Responses of Pea Plants to Elevated CO_2 (*in situ*) and Mesophyll Cell Protoplasts to Supra-optimal Bicarbonate (*in vivo*)

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Plant Sciences

By

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DECLARATION

I, Raj Sheel Pidakala, hereby declare that this thesis entitled **"Responses of Pea Plants to Elevated CO_2 (*in situ*) and Mesophyll Cell Protoplasts to Supra-optimal Bicarbonate (*in vivo*)"** submitted by me under the supervision of Professor A. S. Raghavendra in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date: Name: Raj Sheel Pidakala

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CERTIFICATE

This is to certify that this thesis entitled **"Responses of Pea Plants to Elevated CO_2 (*in situ*) and Mesophyll Cell Protoplasts to Supra-optimal Bicarbonate (*in vivo*)"** is a record of bonafide work done by Mr. Raj Sheel Pidakala, a research scholar for Ph. D. programme in Plant Sciences, Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. The work presented in the thesis is original and plagiarism free. We recommend his thesis for submission for the degree of Doctor of Philosophy of the University.

Prof. A. S. Raghavendra
(Supervisor)

(Head of the Department)

(Dean of the School)

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Raj Sheel

Abbreviations

2-DE	:	two dimensional gel electrophoresis
APX	:	ascorbate peroxidase
ATP	:	adenosine-5-triphosphate
BSA	:	bovine serum albumin
CAT	:	catalase
Chl	:	chlorophyll
EDTA	:	ethylenediaminetetraacetic acid
GR	:	glutathione reductase
GSH	:	glutathione (reduced form)
H_2DCFDA	:	2', 7'-dichlorofluorescien diacetate
IRGA	:	infra-red gas analyser
MALDI	:	matrix-assisted laser desorption/ionization
MS	:	mass spectrometry
NADH	:	nicotinamide adenine dinucleotide
NADPH	:	nicotinamide adenine dinucleotide
NO_2^-	:	nitrite ion
PGA	:	3-phosphoglyceric acid
Pi	:	inorganic phosphate
PMSF	:	phenyl methane sulfonyl fluoride
PQ	:	plastoquinone
PSI	:	photosystem I
PSII	:	photosystem II
Q_A	:	quinone, primary electron acceptor
ROS	:	reactive oxygen species
RuBisCO	:	ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	:	ribulose-1,5-bisphosphate
triose-P	:	triose-phosphate

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

Contents

Chapter 1
Introduction and Review of Literature

Importance and impact of elevated CO_2

One of the current environmental challenges is the marked increase in the carbon dioxide (CO_2) concentration of air and subsequent warming up of global climate. The CO_2 is a major component of greenhouse gases, and is known to capture radiation along with other gaseous forms like methane. Human dependence on fossil fuels as sole source of energy has caused the CO_2 concentration to rise in the atmosphere after industrialization. The anthropogenic influence has been mainly due to the consumption of fossil fuels and change in the land usage that caused this drastic change in the composition of atmosphere (IPCC, 2001). The increase in CO_2 in atmosphere has also lead to an increase in dissolved CO_2 in the oceans, leading to the acidification of water in oceans.

The rise of CO_2 in atmosphere will also lead to an increase of global temperatures, which in turn affects precipitation rates and aggravates global climate. Further these global climate changes can influence the monsoons and river runoff patterns. In some regions, the fall in water availability of soil and can cause serious drought conditions. In the last 250 years, the atmospheric CO_2 level of 280 µmol mol^{-1} (in the year 1780) rose to 400 µmol mol^{-1} till 2014. In a similar trend, CO_2 in atmosphere is expected to be 700 µmol mol^{-1} by year 2100, if there are no cuts in CO_2 emissions. Efforts by governments to place regulations in using fossil fuels might bring down the CO_2 concentration to 550 µmol mol^{-1}, or else the CO_2

concentration surpasses this mark by the middle of this century itself (IPCC, 2007; Keeling et al., 1995).

Short term and long term responses to elevated CO_2

Plants need CO_2 for photosynthetic carbon assimilation. The increase in CO2 levels of atmosphere is therefore of some benefit. The exposure of plants to elevated CO_2 during short term increases the rate of carboxylation by Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). This key enzyme, RuBisCO also catalyses the oxygenation and causes the loss of CO_2 through photorespiration. As CO_2 concentration in atmosphere is doubled, the carboxylation by RuBisCO is expected to increase two folds. However in reality, the actual rise in photosynthesis due to a 2-fold rise in CO_2 concentration is only <60 percent. This anomaly is due to other factors lowering RuBisCO capacity, such as decrease in the rate of RuBP regeneration, electron transport limitation and insufficient Pi regeneration. The short term response to elevated CO_2 have been mostly observed in a span of seconds to hours (Makino and Mae, 1999). Another short term response to elevated CO_2 is decreased stomatal conductance, due to the lowering of internal CO_2 concentration, in the intercellular spaces. These observations point out that the regulation of stomatal aperture might be mediated by the rate of CO_2 consumption in photosynthesis (Long et al., 2004).

When the plants are exposed to elevated CO_2 for a longer term, i.e. months to years, the rise in initial photosynthesis is not sustained and may even decrease, due to photosynthetic acclimation. The decrease observed in long term photosynthesis is due to the over-accumulation of carbohydrates such as starch or sugars can down-regulate photosynthesis. The down-regulation of photosynthesis is associated with

changes in RuBisCO content along with changes in expression of mRNA levels. Such feedback signal is developed often by the limitation of Pi regeneration on photosynthesis, as Pi is not recycled. The lowering of leaf nitrogen content can also be reason for the decrease of photosynthesis under CO_2 enrichment. Plants with higher photosynthesis at elevated CO_2 conditions had higher sink-source ratio (Long et al., 2004). In prolonged elevated CO_2 treatments, stomatal patchiness is also seen restricting the CO_2 entry into the mesophyll.

Direct and indirect effects of elevated CO_2

The effects of elevated CO_2 could be direct or indirect. The most important among the direct effects are the increase in carbon assimilation rate and decrease in stomatal conductance. The indirect effects are due to rise in ambient CO_2 include those from environment as well as those that occur within plants. High CO_2 in atmosphere could decrease the extent of solar radiation reaching the earth surface, and limit the leaf photosynthesis. Leaves at top of the plants might not face such reduced solar radiation. However, the light radiation reaching to the bottom of plant gets canopy, could limit the photosynthesis, as the plant canopies are multi-layered (Stanhill and Cohen, 2001).

Another effect of the increase in CO_2 concentration is the increase the global temperatures. This would result in lowering the availability of soil water and cause drought conditions. Increase in temperature would favour the carboxylation of RuBisCO compared to the oxygenation due to increase in affinity of RuBisCO to CO_2 but reduces the amount of dissolved CO_2 compared to O_2. The responses of plant metabolism to interact with the stressful conditions caused by elevated CO_2 along with the expected temperature rise need to be understood (Sage, 2002; Jensen,

2000; Hamilton et al., 2008; Prior et al., 2011). The lowering of transpiration can lead to insufficient uptake of essential minerals along with nitrogen. Further, CO_2 enrichment can cause an imbalance of carbon-nitrogen ratio. Lower availability of nitrogen can lead to lowering of enzyme levels and decrease the plant metabolism, productivity and growth metabolic activities like respiration. Dilution of nitrogen content in the plant due to CO_2 enrichment force increased grazing of pests compensate for low tissue nitrogen content in high CO_2 grown plants and causes the more damage to plants and reduces the yield (Calfapietra et al., 2009; Coleman et al., 1993; Erickson et al., 2013).

Interactions of Pi with CO_2 assimilation

Availability

Phosphorus, one of the most essential elements like carbon and nitrogen, is available to the plants in the form of inorganic phosphate (Pi). Plants take-up Pi via root system and assimilate into metabolites. The total amount of Pi taken up by plants in the soil (nearly 2 μM) is several fold less than Pi in soil (20 mM). This is because most of the Pi is not readily available, due to multiple reasons. For e.g. Pi in soils is used by microorganisms around the rhizosphere, which might compete for the available Pi or convert the available Pi to unavailable organic compounds, such as phytic acid. Apart from these, soil contains minerals like aluminium and iron which chelate phosphate and make them unavailable for the acquisition. To overcome the limitation of Pi availability, some plant species release root exudates, which help in mobilization of Pi along with other nutrients (Chiou and Lin, 2011; Schachtman and Shin, 2007). The availability of phosphorus is crucial to the growth of the plant as

limitation of phosphate in soil triggers several molecular and adaptive strategies to achieve optimal uptake/recycling of phosphorus.

