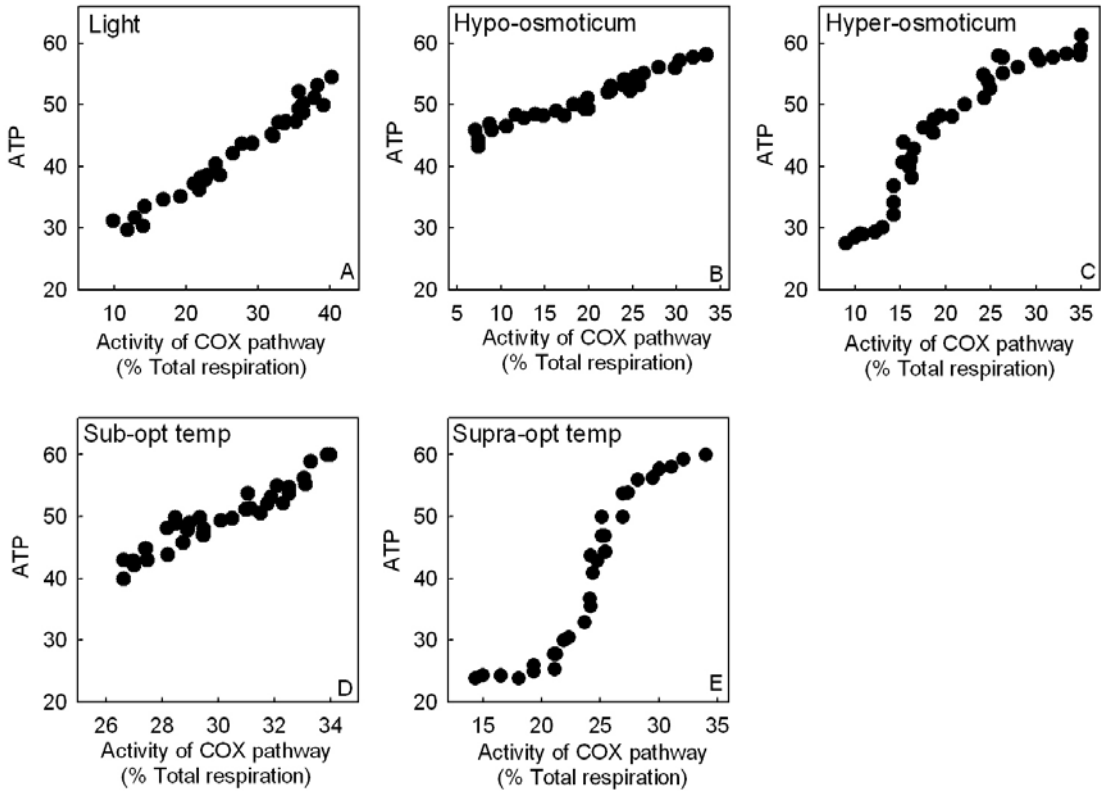

Figure 6.7. Positive correlation between the intracellular levels of ATP and the relative activities of COX pathway (as percent of total respiration) in mesophyll protoplasts of pea during light, osmotic and temperature stress. These are collective data attained at increasing light intensities: 1000 – 3000 μE m^{-2} s^{-1} (A), hypo-osmotic stress: 0.1 M to 0.4 M sorbitol (B), hyper-osmotic stress: 0.4 M – 1.0 M sorbitol (C), sub-optimal temperature10 ^{o}C – 25 ^{o}C (D) and supra-optimal temperature 25 ^{o}C – 50 ^{o}C (E).

As the electron partitioning to AOX pathway increased irrespective of the light, osmoticum or temperature stress, we also examined the effects of corresponding stresses on the AOX protein abundance in mesophyll protoplasts (Fig. 6.8). There was a remarkable increase in the monomeric active form (36 kDa) of AOX in mesophyll protoplasts on transition from darkness to saturating light (1000 μE m^{-2} s^{-1}) and high light (3000 μE m^{-2} s^{-1}), while the changes in the dimeric inactive form (72 kDa) of AOX were marginal at normal and high light when compared to darkness (Fig. 6.8A).