Uptake

Plants have developed multiple ways to overcome the Pi limitation by improving their capacities of uptake, assimilation and partitioning. The uptake of Pi is facilitated by transporters, which mediate co-transport of Pi along with protons. The uptake of Pi by transporters is energy dependent (Bucher, 2007). The phosphate transporters are divided into high affinity Pi transporters (PHT1) and low affinity Pi transporters (PHT2). Higher expression of these transporters around the vascular bundles helps in the uptake of Pi by plants (Bucher et al., 2001). The uptake of phosphorus is facilitated by also modification of root system architecture by regulating genes, like LPR (low phosphate root), PRD (phosphate root development), SCR (scarecrow) and SHR (short root) for inducing the growth of lateral roots. Under starvation, induction of relevant genes encoding acid phosphatase or phosphate responsive pathway and phosphate transporter, help in Pi uptake by plants (Peret et al., 2011; Rouached et al., 2010).

Metabolism

Taken up phosphate is assimilated and is utilized in several metabolic functions in the plants. Besides, being a component of ATP/sugar phosphates, phosphate is involved in many enzyme reactions. It has a crucial role to play in the cellular processes of signal transduction and photosynthesis, being a component of membranes and enzyme reactions. When the phosphate is low, bypasses, like adenylate independent and Pi independent glycolytic enzymes, operate. These bypasses use PPi instead of ATP and conserve the Pi in the cell. (Plaxton and Tran,

2011). Similarly, when phosphate is low, enzymes like nucleases, phosphatases and phospholipases attempt to release Pi from the internal sources (Lopez-Arrendondo et al., 2014).

Regulatory role

Under elevated CO_2 conditions, plants have been shown to increase the uptake of phosphorus from the soil and in some instances by releasing acid phosphatases (Conroy et al., 1990; Barrett et al., 1998). Sucrose feeding led to the same responses as Pi starvation, for e.g. alteration of lateral root density, root hair density and length (Rouached et al., 2010; Yuan and Liu, 2008). These responses suggest that increased accumulation of sugar by elevated CO_2 might be leading to such Pi starvation responses.

There is a marked coordination between photosynthesis and Pi homeostasis, mediated by phosphate transporters on chloroplast inner envelope membrane. Triose-P is the major metabolite exported out of chloroplasts in equilibrium to the cytosol by a triose phosphate translocator (TPT) situated on chloroplast (Brautigam and Weber, 2011). A high concentration of Pi in the cytoplasm accelerates the movement of triose-P from chloroplast and can cause deprived levels of Calvin cycle metabolites, ultimately leading to the inhibition of photosynthesis. The presence of high Pi within the chloroplast can inhibit ADP glucose pyrophosphorylase (AGPase) reaction and reduce the starch synthesis in chloroplasts. Reduction of Pi and increase in PGA:Pi ratio due to increased CO_2 fixation and sucrose accumulation activates AGPase (Stitt et al., 2010).

Interactions of nitrogen metabolism with CO_2 assimilation

Availability

Nitrogen, which accounts for nearly 2% of plant ash, is an essential mineral nutrient. The assimilation and metabolism of N drives the biosynthesis of amino acids as well as several structural components of cell. Although, N is the abundant in the atmosphere, N cannot be directly assimilated by plant cells. Nitrogen recycled in the soil is made available to the plant, by organic matter decomposition by microorganisms. The elemental nitrogen can be fixed by microorganism alone or in symbiotic associations.

Uptake

Plants take up nitrogen from the soil in the form of nitrate or ammonia. Roots, take up nitrate through membrane transporters. Among them, NRT 1.1, a dual affinity transporter, switches from low to high affinity transporter by phosphorylation when the nitrate is low in the vicinity of roots. Another nitrate transporter (NRT2.1), is a high affinity transport system, induced by the supply of nitrate and gets repressed under high nitrogen status (Miller et al., 2007; Krapp et al., 2014). Another form of nitrogen that can be taken up by plants is ammonia.

Similarly, the uptake ammonia is facilitated by ammonium transporter (AMT1), which can be controlled by phosphorylation in a concentration dependent manner, as high level of ammonium is toxic to cells (Yuan et al., 2007; Lanquar et al., 2009). AMT1.3 promotes the lateral root development (Lima et al., 2010).

Metabolism

The two forms of N, namely N and NO_3, are actively reduced to NH_4 for further assimilation into amino acids. Nitrate in the plant cells undergoes a series of reduction reactions. For e.g. nitrate is converted to nitrite by nitrate reductase, with the help of reducing equivalents (NADH) available in the cytosol. The required NADH is provided by malate shuttle from chloroplasts and mitochondria, nitrite thus formed enters the leaves and reaches chloroplasts and it is reduced by ferredoxin system.

Ammonia is formed in plant tissues by different routes. It can be taken up directly by roots or regenerated during their action of glycine decarboxylase. Ammonia is also released from the degraded proteins or amino acids, particularly during senescence. (GS)-glutamine-2-oxoglutarate aminotransferase (GOGAT) facilitates the incorporation of ammonia into 2-oxyglutarate. Generation of 2-oxyglutarate was performed by isocitrate dehydrogenase, hence linked with carbon metabolism. This glutamate serves as the N-source for the synthesis of several amino acids, to be assembled into proteins (Foyer et al., 2011).

Regulation of N-metabolism

Reduction of nitrogen is a highly energy dependent process. The necessary ATP and NAD(P)H are generated in the photosynthesis as well as in respiration. Generation of ammonium from nitrate utilizes one NADH and six reduced ferredoxin. In light, the rate of N-assimilation is higher than that in dark. In elevated CO_2 nitrate assimilation is inhibited in wheat and Arabidopsis plants, which would lower organic N production. The possible mechanism for this effect is that under increased CO_2 concentration, photorespiration is expected to decrease. This might lead to the lower levels of NADH, which may affect the rates of nitrate reduction (Bloom et al., 2010).

High accumulation of carbohydrates in elevated CO_2 conditions is often associated with reduced N-assimilation, when nitrogen is low. This leads to inhibited photosynthesis and can be reversed when nitrogen supply was adequate. Thus, increased rate of growth in elevated CO_2 requires higher rates of nitrogen uptake and assimilation (Stitt and Krapp, 1999). Nitrogen assimilation and CO_2 enrichment are related by another factor. The increase in CO_2 can cause a competition for reductant, such as NADPH in the chloroplastic fraction, which will affect nitrate assimilation. Many metabolic processes in the stroma of chloroplast, include those of carbon fixation, nitrate or NH_4-assimilation, and fat metabolism are all affected by elevated CO_2 levels (Bloom et al., 2010).

Role of nitrogen under elevated CO_2

Importance of C/N equilibrium and its alteration at high CO_2

A coordinated balance between carbon and nitrogen is essential for optimal growth and development of plants. Low CO_2 causes lowering of photosynthesis/productivity due to not only limiting substrate for RuBisCO carboxylation, but also low leaf nitrogen and low RuBisCO levels compared to those at normal CO_2 (Sage and Coleman, 2001). Nitrogen use efficiency and biomass production are low in plants of *Bromus tectorum* and *Prosopis glandulosa* grown at low CO_2 along with increased requirement of water (Gerhart and Ward, 2010).

Growth of the plants in the elevated CO_2 causes a relative decrease in their N concentration, possibly due to the increase in non structural carbohydrates or secondary compounds. Such reduction in N, compared to the non structural carbohydrates has been reported for e.g., in *Betula pendula, Xanthium strumarium*

(Pettersson and McDonald, 1992; Lewis et al., 2002). Compared to the increase in shoot photosynthesis, the decrease in uptake of N may be another reason, besides possible involvement of other unknown factors (Taub and Wang, 2008).

High CO_2 is also a type of stress – Importance of ROS and antioxidants under elevated CO_2

Generation of reactive oxygen species (ROS) in plant cells is unavoidable, as our atmosphere is rich in oxygen and even the chloroplasts evolve oxygen. Further, the bioenergetic reactions in chloroplasts as well as mitochondria tend to produce ROS. Any imbalance of electron transfer across the membranes causes the electrons to react with freely available oxygen to generate free radicals, which are harmful (Apel and Hirt, 2004). Although many biotic and abiotic stress factors cause an increase in cellular ROS levels, the diverse antioxidant systems in cells efficiently scavenge the ROS and try to keep ROS low. These include low molecular antioxidants like tocopherols, carotenoids, flavonoids, sugars and sugar alcohols along with well known ascorbate and glutathione, which are non enzymatic. Enzymes involved in detoxification of ROS are three types of superoxide dismutase, catalase, ascorbate peroxidase glutathione peroxidase and peroxiredoxins (Foyer and Noctor, 2009).

Chloroplasts are active sites of ROS generation through light dependent electron transport and resultant generation of singlet oxygen and superoxide radicals. Yet, chloroplasts withstand high levels of ROS due to the operation of ascorbate-glutathione cycle (Asada, 2006). The excess reductants, exported out of the chloroplast through malate/oxoloacetate shuttle and triose-P/PGA shuttle can also be a source of ROS (Foyer and Shigeoka, 2011). The process of

photorespiration is another source of producing ROS. H_2O_2 is produced at the site of glycolate oxidase in the peroxisomes, while the synthesis of glycolate is initiated in chloroplasts by combining O_2 to RuBP. Often, under the conditions of low CO_2 where the stomata close under the water stress the partial pressure of O_2 increases compared to CO_2, the photorespiration increases. This eventually leads to the increase in ROS. Under elevated CO_2 conditions the partial pressure of CO_2 increases in the cells, this event favours the carboxylation of RuBisCO over oxygenation and thereby decreases photorespiration and H_2O_2 generation (Foyer et al., 2009).

There seems to be a disagreement on the effects of elevated CO_2 on ROS status in plant cells. Some of the reports suggest that elevated CO_2 can mitigate stress conditions, while others suggest that elevated CO_2 can cause a further increase in ROS (Farfan-Vignolo and Asard, 2012; Qiu et al., 2008). Oxidative stress was low in elevated CO_2 exposed plants and was able to counter other stresses. This is associated with alteration of one or more antioxidant enzymes like SOD, peroxidase, GR, APX, catalase, MDAR, DHAR in wheat, maize, oak, pine and barley plants (Baczek-Kwinta and Koscielniak, 2003; Pinto-Marijuan et al., 2013). Elevated CO_2 caused oxidative stress in plants and lead to increased leaf protein carbonylation, which also lead to loss of chlorophyll and decreased photosynthetic rate (Qiu et al., 2008; Gillespie et al., 2011).

Chlorophyll a fluorescence: Useful tool

Plants harvest the light with the help of protein-pigment complexes containing carotenoids and chlorophylls. In plants chlorophyll a (Chl) is the predominant and main pigment. The primary reaction in photosynthesis is the excitation of special Chl

molecules (Chl*). Since the energy transfer is not always 100%, a part of excited molecules undergo decay process leading to loss of energy as fluorescence and heat. Thus, Chl in PSI, PSII and light harvesting complexes, emit fluorescence, when excited by light. The fluorescence from PSI is weak and is better observed at low temperature (77k) than at room temperature. On the other hand, fluorescence signal from green tissues at room temperatures comes predominantly from PSII and surrounding antennae. Chl fluorescence has been extensively used to measure photosynthetic performance of leaves and green tissues. Several plant physiologists and ecologists employ this technique as it provides quick estimation of the physiological condition of the plant sample. It is non-invasive, does not create any mechanical stress and does not affect the physiological condition during the assay.

The fluorescence signal from Chl can be measured by detector and employing either simple single flash of high light (For e.g. Handy PEA by Hansatech) or by applying multiple pulses of saturated light at different time points (Pulse-amplitude modulated Chl fluorometry by Walz). When dark-adapted photosynthetic material is transferred or exposed to light, the fluorescence emitted from Chl has a characteristic pattern known as Kautsky curve. This reflects the quantum yield changes by quenching or de-quenching of Chl* antenna by photosynthetic electron transport. The excited Chl fluorescence comes down gradually to original levels by photochemical quenching, non-photochemical quenching and state transitions. Photochemical quenching or its de-quenching is determined by the steps involved in primary charge separation process of the reaction center of PSII (Papageorgiou et al., 2007).

A typically dark-adapted leaf has minimum fluorescence at origin, designated as F_o. On illumination, the Chl fluorescence rises to a maximal peak (F_m), through a polyphasic transient rise (Fig. 1) with two inflections at J and I or I_1 and I_2 (Strasser et al., 1995) gives the information of metabolic activity. The typical OJIP or OI_1I_2P fast fluorescent transients alter when exposed to different kinds of stresses. The signature of this fluorescent transient can indicate the status of the sample, i.e. either stressed or normal. The parameters obtained from Chl fluorescence transients can be analysed with suitable software and the calculations by JIP test reveal several phenomenological and biophysical differences in the normal and treated/stressed samples.

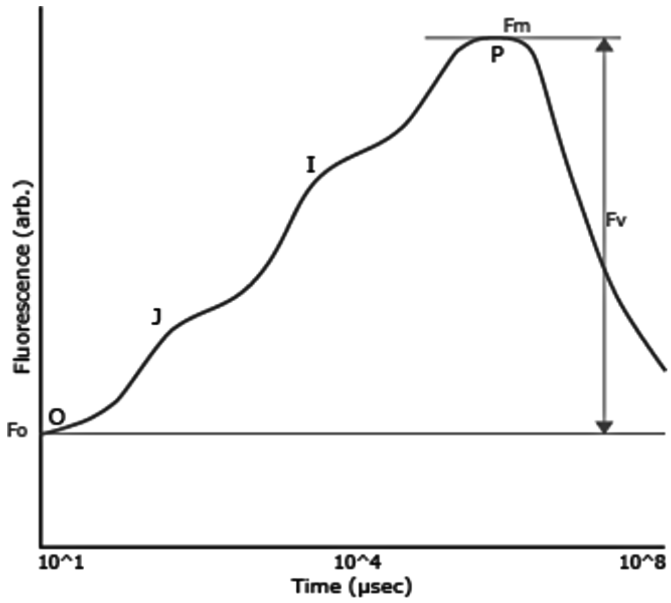

Fig.1. Typical pattern of induction of Chl fluorescence in the dark- adapted leaf upon illumination. The measurement by Handy PEA (Hansatech) shows up different points of inflections (OJIP). Fm is maximum fluorescence observed at P, Fo is the initial fluorescence at O. Fv is the variable fluorescence calculated by difference between Fm and Fo. Source: Web-resource material (http://hansatech-instruments.com)

To gain an insight of the photosynthetic performance of the sample from the Chl fluorescence measurements, the processes of photochemical quenching and non-photochemical quenching need to be distinguished. As there is an increasing reduction in electron acceptors of photosynthetic pathway, due to the flow of electrons, when excited by light, the reaction centres are converted from open to closed state, reducing Chl fluorescence from PSII. The quenching of fluorescence by electron transport to ferredoxin/NADP is termed as photochemical quenching. During this process some amount of energy is converted to heat and the quenching of fluorescence because of this process is known as non-photochemical quenching.

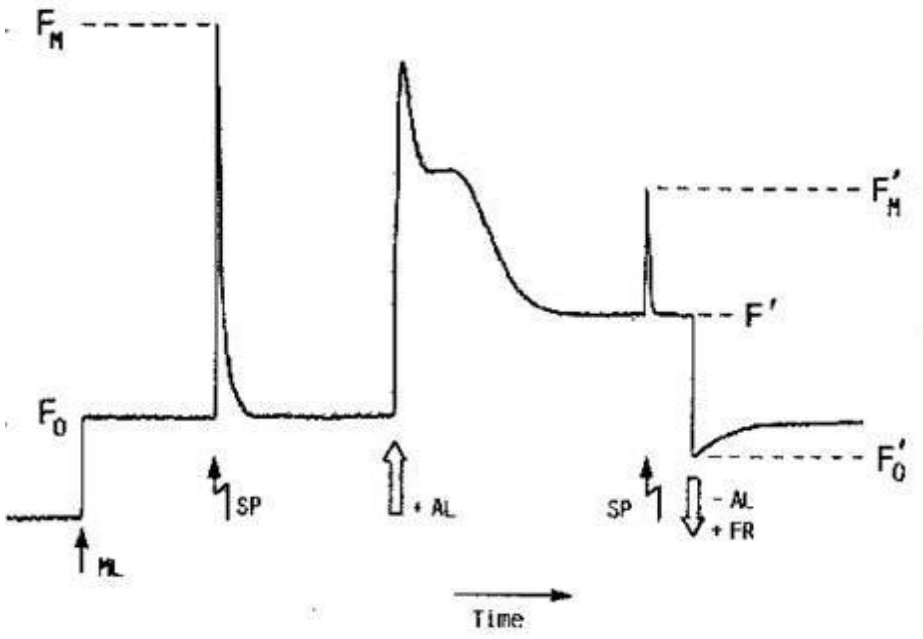

Fig. 2. Fluorescence trace induced by saturating pulses of high intensity of light, called as pulse-modulated light. This curve is typically obtained with a PAM machine (e.g. Walz). When measuring light (ML) is turned on, zero level fluorescence is measured F_o. Saturating pulse (SP) of approximately, 10000 µmol m^{-2} s^{-1} light intensity is applied for 0.5 to 2 seconds, on to a dark-adapted leaf/tissue causing induction of maximum fluorescence F_M. Actinic light (AL) is switched on to drive photosynthesis, another saturating pulse in light allows the measurement of F'_M. F' indicates the steady state level fluorescence in presence of AL. Turning off the AL in far-red light (FR) retrieves the fluorescence to zero level (F'_o). Adapted from Kooten and Snel (1990).