The effects of osmotic stress on AOX protein abundance varied under 0.3 M sorbitol (hypo-osmoticum) and 1.0 M sorbitol (hyper-osmoticum) when compared to 0.4 M sorbitol (iso-osmoticum). Both monomeric (active) and dimeric (inactive) forms of AOX increased remarkably under 1.0 M sorbitol. However, the changes in both forms were marginal under 0.3 M sorbitol (Fig. 6.8B). The effects of temperature on AOX abundance were quite different from those of light and osmotic stress (Fig. 6.8C). Treatment of mesophyll protoplasts with 10 ^{o}C (sub-optimal temperature) resulted in a significant decrease in dimeric (inactive) form of AOX. Interestingly, there was a corresponding increase in the monomeric (active) form of AOX under these conditions (Fig. 6.8C). At supra-optimal temperatures (40 ^{o}C), while there was an increase in dimeric (inactive) form, the monomeric (active) form decreased marginally when compared to their control (25 ^{o}C) (Fig. 6.8C).

The engagement of AOX pathway has also been suggested to be dependent on the pyruvate levels apart from dependence on reduction state of AOX and the amount of AOX protein. The total cellular levels of pyruvate increased remarkably under high light (60%), hypo-osmotic (43%), hyper -osmotic (121%), sub-optimal (55%) and supra

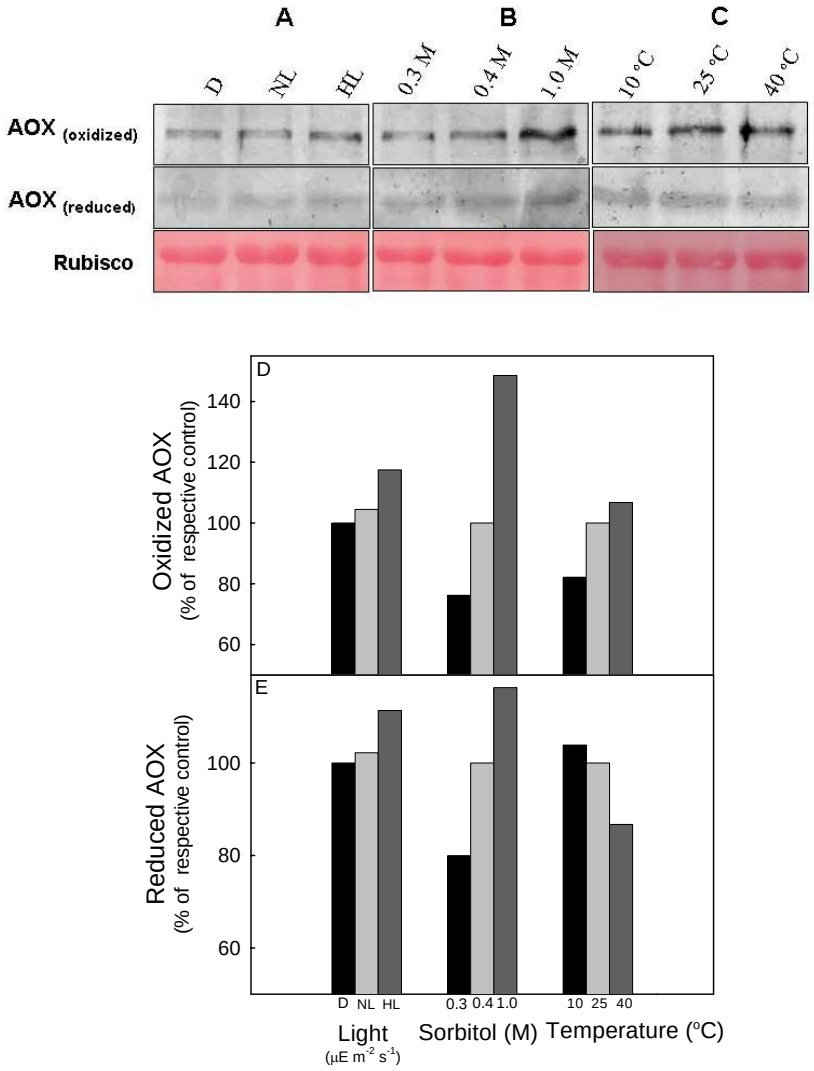

Figure 6.8. Immunodetection of reduced and oxidized forms of AOX as shown by western blots. AOX abundance in mesophyll protoplasts of pea during light (A), osmotic (B) and temperature (C) stress. Mesophyll protoplasts after exposure to stress treatments (10 min) as described in 'Materials and Methods' were separated by SDS-PAGE on 12.5% acrylamide gels (50 μg protein per lane). After electrophoretic transfer to PVDF membranes, proteins were probed with anti-AOX antibody and detected using NBT and BCIP. Equal loading of total protein was checked by Ponceau–staining of Rubisco after transfer of proteins to PVDF membrane. Quantification of oxidized (E) and reduced (F) forms of AOX was performed by Densitometry using Image J software 1.37 V, National Institute of Health, USA. Controls (without stress) were treated as 100% and the increase or decrease in the density of band with stress treatment were shown as % of respective control.