When a flash is given, the fluorescence yield reaches to the maximum (F_M) due to the absence of photochemical quenching. Comparing this to the fluorescence yield in presence (F') and absence (F_o) of actinic (photosynthetic) light gives the information about the efficiency of photochemical quenching or the performance of PSII (Fig. 2). As it is impossible to inhibit heat dissipation and thereby non-photochemical quenching, it is estimated based on the changes between F_M and F'_M. So, it is crucial to measure the actual dark adapted reference point F_M (Maxwell and Johnson, 2000).

These expressions can be used to calculate the fluorescence parameters, such as yield of PSII photochemistry (Y_{II}), yield of non-photochemical quenching (Y_{NPQ}) and yield of non-regulated energy loss (Y_{NO}). The following formulae are described by Kramer et al. (2004).

$$\phi_{II} = (F'_M - F')/ F'_M$$

$$\phi_{NPQ} = F'/ F'_M - F'/F_M$$

$$\phi_{NO} = F'/F_M$$

In view of the versatility and non-invasive use, Chl fluorescence has been used to assess the photosynthetic responses of plants under elevated CO_2. Fluorescence parameters observed in *Pinus radiata* indicate the electron flow subsequent to the PSII was affected at elevated CO_2 (Conroy et al., 1986). Photosynthetic activity, as measured by yield of PSII in elevated CO_2 was higher than that in ambient CO_2 in spruce and barley (Kitao et al., 2012; Robredo et al., 2010). Alfalfa and rose plants grown in high CO_2 had high non-photochemical quenching (Urban et al., 2001; Aranjuelo et al., 2008).

Studies at different levels of organization

Elevated CO_2 has been shown to affect the physiology of individual plants at different levels of organization: ecosystems, crop stands, individual plants and leaves. Reich et al. (2001) observed that the biomass of plants, grown with a combination of elevated CO_2 and nitrogen, was better than that in either elevated CO_2 or nitrogen. They also observed that species rich with diversity had shown better response in all the grown conditions. Such performance could be due to favorable plant microbe interaction, promoted by reduced N availability for microbes for e.g. in a grassland ecosystem (Hu et al., 2001). Another study in herbaceous brackish wetland proved the concept of progressive nitrogen limitation, as addition of nitrogen had enhanced further the biomass, that was stimulated by elevated CO_2 (Langley and Megonigal, 2010).

Most of the grains for human food, are from C_3 crops, which include rice, wheat, barley and oats. The C_3 and C_4 crops respond differently to elevated CO_2. The relative advantage, in the conversion of solar energy to biomass by C_4 over C_3 crops, is lost above 700 ppm of CO_2, possibly due to the lowering of photorespiration (Zhu et al., 2008). In general, elevated CO_2 helps the C_3 crops due to a combination of increased carbon uptake, improved nitrogen use efficiency, and reprogramming of respiration along with better water relations (Leakey et al., 2009). The expected theoretical increase in photosynthesis is not seen in crop plants in free air carbon dioxide enrichment (FACE) studies compared to the woody plants (Long et al., 2006; Leakey et al., 2009).

Individual plants grown in controlled conditions have shown a good short term response of photosynthesis, with an increase in the rate by 25-75%. However, such

photosynthetic response is not sustained when plants are grown in the field, at elevated CO_2. Photosynthesis gets acclimated on long term exposure to high CO_2, possibly due to the absence of higher sink demand (Stitt, 1991) and limitation of nutrients that was caused by pot size (Sage, 1994). Lowering of the nitrogen concentration in the plant is also due to the limitations on nitrogen uptake at CO_2 enriched conditions.

Experiment on plant responses to elevated CO_2 were also performed with leaves, with reference to their CO_2 compensation point, photosynthesis, photorespiration and respiration (Smith et al., 1976; Lester and Goldsworthy, 1973). Both detached and intact leaves were used to study their responses to elevated CO_2. Leaf respiration increased on long term exposure to high CO_2 possibly due to the increased availability of carbohydrates within leaf (Jahnke and Krewitt, 2002; Davey et al., 2004).

Relevance of *Pisum sativum*

The research on elevated CO_2 effects on plants was extensive with grassland species and trees. These studies were extended to assess the relative performances of C_3 and C_4 species, again frequently with grasses. However, the focus of studies on elevated CO_2 has shifted to legumes, which are complimented with symbiotic nitrogen fixation and would be able to cope up with elevated N-requirement under elevated CO_2 conditions (Rogers et al., 2009; Leakey et al., 2009). Apart from benefitting from the nitrogen fixation from symbiotic association, the presence of root nodules increased the sink capacity for carbon under elevated CO_2, as in *Glycine max* (Ainsworth et al., 2004; Aranjuelo et al., 2013). Interestingly, non-nodulated pea plants have shown a down-regulation of photosynthesis in younger leaves with

decreased RuBisCO content at high CO_2 (Xu et al., 1994). Thus, several reports emphasize the relevance of legume plants, such as pea (*Pisum sativum*) for further studies on metabolic responses to elevated CO_2. It would be appropriate and essential to examine the changes at leaf or cellular level. These studies would explain the basic mechanism of optimization and metabolic adjustment to increased CO_2 concentration.

We have therefore chosen to work with pea plants at three different levels of organization: whole plants and leaves collected from the field grown at ambient or elevated CO_2, besides the mesophyll protoplasts were exposed to supraoptimal bicarbonate (mimicking high CO_2).

Relevance of protoplasts

Isolated intact cells and protoplasts are among excellent experimental systems for studying the plant metabolism, compared to leaves, plants and plant stands. Protocols for isolation of protoplasts from the leaves, referred to as mesophyll cell protoplasts are well documented. Mesophyll protoplasts have been extensively used for characterizing the cellular metabolism like photosynthesis and respiration (Saradadevi et al., 1996; Covey-Crump et al., 2007).

Protoplasts, which are free of cell wall, are ideal for modulating their function by altering their medium components. The requirement for CO_2 for optimal photosynthesis, in mesophyll protoplasts was lower than that in chloroplasts (Riazunnisa et al., 2006) and was found to be due to the recycling of the CO_2 that is released in the photo-respiratory events. Protoplasts isolated from leaves of wheat by Edwards et al. (1978) exhibited high rates of photosynthesis and short lag period,

even in the absence of exogenous inorganic phosphate, compared to the chloroplasts. In contrast, chloroplasts needed higher levels of sodium bicarbonate, additional components such as EDTA, PPi and ATP for decreasing the time of induction, which compensate the metabolites of Calvin cycle that were lost.

Although, the rapid isolation protocols for protoplasts from pea and arabidopsis leaves provided a versatile experimental system (Devi et al., 1992; Riazunnisa et al., 2007; Yoo et al., 2007; Wu et al., 2009), there were also reservations that the protoplasts and their response might be different from those of intact leaves/ plants (Faraco et al., 2011). The isolation of protoplasts is stressful process as it involves in enzymatic digestion of cells at acid pH. However, the isolated protoplasts isolated from different sources are quite viable and totipotent as they can undergo dedifferentiation and regenerate shoots, roots and entire plants (Eeckhaut et al., 2013). The interdependence of both organelles i.e. chloroplasts and mitochondria could be studied by isolating the organelles form the protoplasts or by using specific inhibitors for the respective pathways (Raghavendra and Padmasree, 2003). The protoplasts isolated from *Pisum sativum* are found to be as photosynthetically active as leaves, when analysed by non-invasive Chl fluorescence measurements (Sunil et al., 2008). Based on the above available information, we have used leaves as well as protoplasts from a legume plant, pea.

The objectives and approaches of the present work are described in the next chapter.

Chapter 2
Objectives and Approaches

Specific objectives of the present study

The atmospheric concentration of CO_2 is increasing significantly, despite the efforts to curb the CO_2 emissions into the environment. There is growing evidence that this rise in the CO_2 would cause both short-term and long-term changes in the plants. The increased ambient CO_2 can raise the rates of photosynthetic carbon assimilation in the plants. This initial increase in photosynthesis in a long term is dependent on also the nitrogen and water availability (McMurtrie et al., 2008; Leakey et al., 2009). The information about responses of C_3 plants to elevated CO_2 is vast but most with crop plants or trees, while the studies on responses of a legume to elevated CO_2 are quite limited. The legumes can fix nitrogen and might overcome the responses occurring due to nitrogen limitation (Rogers et al., 2009). If so, it is interesting to know what happens to the physiology and protein profile in typical legume plants, along with the importance of phosphate in regulation of photosynthesis. Similarly, the other interesting points are to consider if oxidative stress occur and if there are changes in the energy transfer mechanisms during photochemistry. To answer some of these questions, we have framed our objectives as follows. Experiments were planned to be executed with leaves as well as mesophyll protoplasts.

1. To assess the physiological characteristics and proteomics of pea leaves from plants exposed to varied CO_2 in field.

2. To check the response of photosynthetic O_2 evolution and Calvin cycle metabolites in mesophyll protoplasts of pea to varied bicarbonate (mimicking CO_2) and inorganic phosphate.
3. To determine the effect of varying bicarbonate (mimicking CO_2) on nitrogen assimilation, reactive oxygen species and antioxidant enzymes in mesophyll protoplasts of pea.
4. To examine the effect of varying bicarbonate (mimicking CO_2) on chlorophyll fluorescence in pea mesophyll protoplasts.