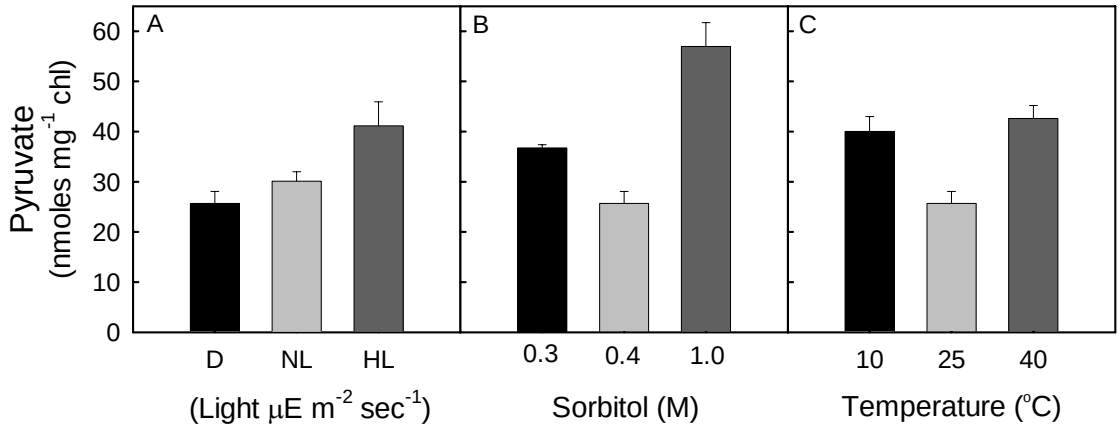

Figure 6.9. Effect of light (A), osmotic (B) and temperature (C) stress on intracellular levels of pyruvate in pea mesophyll protoplasts. At the end of the stress treatments (10 min) HClO4 was added to the reaction medium and the samples were frozen dry in liquid nitrogen for analysis of pyruvate as mentioned in materials and methods.

optimal temperature (66%) when compared to their controls without stress (Figure 6.9A-C).

Discussion

The initial experiments established that antimycin A was as effective as KCN in inhibiting the COX pathway (Fig. 6.1). Antimycin A was often used to restrict complex III of the mitochondrial oxidative electron transport chain in plants (Millar and Day, 1996; Chew et al., 2003, Bykova et al., 2005; Amirsadeghi et al., 2006; Sweetlove et al., 2007; Naydenov et al., 2008). Cyanide has been used as an inhibitor in several in vivo studies in classic titration experiments to determine the relative contribution/partitioning of electrons to the COX and AOX pathways (Vani and Raghavendra, 1994; Dwivedi et al., 2003). The titration values obtained in the presence of cyanide or antimycin A give the maximum possible flux through the AOX pathway in the presence of a given SHAM concentration. The values obtained in the absence of KCN or antimycin A give the total flux of electrons at each SHAM concentration (Fig. 6.2; Lambers, 1982). Also, treatment with antimycin A can divert the electron flow to alternative pathway and can show an increased O_2 uptake if the capacity of alternative pathway is high (Vanlerberghe and McIntosh 1992b).

Under the given set of experimental conditions, the contribution of COX pathway, AOX pathway (and its engagement) to total respiration were identical in mesophyll protoplasts of pea by using either KCN or antimycin A in titration experiments along with SHAM (Table 6.1). Due to the highly toxic nature of KCN to mankind, we suggest that KCN can be avoided and antimycin A can be successfully employed in its place to assess the partitioning of electrons between COX and AOX pathways. Atkin et al. (2002) used antimycin A to assess the alternative pathway activity

in soya bean cotyledon mitochondria at various temperatures. However, Antimycin A (1.0 mM) has to be used at a ten-fold higher concentration than that of KCN (0.1mM) during the titration experiments while determining the relative activities of COX and AOX pathways to total respiration (Figs. 6.1 & 6.2, Table 6.1). Throughout this study, antimycin A was successfully used to assess the relative activities of COX and AOX pathways under HL, hypo- / hyper- osmoticum or sub- / supra-optimal temperatures (Figs. 6.3-6.5; Tables. 6.2-6.4).

On transition from darkness to optimal light intensities (500-1000 $\mu E\ m^{-2}\ s^{-1}$) where maximum photosynthetic rates were observed (Fig. 5.1, Chapter 5), the activities of both COX and AOX pathways increased in mesophyll protoplasts once again confirming their importance in optimizing photosynthesis (Table 6.2). However, on exposure of mesophyll protoplasts to high light intensities where photoinhibition of photosynthesis occurred, the relative contribution of AOX pathway activity to total respiration increased significantly up to $\geq$66%, while the contribution of COX pathway activity decreased to 11% (Fig. 5.1, Chapter 5 and Table 6.2). Further, the increase in 'ρ' value from 0.808 (in darkness) to 0.99, which is almost closer to 1.0 indicates the complete engagement of alternative pathway under HL (3000 $\mu E\ m^{-2}\ s^{-1}$) (Table 5.2). Our results were in agreement with those of Feng et al. (2007) who noted increased capacity of AOX pathway and *Aox1* transcript levels in rice leaves under constant illumination where CO_2 fixation rates were high. AOX activation in light has been postulated to have important benefits for dissipation of excess reducing equivalents from chloroplasts to optimize photosynthetic carbon assimilation (Hoefnagel et al., 1998, Padmasree et al., 2002, Raghavendra and Padmasree, 2003, Svensson and Rasmusson, 2001). Ribas-Carbo et al. (2000) observed a significant increase in AOX pathway

activity when soyabean cotyledons grown in the dark were exposed to light for 2 h. Using oxygen isotope fractioning technique it was further showed that the red light causes shift in partitioning of electrons to AOX pathway in seedlings of soybean (Ribas-Carbo et al., 2008).

The significant increase in the contribution of AOX pathway activity to total respiration and its engagement to full capacity under both hypo- and hyper-osmotic media indicates its importance in protecting photosynthesis under osmotic stress (Fig. 5.1, Chapter 2 and Table 6.3). Ribas-Carbo et al. (2005) showed an increase in the activity of AOX pathway with concomitant decrease in COX pathway activity in soyabean leaves under water stress. The increase in AOX pathway with concomitant decrease in COX pathway was due to direct inhibition of the cytochrome oxidase under water stress. Dwivedi et al. (2003) showed an increase in AOX pathway activity and decrease in COX pathway activity in mesophyll protoplasts under hyper-osmotic stress. While the leaf discs of *Saxifraga cernua* did not show any differential response in COX and AOX pathways on exposure to a range of sorbitol osmotic potentials from 0.0MPa to 4.0MPa (Collier and Cummins 1993), in wheat the COX pathway increased under water stress (Zagdanska, 1995).

Along with light and osmotic stress, any deviation from optimal temperature also lead to decrease in the COX pathway activity and increase in the AOX pathway activity and its engagement in mesophyll protoplasts (Table.5.4). Using oxygen isotope fractionation technique, electron partitioning studies between the COX and AOX pathways in two cultivars of maize differing in chilling sensitivity showed that although total leaf respiration was not affected by the chilling treatment in either of the two cultivars, electron partitioning to the AOX pathway was significantly increased in the

more stressed chilling–sensitive plant. The results from these experiments suggested a possible role of the AOX pathway in plants under stress conditions rather than its specificity to contribute to resistance during chilling (Ribas-Carbo et al. 2000). A postulated role of the AOX pathway in plants is the maintenance of mitochondrial electron transport at low temperatures that would otherwise inhibit the main phosphorylating pathway and prevent the formation of ROS (Gonzalez-Meler et al., 1999).