The rationale of our experiments are described below

Plant material: Pea, Pisum sativum

Pea (*Pisum sativum*) plant, offers an advantages to decipher the effects of high CO_2 in legume besides the ease of growing them in green house chambers (Sunil et al., 2008; Puli and Raghavendra, 2012). The leaves of pea are also quite ideal for the process of protoplast isolation.

Systems of study: Leaves and mesophyll protoplasts

Plant leaves harbour the photosynthetic machinery, so they perform the carboxylation reactions in presence of light. The epidermal layers of a leaves are also the sites where stomata can be seen which would allow the gaseous exchange. These factors enable us to perform analysis of physiological measurements like photosynthesis and transpiration.

Mesophyll cell protoplasts on the other hand offer the benefit of facilitating the external addition of metabolites in the medium. As these protoplasts lack cell wall and devoid of intercellular spaces as in leaves, the conductance/movement of

CO_2/metabolites is much better and effective. The responses of protoplasts are also quick and can be obtained within five-ten minutes (Saradadevi and Raghavendra, 1992).

Treatments or usage of modulators

High levels of CO_2 were maintained while growing plants in the field, by pumping additional CO_2 mixed with ambient air (Reddy et al., 2010). Parallely, in the lab, experiments with protoplasts, the CO_2 levels were increased by raising the sodium bicarbonate concentration in the medium. To increase the phosphate levels, Pi buffer of same pH but increasing Pi was used. Mannose was used to sequester the Pi. To alter the nitrate content in mesophyll cells, potassium nitrate was used as the external source.

Several experimental techniques were used in our study to monitor physiological observations at field level and at cellular level in the lab. These include photosynthetic measurements, proteomic analysis, metabolite estimations and determination of oxidative status. The results and discussion are presented as four chapters, corresponding to the four objectives mentioned earlier.

Chapter 3
Materials and Methods

Plant material

Pea (*Pisum sativum* L cv. Arkel.) seeds were purchased from National Seeds Corporation Ltd. New Delhi, India. The seeds were soaked in water overnight, surface sterilized with 0.2% (v/v) sodium hypochlorite solution for 15 min and then washed for 1 h under running tap water. The washed seeds were kept covered in a moist black cloth/filter paper at 25^{o}C, until they germinated (usually 3-4 days). The germinated seeds were then sown in plastic trays filled with soil + farmyard manure (3:1) and were watered twice daily. The plants were grown in a green house at average temperatures of 33^{o}C day/25^{o}C night and a natural photoperiod of approximately 12 h.

Growth at normal or elevated CO_2

When the plants were one week old, trays containing the plants were placed in the open top chambers (OTC), either with CO_2 injection (Elevated) or without CO_2 injection (Ambient). The average CO_2 concentrations in the elevated OTC and ambient OTC were 550 ppm and 380 ppm, respectively (Reddy et al., 2010). The second fully expanded leaves from the top were picked from plants exposed to CO_2 for the duration of weeks.

Measurement of gas exchange parameters by infrared gas analyser (IRGA)

The gas exchange parameters were measured between 10.00-11.00 a.m. under an artificial LED light source (700 μmol m^{-2} s^{-1}), using portable infrared gas

analyser (LCpro+, ADC Bioscientific Ltd., Great Amwell, Hert, UK). The parameters included: Net photosynthesis (P_n), stomatal conductance to CO_2 (g_s) transpiration rates (E) and internal CO_2 concentration (C_i). Second fully formed leaf from the top was taken for measurement.

Protein extraction and two-dimensional electrophoresis (2-DE)

Leaves from field grown plants at either normal or elevated CO_2, were collected, immediately frozen in liquid nitrogen and stored at -80°C, until used. The leaf proteins were extracted as described by Sengupta et al. (2011). Frozen leaf tissue of 1g was ground to fine powder in liquid nitrogen and suspended in 4 ml of the extraction buffer. Detailed composition of the buffer is mentioned in Table 3.1. Equal volume of phenol saturated with Tris–HCl (pH 7.5) was added, mixed for 30 min at 4°C and the mixture was centrifuged at 5,000g for 30 min at 4°C. The upper phenolic phase was collected and an equal volume of extraction buffer was added to it. The above step was repeated and the upper phenolic phase was re-extracted. Four volumes of 0.1 M ammonium acetate in methanol was added to the collected phenolic phase and kept overnight at -20°C for protein precipitation. The samples were then centrifuged at 10,000g at 4°C for 30 min and the precipitate was washed thrice in ice cold methanol, twice in ice cold acetone and air dried for few minutes to collect the pellet.

The final pellet was solubilised in 200 µL of the rehydration solution (Table 3.1) and the protein concentration was determined by using RC–DC protein assay kit (Bio-Rad, Hercules, CA, USA) using BSA as standard. Aliquots of 600 µg protein were mixed with rehydration solution with 0.004% bromophenol blue to a final volume of 320 µL and used for 2-DE (Rasineni et al., 2010). Active rehydration of

protein (600 µg) was done on IPG strips (18 cm, 4–7 pH linear gradient; GE Healthcare) for 12 h at 50 V. Rehydration and focusing was carried out in Ettan IPGphor II (GE Healthcare) at 20°C, using the following program: 30 min at 500 V, 3 h to increase from 500 to 10,000 V and 6 h at 10,000 V (a total of 60,000 Vh). After IEF, strips were equilibrated twice for 30 min with gentle rocking at room temperature (25 ± 2°C) in equilibration buffers1 and 2 respectively (Table 3.1).

The proteins were separated in the second dimension SDS–PAGE (12% vertical polyacrylamide slab gels) at 10 mA gel^{-1} for 1 h and then 38 mA gel^{-1} for 6 h, using an EttanDalt6 chamber (GE Healthcare). The gels were stained by colloidal coomassie staining procedure. The protein patterns in the gels were scanned and recorded as digitized images using a calibrated densitometric scanner (GE Healthcare). In-gel digestion and matrix-assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) analysis were conducted with a MALDI-TOF/TOF in the proteomics facility of our UoH Life Sciences (Sengupta et al., 2011).

Protein identification: peptide mass fingerprinting and MS/MS analysis

Protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (http://www.matrixscience.com) employing Biotools software (Bruker Daltonics).The similarity search for mass values was done with existing digests and sequence information from NCBI non-redundant database and Swiss-Prot database. The taxonomic category was set to Viridiplantae (green plants). The other search parameters were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1^+ and monoisotropic. According to the MASCOT probability analysis (P <0.05), only significant hits were accepted for protein identification.

All the statistical analyses were performed by Sigma Plot 11.0. For proteomic analysis, three independent experiments were done with three replications of both control and treated samples at each time point. Each replication comprised of 10–15 pooled plants. The spots were analyzed using Image Master 2-D Platinum image analysis software (GE Healthcare).The normalized volume (% vol) of each spot was automatically calculated by the software as a ratio of the volume of a particular spot to the total volume of all the spots present on the gel.

Isolation of mesophyll protoplasts of *Pisum sativum*

One to two week old leaves were collected from the green house grown plants. Lower epidermis of the leaves was peeled off with forceps and the naked leaf pieces were incubated in a petriplate containing pre-plasmolysis medium for 15 min. The pre-plasmolysis medium was removed from petriplates. Digestion medium was added, containing cellulase and macerozyme enzymes (cellulase for digesting cellulose and macerozyme for digesting pectin layer of cell wall). The mesophyll protoplasts released from leaves were centrifuged thrice (at 500g for 5, 4, 3 min respectively) to obtain pure protoplasts. The protoplasts were then kept in suspension medium (Devi et al., 1992). Details of the media components are all given in Table 3.1.

Chlorophyll was estimated after extracting protoplasts with 80% acetone (Arnon, 1949). Protoplast suspension of 12.5 µl was mixed with 5 ml of 80% (v/v) acetone by vortex. Absorbance of this solution was measured at 652 nm, against correction for turbidity at 710 nm.

$$\text{Chl (mg ml}^{-1}\text{ of protoplast suspension)} = (A_{652}\text{-}A_{710}) \times 11.11$$

Table 3.1. Buffers and media mentioned in the Chapter-3: “Materials and Methods”

Buffer/Medium/Experiment	Components	Concentration/pH
Extraction buffer (For extracting proteins from leaves)	Tris–HCl	0.5 M (pH 7.5)
	Sucrose	0.7 M
	KCl	0.1 M
	EDTA	50 mM
	β-mercaptoethanol	2% (v/v)
	PMSF	1 mM
Rehydration solution (For solubilising the protein)	Urea	8 M
	Thiourea	2 M
	CHAPS	4% (w/v)
	DTT	30 mM
	Immobilized pH gradient (IPG) buffer (GE healthcare)	0.8% (v/v)
Equilibration buffer 1 (For IEF of leaf proteins)	Urea	6 M
	Tris-HCl buffer	50 mM (pH 8.8)
	Glycerol	30% (v/v)
	SDS	2% (w/v)
	DTT	2% (w/v)
Equilibration buffer 2 (For IEF of leaf proteins)	Equilibration buffer 1, with	
	Iodoacetamide	2.5% (w/v)
Pre-plasmolysis medium (For pre-plasmolysis of naked leaf pieces)	Sorbitol	0.3 M
	$CaCl_2$	1 mM
	MES-KOH	10 mM (pH 6.0)
Digestion medium (For isolation of MCP)	Sorbitol	0.4 M
	$CaCl_2$	1 mM
	EDTA	0.25 mM
	MES-KOH	10 mM (pH 5.5)
	Cellulase Onozuka R-10	2.0% (w/v)
	Macerozyme R-10	0.2% (w/v)
	BSA	0.25% (w/v)
	Sodium ascorbate	10 mM
Washing medium (For purifying isolated MCP)	Sorbitol	0.4 M
	$CaCl_2$	1 mM
	MES-KOH	10 mM (pH 6.0)
Suspension medium (For all enzymatic/metabolic studies with MCP)	Sorbitol	0.4 M
	$CaCl_2$	1 mM
	$MgCl_2$	0.5 mM
	HEPES-KOH	10 mM (pH 7.0)
Reaction medium (For measurement of photosynthesis by MCP)	Sorbitol	0.4 M
	$CaCl_2$	1 mM
	$MgCl_2$	1 mM
	HEPES-KOH	10 mM (pH 7.5)

Measurement of photosynthesis and respiration of mesophyll protoplasts

Photosynthetic activity of protoplasts was measured in terms of O_2 evolution on illumination, while respiration was measured by monitoring O_2 uptake in darkness, both by using a Clark type oxygen electrode (Hansatech Instruments Ltd., King's Lynn, England). The measurements were made at a temperature of 25^{o}C. A 35 mm slide projector (Kindermann diafocus 1500 E) containing a halogen lamp (150W, 24V, Philips) provided the illumination of 700 µmol m^{-2} s^{-1}. Changes in light intensity, if any, are described wherever relevant. The reaction mixture containing the protoplasts was placed in the oxygen electrode chamber and dark O_2 uptake, or O_2 evolution upon illumination, was measured. The reaction medium (1 ml) for monitoring photosynthesis by mesophyll protoplasts contained protoplasts equivalent to 10 µg of Chl. Test compounds or different bicarbonate levels were included in the reaction medium (Table 3.1).

Metabolite Estimation

Aliquots (750 µl) of reaction medium containing protoplasts equivalent to 100 µg Chl were withdrawn after illumination at 700 µmol m^{-2} s^{-1}, added to 70% (v/v) $HClO_4$ (final concentration of $HClO_4$ was 3%, v/v) and were frozen in liquid nitrogen. When required (usually, the next day) the samples were thawed and centrifuged at 10000 rpm for 10 min. The supernatant was neutralized with KOH/triethanolamine and left on ice for 30 min. The neutralized samples were centrifuged again at 10000 rpm and the cleared supernatant was used for estimation of metabolites (Padmasree and Raghavendra1999a). The metabolite levels were measured by enzymatic assays coupled to NADH oxidation at 340 nm using a spectrophotometer (Shimadzu UV-1601).

The reaction medium (1 ml) for the determination of PGA contained 100 mM Tris–HCl pH 8.1, 10 mM $MgCl_2$, 1 mM ATP and 0.1 mM NADH. An aliquot of extract containing 20 μg Chl was pre-incubated in the reaction medium for 20 min at 25°C. The reaction was initiated by the sequential addition of 2 U glyceraldehyde-3-P dehydrogenase and 2 U phosphoglycerate kinase. The decrease in absorbance of NADH at 340 nm was used to calculate the amount of PGA present.

Triose-P levels were estimated by using 1 ml of reaction medium containing 20 μg Chl equivalent of extract, 100 mM Tris–HCl pH 8.1,10 mM $MgCl_2$, and 0.15 mM NADH. The assay was initiated by the addition of an enzymatic mixture of glycerol-3- P dehydrogenase/triose-P isomerase (0.8 U and 2.3 U). The decrease in absorbance of NADH at 340 nm was used to calculate the Triose-P.

RuBP levels were determined by using 1 ml of reaction medium containing 20 μg Chl equivalent of extract, 100 mM Tris–HCl pH 8.1, 10 mM $NaHCO_3$,10 mM $MgCl_2$, 1 mM ATP and 0.1 mM NADH. The extract was preincubated in the reaction medium for 20 min at 25°C. The reaction was initiated by the sequential addition of 2 U glyceraldehyde-3-P dehydrogenase and 2 U phosphoglycerate kinase. After an equilibration period of 2-3 min 50 μg of rubisco was added. The decrease in absorbance of NADH at 340 nm was used to calculate amount of RuBP (Padmasree and Raghavendra 1999b).

Enzymes of Nitrogen assimilation

Nitrate reducatse (NR; EC 1.6.6.1) was assayed, as described by Heidari et al. (2011). The NR activity was measured in terms of NO_2^- formation. The protoplasts were homogenized in extraction buffer (0.1 M HEPES pH 7.5, 1 mM EDTA, 3.33%

(w/v) PVP, 0.06% (w/v) Cysteine) and centrifuged at 10000 rpm for 10 min at 4°C. An aliquot was taken out for estimating total protein content by the method of (Lowry et al., 1951). Bovine serum albumin (BSA) was used as a standard for protein. The reaction mixture for actual activity contained extract equivalent to 25 μg protein, 50 mM HEPES pH 7.5, 2 mM KNO_3, 200 μM NADH, 6 mM $MgCl_2$. For measuring total activity $MgCl_2$ was replaced by 2 mM EDTA. The total and actual activities were determined.

The activity of nitrite reducatse (NiR; EC 1.7.7.1) was measured as reduction in the amount of NO_2^- in the reaction mixture by Debouba et al. (2007). The extract was incubated in a solution containing extract equivalent to 25 μg protein, 100 mM potassium phosphate buffer (pH 7.4), 15 mM sodium nitrite, 5 mM methyl viologen, 86.2 mM sodium dithionite in 190 mM $NaHCO_3$. Nitrite was estimated at 540 nm by spectrophotometer due to diazotation of nitrite ions with sulfanilamide and N-(1-naphthyl)-ethylenediamine-dihydrochloride.

Glutamine synthetase (GS; EC 6.3.1.2) activity (transferase) was determined by the method of Debouba et al. (2007). Samples were homogenized in a cold mortar and pestle with grinding medium (25 mM Tris–HCl buffer, pH 7.5, 1 mM $MgCl_2$, 1mM EDTA, 14 mM β-mercaptoethanol and 1% (w/v) PVP). The homogenate was centrifuged at 13000 rpm for 30 min at 4° C. The GS activity was determined in the reaction medium, containing the extract equivalent to 25 μg protein, 50 mM imidazole pH 7.5, 10 mM ATP, 50 mM $MgSO_4$, 6 mM NH_2OH and 100 mM monosodium glutamate. The formation of γ-glutamylhydroxamate was determined with acidified ferric chloride against standard at 540 nm after 5 min.

Fd-GOGAT activity was measured by grinding protoplast pellet at 4^0C in extraction buffer (50mMHEPES, pH 7.5, 15 mM KCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF) and centrifuged at 13000 rpm. The activity was measured by the decrease in absorbance of NADPH at 340 nm in the reaction mixture containing the extract equivalent to 25 μg protein, 50 mM HEPES buffer, pH 8.5, 1% (v/v) 2-mercaptoethanol, 3.65 mM glutamine, 3 mM 2-oxoglutarate, 0.2 mM NADPH, 4 mM ferredoxin (Jamai et al., 2009).

Antioxidant enzyme assays

Protoplasts were collected from oxygraph chamber after the treatment with light and/or bicarbonate. They were pelleted down by spinning at 13000 rpm (in an Eppendorf centrifuge) and frozen in liquid nitrogen, until use. When required, the frozen protoplast samples were homogenized in 50 mM potassium phosphate buffer (pH 7) and 1 mM PMSF in ice. These are centrifuged at 12000 rpm for 10 min at 4^oC. The supernatant obtained was used for further enzyme assays (Jiang and Zhang, 2001). An aliquot was used for estimating total protein content, by the method of Lowry et al. (1951), BSA was used as standard for protein.

The activity of CAT (EC 1.11.1.6) was measured by observing the decrease in absorbance of H_2O_2 at 240 nm (extinction coefficient 39.4 $mM^{-1}cm^{-1}$). The reaction mixture contained 25 μg equivalent of protein extract, 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2.