The COX pathway comprising complex III and complex IV is strongly regulated by the proton gradient between the matrix and the intermembrane space, which is in turn directly affected by the mitochondrial respiratory control or ATP/ADP ratio (Millenaar and Lambers, 2003). On the other hand, AOX pathway does not itself contribute to the transmembrane pH gradient and ATP synthesis, since it accepts electrons directly from reduced ubiquinone bypassing the cytochrome bc1 complex and cytochrome c oxidase of the COX pathway (Molen et al., 2006). Instead the energy released through this pathway is lost as heat. AOX therefore is generally considered as an energy-dissipating by pass or an overflow of the excess electrons when the COX pathway is saturated (Lambers, 1982). Therefore, any increase in AOX pathway activity at the expense of COX pathway implies a lower ATP yield. Under all abiotic stresses examined in the present study the decrease in COX pathway activity was associated with remarkable decrease in cellular ATP levels (Figure 6.6 A-C). Ribas-Carbo et al. (2005) observed a significant decrease in mitochondrial ATP synthesis in Soybean leaves under water stress. A similar response was also observed by Flexas et al. (2004) in C_3 plants and suggested that the reduction in leaf ATP concentration under severe water stress, at least partially, reflecting the changes in the partitioning of electrons

between the COX and the AOX pathways in the mitochondria. The results from Fig. 6.7 (A-C) also showed a strong positive correlation between cellular ATP levels and COX pathway under various abiotic stresses.

The common substrate for the COX and AOX pathways is reduced ubiquinone. Early experiments with the "Q electrode" method showed that partitioning of electrons to the COX pathway varied linearly with the degree of ubiquinone reduction and AOX was not active until the level of reduced ubiquinone reached a high threshold value (Dry et al., 1989; Moore et al., 1988). This nonlinear response of the AOX pathway was confirmed by numerous studies on both isolated mitochondria and whole tissue, which suggested that AOX would function as an "energy overflow," and become active in O_2 consumption only when the COX pathway was saturated with electrons (Lambers, 1982, 1985). Such conditions were envisioned to arise when respiratory substrate was plentiful or when cellular adenylate energy charge was high, thus restricting oxidative phosphorylation (Azcón-Bieto et al., 1983: Day and Lambers, 1983).

However, the following two mechanisms of biochemical control of the AOX now indicate that the regulation of electron partitioning to the AOX is considerably more sophisticated than that of a simple overflow of the COX pathway. First, it has been shown that certain α-keto acids (particularly pyruvate) stimulate AOX activity by directly interacting with the AOX protein (Day et al., 1994, Millar et al., 1993). The second is a reversible covalent modification of AOX activity. AOX exists in the inner mitochondrial membrane as either a covalently or noncovalently linked dimer. The relative amounts of the two forms could be visualized using nonreducing SDS-PAGE in which the covalently bound AOX has an apparent molecular mass approximately twice that of noncovalently bound AOX. The dimer, when covalently linked by putative

intermolecular disulfide bond(s), was a less active form of the enzyme. Reduction of the disulfide bond(s) in isolated mitochondria by dithiothreitol produced the more active form while oxidation of the thiol(s) with diamide produced the less active form. The more active reduced form of AOX was susceptible to pyruvate activation through generation of a thiohemiacetal (Millar et al., 1993; Umbach and Siedow, 1993; Umbach et al, 1994; Rhoads et al., 1998).

In our studies, we observed an increase in active monomeric form of AOX under HL, hypo-osmotic stress (1.0M sorbitol) and sub-optimal temperature (10 ^{o}C) (Fig. 6.8 A-C) and this increase in active AOX monomeric forms correlated well with an increase in cellular pyruvate levels, which is highly significant at 1.0 M sorbitol (Fig. 6.9 A-C) and increase in engagement of alternative pathway (Table 6.2-6.4). The electron partitioning between the COX and AOX pathways was also subject to regulation by the amount of total AOX present as well as the redox status of the AOX (Siedow and Umbach, 1995). Reduction of AOX is known to be mediated by oxidation of specific TCA cycle substrates, isocitrate and malate (Vanlerberghe, 1995). All these suggested biochemical factors are known to interact to different extents, in different mitochondrial types isolated from different tissues in soybean to affect AOX pathway activity and its ability to compete for electrons with the COX pathway (Ribas-Carbo et al., 1997).