APX (EC 1.11.1.11) activity was determined by the decrease in absorbance of ascorbate at 290 nm (extinction coefficient 2.8 $mM^{-1}cm^{-1}$). The reaction mixture

contained 50 μg equivalent of protein extract, 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2.

GR (EC 1.6.4.2) activity was estimated by the decrease in absorbance of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) The assay mixture containing 50 μg equivalent of protein extract, 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM GSSG.

Estimation of reactive oxygen species (ROS) by H_2DCF-DA and DAB

The levels of ROS were determined by using 2, 7-dichlorofluorescein diacetate (20 μM H_2DCF-DA). The dye was loaded into the protoplasts for 30 min in dark. Excess dye was removed by washing the protoplasts with suspension media (Dinakar et al., 2010). An equivalent of protoplasts containing 100 μg Chl was treated with different bicarbonate concentrations at 25°C and 700 μmol m^{-2} s^{-1} light intensity for about 10 min. Fluorescence intensities were measured (with excitation at 488 nm and emission at 525 nm) by using microplate reader (Tecan infinite M200).

For estimating H_2O_2, 3, 3-diaminobenzidine (1 mg ml^{-1} DAB pH 3.8) was infiltrated to the treated protoplasts for 5 min. The mixture was fixed with ethanol: lactic acid: glycerol mixture (3:1:1). The supernatant was discarded after centrifugation and pellet was crushed using 0.2 M $HClO_4$. The extracts were centrifuged at 10000 rpm for 10 min at 4^o C. The absorbance of supernatant was measured at 450 nm (Kwon et al., 2013).

Table 3.2. Parameters of fluorescence transients and formulae used in JIP-test parameters. The measurements were made with either Handy PEA or Mini-PAM.

Instruments	Parameters/Points	Remarks
Handy PEA	F_o	Minimal fluorescence (at ~ 50 μs) when reaction centres (RC) are open
	F_{300}	Fluorescence intensity at 300 μs
	F_J	Fluorescence intensity at J-step (at ~ 2ms)
	F_M	Maximal fluorescence intensity
	V_J	$(F_J - F_o) / (F_M - F_o)$
	M_o	$4 . (F_{300} - F_o) / (F_M - F_o)$
	ABS / RC	$M_o . (1 / V_J) . (F_M / 1 - F_o)$
	TR_o / RC	$M_o . (1 / V_J)$
	ET_o / RC	$M_o . (1 / V_J) . (1 - V_J)$
	DI_o / RC	(ABS / RC) - (TR_o / RC)
Mini-PAM	F_M	Maximal fluorescence intensity
	F'	Steady state level fluorescence when RC are closed due to light
	F'_M	Maximal fluorescence induced by saturating pulse when RC are closed due to light
	ϕ_{II}	$(F'_M - F') / F'_M$
	ϕ_{NPQ}	$F' / F'_M - F' / F_M$
	ϕ_{NO}	F' / F_M

Chl *a* fluorescence kinetics

Measurement by Plant Efficiency Analyser

Chl *a* fluorescence transients were measured by using Plant Efficiency Analyser (Handy PEA, Hansatech Inst. Ltd. King's UK) as described by Sunil et al. (2008). Transients obtained by Handy PEA comprised of O-J-I-P-S-M-T phases. Dark adapted protoplasts were exposed to different bicarbonate concentrations. The pre-treated protoplast suspension was poured into the flat transparent cuvette. The cuvette was placed between the clips provided with the instrument.

The transients were induced by illumination with strong light for 1 s. The light of ~3000 μmol m^{-2} s^{-1} was provided by an array of three LEDs focussed on the surface of the sample. A RG9 long pass filter prevented the light from LEDs reaching the PIN photodiode detector. The fluorescence signal perceived by control box and was digitized by fast analogue/digital convertor (12 bit resolution). Fluorescence transients obtained from different treatments for nearly 200 s were assessed for changes in the OJIPSMT phases. These transients were normalized at F_o/F_m for further analysis.

Analysis of Chl a fluorescence transients using JIP test

The fluorescence induction curves were examined by JIP test. Information from the induction curves from Handy PEA was analysed for JIP test parameters by using Biolyzer software according to Sunil et al. (2008). These parameters explain the stepwise flow of energy through PSII. Different variables such as F_o, F_m, $F_{300\mu s}$, F_J, V_J, M_o obtained by fluorescent transients were used to derive specific fluxes of energy partitioning. Specific fluxes derived are absorption per reaction centre (RC),

trapping per RC, electron transport per RC and dissipation per RC. These variables and calculations are listed in Table 3.2. Detailed explanation about these parameters was given in Strasser et al. (2000).

Measurement by PAM fluorometer

Mini-PAM (Walz GmbH, Effeltrich, Germany) was used to obtain fluorescence transients as described by Vishwakarma et al. (2014). After the different bicarbonate treatments protoplasts were dark adapted for 30 min. Chlorophyll fluorescence was measured using an automated induction curve. The minimum fluorescence (F_o) and maximum fluorescence (F_m) were measured in a dark-adapted sample with a saturation pulse (SP) of 3000 μmol m^{-2} s^{-1} for 800 ms. After a delay of 40 s, actinic light (AL) of 700 μmol m^{-2} s^{-1} was switched on along with SP of 3000 μmol m^{-2} s^{-1} for 800 ms, followed by repetitive application of SPs at every 20 s for 5 min. With each SP the relative changes in yield, photochemical and non-photochemical quenching and other non-photochemical losses were recorded (Table 3.2). The information on different parameters was derived automatically by the calculations from PamWin software. These chlorophyll fluorescence parameters were calculated according to Kramer et al. (2004).

Replications

The data presented are the average values, with ± standard error. Each experiment was an average of at least 3 replications, from different experiments conducted on 3 different days.

Chemicals and Materials

Cellulase (Onozuka R-10) and Macerozyme R-10 (pectinase) were procured from Yakult Honsha Co. Ltd., Tokyo, Japan. All enzymes and standard metabolites were procured from Sigma-Aldrich Corporation, USA. Immobiline IPG strips were procured from GE Healthcare Life sciences Pvt. Ltd. Sweden. Other chemicals and materials were purchased from the following companies: Sisco Research Laboratories, Merck Ltd., Loba Chemie, Himedia Laboratories and Qualigens, all from Mumbai.

Chapter 4

Physiological Characteristics and Proteomics of Leaves from Pea Plants Exposed to Elevated CO_2 in Open Top Chambers

Introduction

It is now well-established that there is an alteration in physiological characteristics of plants exposed to high CO_2 environment. The alterations occur in photosynthesis (due to increased carboxylation) and transpiration (due to decreased stomatal conductance) (Ainsworth and Rogers, 2007). The advantage of CO_2 availability at elevated CO_2 is subject to other factors like nitrogen or phosphorous limitation (discussed earlier in Chapter 1). Legumes, being capable symbiotic nitrogen fixation, are expected to benefit from elevated CO_2, unlike non-symbiotic. Pea plant being a legume, capable of symbiotic nitrogen fixation, it would be interesting to know if elevated CO_2 affects the physiology and performance of pea plants. The increased requirement of N to keep in tune with high carbon fixation in elevated CO_2, is to optimize the availability and functioning of key proteins like RuBisCO. During this adjustment the reallocation of nitrogen to other requirements, for a balance between C/N assimilation pathways is often reflected in protein profiles (Li et al., 2007). While the physiological studies of C_3 plants at elevated CO_2 are quite extensive, proteomic analysis have been very few.

In *Chlamydomonas reinhardtii* the extracellular proteins were increased at high CO_2, compared to the intracellular soluble and insoluble fractions (Baba et al., 2011). In C_3 plants, proteomic analysis in rice plants indicated a decrease in levels of enzymes related regenerative phase of Calvin cycle (Bokhari et al., 2007). In a C_3 halophyte, *Aster tripolium,* there was an increased expression of ATPase protein

under elevated CO_2 treatment, possibly conferring protection against salinity stress (Giessler et al., 2010). The changes in proteome of legumes grown at elevated CO_2 have not been studied to date. However there have been basic studies on proteomics of leaves of certain legumes and these can provide proteomic references map. For example, such proteomic studies are available with soyabean (Xu et al., 2006) and pea (Schiltz et al., 2004).

This chapter describes and discusses our experiments, with pea which is capable of symbiotic nitrogen fixation. The open top chambers render different levels of CO_2 treatment i.e. ambient CO_2 (380 ppm) and elevated CO_2 (550 ppm) by pumping pure CO_2 mixed with air. Measurement of photosynthesis, stomatal conductance, along with proteomic analysis allows us to assess the physiological responses and relate to altered protein profiles under CO_2 treatments.