AOX expression and protein has also been shown to increase in response to different growth conditions, biotic and abiotic stresses such as drought, low temperature (Stewart et al., 1990a, b; Vanlerberghe and McIntosh, 1992a; Millenaar and Lambers, 2003; Fung et al., 2004; Bartoli et al., 2005; Lambers et al., 2005; Noguchi et al., 2005; Sieger et al., 2005), inhibition of COX pathway (Wagner et al., 1992; Rizhsky et al.,

2002; Seki et al., 2002; Bartoli et al., 2005) and by the application of inhibitors of mitochondrial protein synthesis (Day et al., 1996). Further, the importance of the regulatory mechanisms for AOX engagement and for the ability of AOX to compete with the COX pathway for electrons has been well documented using isolated mitochondria (Hoefnagel et al., 1995; Ribas-Carbo et al., 1995). Under *in vivo* conditions, the AOX protein was often (but not always) shown to be present exclusively in its highly active reduced form, and estimated matrix levels of pyruvate were always suggested to be high enough to fully activate AOX. If this is the case one might expect that, under a given set of metabolic conditions, AOX engagement might indeed be strongly dependent upon cellular pyruvate levels and reduction state of AOX rather than AOX protein level (Guy and Vanlerberghe, 2005). A controversy to this proposal is also observed in some isotope discrimination studies (Millenaar et al., 2002; Gonzalez-Meler et al., 1999). The results of these experiments suggest that AOX protein itself has little influence over engagement of AOX, which is known to be potentially modulated by both the redox state of the mitochondria controlling AOX reduction and carbon status which is defined by the levels of activating α-ketoacids like pyruvate mentioned above (Millenaar et al., 2002; Gonzalez-Meler et al., 1999).

Taken together these results suggest that though the relative contribution of COX pathway activity to total respiration decreased under high light, hypo-/ hyper osmoticum, sub-/ supra- optimal temperature the importance of existing COX activity in sustaining optimal photosynthesis was evident by the significant decrease in photosynthetic O_2 evolution rates in presence of antimycin A (Chapter 5, Fig. 5.1). On the other hand the increased contribution of AOX pathway to total respiration under all abiotic stress conditions examined in the present study suggests that AOX pathway

plays an important and significant role over cyanide sensitive COX pathway in protecting photosynthesis in mesophyll protoplasts of pea. Further these results also emphasize the flexibility of mitochondrial electron transport in meeting the requirements of chloroplastic photosynthesis to optimize photosynthetic carbon assimilation.

Major Conclusions

Major Conclusions

1. ROS and antioxidant system, both play a significant role in fine-tuning the beneficial interactions of mitochondrial oxidative metabolism with photosynthetic carbon assimilation.

2. Inhibitors of both COX pathway (antimycin A) and AOX pathway (SHAM) raised cellular ROS levels, while suppressing the rate of photosynthetic O_2 evolution in intact protoplasts.

3. The pronounced decrease in cellular redox state of antioxidant molecules ascorbate and glutathione and increase in antioxidant enzymes in presence of SHAM when compared to antimycin A indicates that AOX pathway plays a more significant role over COX pathway in maintaining optimal levels of cellular ROS under steady state photosynthesis.

4. A model was proposed indicating the function of ROS and antioxidant system in coordination with other redox systems (Mal/OAA and Triose-P/PGA) to fine-tune the beneficial interactions between chloroplasts, mitochondria and cytosol to optimize photosynthetic carbon assimilation.

5. Mitochondrial oxidative electron transport optimizes photosynthetic carbon assimilation during light, osmotic or temperature stress.

6. Inhibition of COX pathway or AOX pathway under light, osmotic or temperature stress caused marked rise in cellular redox state of malate while decreasing the redox state of ascorbate and glutathione.

7. Inhibition of COX pathway or AOX pathways under light, osmotic or temperature stress caused marked rise in the total cellular activities of SOD and CAT, while decreasing the activities of APX, MDHAR and GR, related to Asc-GSH cycle.

8. The changes in redox couples: malate/OAA, Asc/DHA, GSH/GSSG and antioxidant enzymes observed under light, osmotic or temperature stress are in

agreement with our proposed model of metabolic interactions between chloroplasts and mitochondria to optimize photosynthetic carbon assimilation.

9. Antimycin A is as good as KCN to determine the activities of COX and AOX pathways using inhibitor method.

10. The increased contribution of AOX pathway over COX pathway during high light or hyper/hypo-osmotic stress or sub-/supra- optimal temperatures not only indicates the flexibility of mitochondrial electron transport but also demonstrates the relative importance of AOX pathway over COX pathway in optimizing photosynthetic carbon assimilation.

11. The decreased contribution of COX pathway to total respiration correlated with decreased cellular levels of ATP, while increased contribution of AOX pathway correlated with pyruvate during light, osmotic or temperature stress.

Chapter 7

Literature Cited

Chapter 7

Literature Cited

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Annexure:1

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