Results

Morphological and physiological changes during growth at ambient or elevated CO_2

There was no perceptible change in the morphology of pea plants grown at elevated CO_2 (550 ppm) compared to the ambient CO_2 (380 ppm) at two weeks after exposure. However, by three weeks of exposure, the plants grown at elevated CO_2 looked more robust than those of ambient CO_2 by completion of three weeks of exposure (Fig. 4.1). The biomass of the whole-plant increased by about 30% per plant. When above-ground and below-ground were examined separately for the response to elevated CO_2, the increase in below-ground biomass was three-folds in elevated CO_2 treatments compared to ambient CO_2. The above-ground biomass increased by about 16% in elevated CO_2 (Fig. 4.2).

The physiological characteristics of plants were studied by Infra-Red gas analyser (IRGA). The characteristics were: photosynthesis, transpiration, water use efficiency, stomatal conductance, internal CO_2 concentration and ratio of internal CO_2 to outside atmospheric CO_2 (C_i/C_a). The photosynthetic rate was higher in the elevated CO_2 grown plants after first and second week of exposure (Fig 4.3 A). However, after the third week of exposure in elevated CO_2 grown plants the rate of photosynthesis was less than those at ambient CO_2. Stimulation of photosynthesis was higher after first week but gradually decreased towards the progression of time (Fig. 4.3 A).

Transpiration steadily increased with time, but the extent of increase in plants elevated or ambient CO_2 differed. The rate of transpiration remained low in elevated CO_2 grown plants compared to that in ambient CO_2 grown plants throughout the experimental period of four weeks (Fig. 4.3 B). Water use efficiency decreased by the end of the experiment in ambient CO_2 grown plants. In elevated CO_2 grown plants water use efficiency was high after the first week of exposure but decreased later to the level of those at ambient CO_2 (Fig. 4.3 C).

Stomatal conductance in the plants grown at elevated CO_2 was lower than those at ambient CO_2. Stomatal conductance decreased in both conditions after the exposure of first week. However, the elevated CO_2 grown plants showed marked decrease till the end of second week of exposure (Fig. 4.4 A). Intercellular CO_2 concentration is increased in elevated CO_2 grown plants till the second week of exposure and later decreased by the end of fourth week, while in ambient CO_2 grown plants the intercellular CO_2 concentration did not vary much. The levels of intercellular CO_2 in plants at elevated CO_2 were higher than those in ambient CO_2

upto 3 weeks after exposure (Fig. 4.4 B). The C_i/C_a values did not altered much in ambient CO_2 grown plants when compared to the elevated CO_2 grown plants, where the Ci/Ca values dropped to very low at the durations after first week and after fourth week of exposure (Fig. 4.4 C).

Proteome analysis of leaves from plants grown at elevated or ambient CO_2

The 2-DE analysis of leaf extracts revealed the prominent presence of about 94 protein spots at the start of the experiment (Fig. 4.5) and this pattern was consistent throughout treatments. The typical pictures of the proteome of leaves from pea plants grown at ambient or elevated CO_2 are represented in Fig. 4.6. Among the protein spots, about 70% were unchanged in their levels, even in elevated CO_2. The fraction of unchanged proteins was higher at elevated CO_2 treatment after the first week of exposure. The amount of down regulated proteins was also lower in elevated CO_2 treatments (Fig. 4.7).

The Venn diagram (Fig. 4.8) showed differential expression of protein spots at varied treatment conditions. Exposure of 550 ppm CO_2 for a week resulted in up-regulation ten proteins and down-regulation of eighteen proteins. 550 ppm CO_2 exposed for 3 weeks, up-regulated ten proteins and down-regulated seventeen proteins., The most prominent and five up-regulated proteins treated with 550 ppm CO_2 were included in protein spots to be analysed. A total of 10 protein spots were chosen for further analysis (Fig. 4.9). These selected spots were subjected to MS/MS, which revealed that the most prominent protein spot was RuBisCO large subunit (Table. 4.1). Eight protein spots were indentified to be the proteins belonging to Calvin cycle and nitrogen metabolism in *Pisum sativum*. Other two spots did not match any specific hits that belong to *Pisum sativum* (Table. 4.2).

RuBisCO large subunit did not alter much in its levels under varying CO_2 conditions, while small subunit of RuBisCO increased markedly after the first week of exposure to elevated CO_2 (Fig. 4.10). Similarly, glyceraldehyde-3-phosphate dehydrogenase was up-regulated after the first week of exposure to elevated CO_2. In contrast, fructose-bisphosphate aldolase was up-regulated at both 1st and 3 weeks of elevated CO_2 treatment (Fig. 4.10). Among the other identified protein spots, phosphoglucomutase (known to be involved in starch synthesis) was up-regulated by 2-folds after the first week of exposure to elevated CO_2., Glutamine synthase, an enzyme of nitrogen metabolism was up-regulated at 1st week after exposure to elevated CO_2 treatment. Glycine cleavage system H protein was markedly up-regulated at the first and third weeks of exposure to elevated CO_2, in comparison to the levels in ambient grown plants. Elongation factor Tu (involved in protein synthesis) was also up-regulated in elevated CO_2 exposure after the first week (Fig. 4.11).

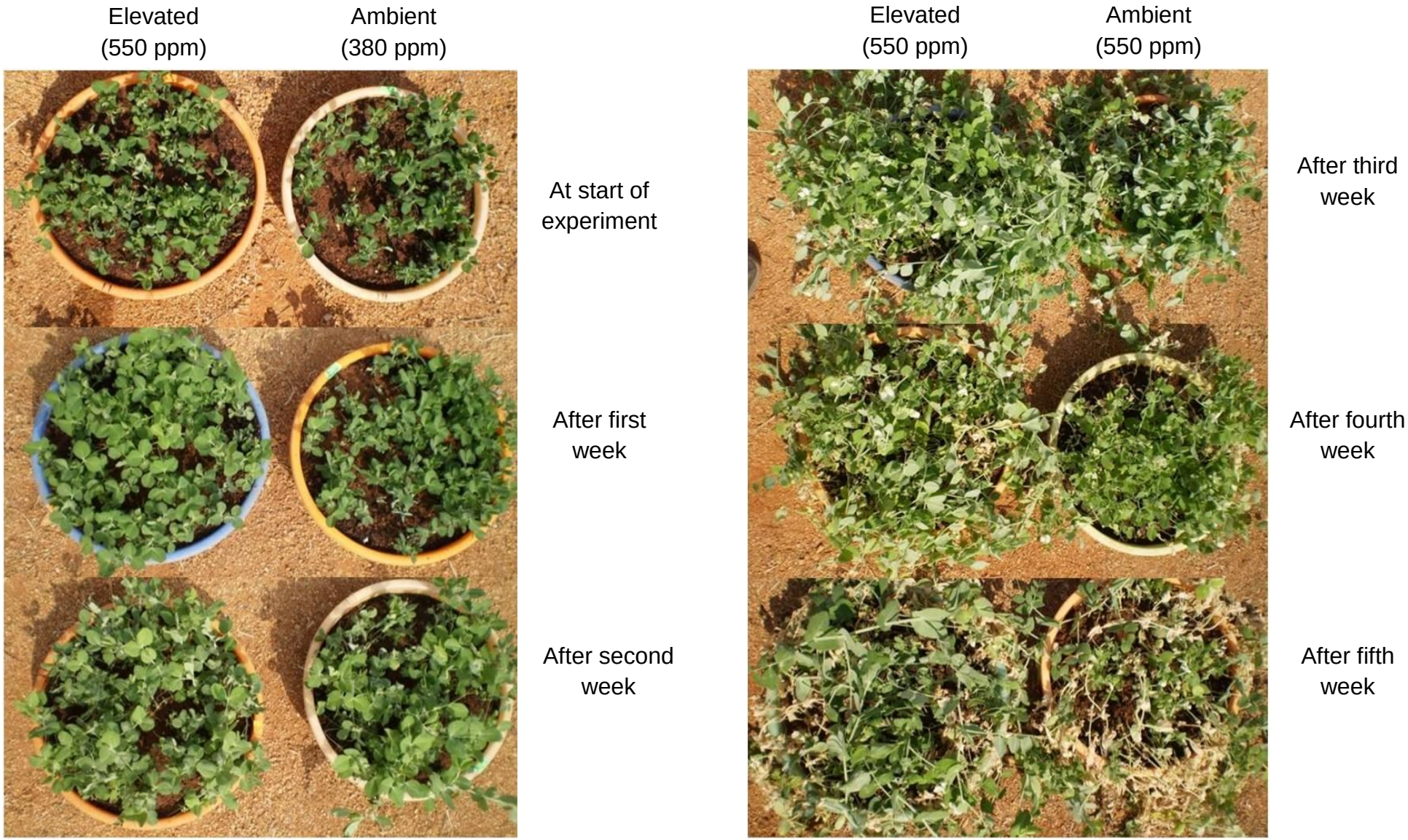

Fig. 4.1. The appearance of plants grown at either elevated (550 ppm) or ambient (380 ppm) CO_2 in open top chambers for five weeks. Before the CO_2 treatment, the plants were in green house.

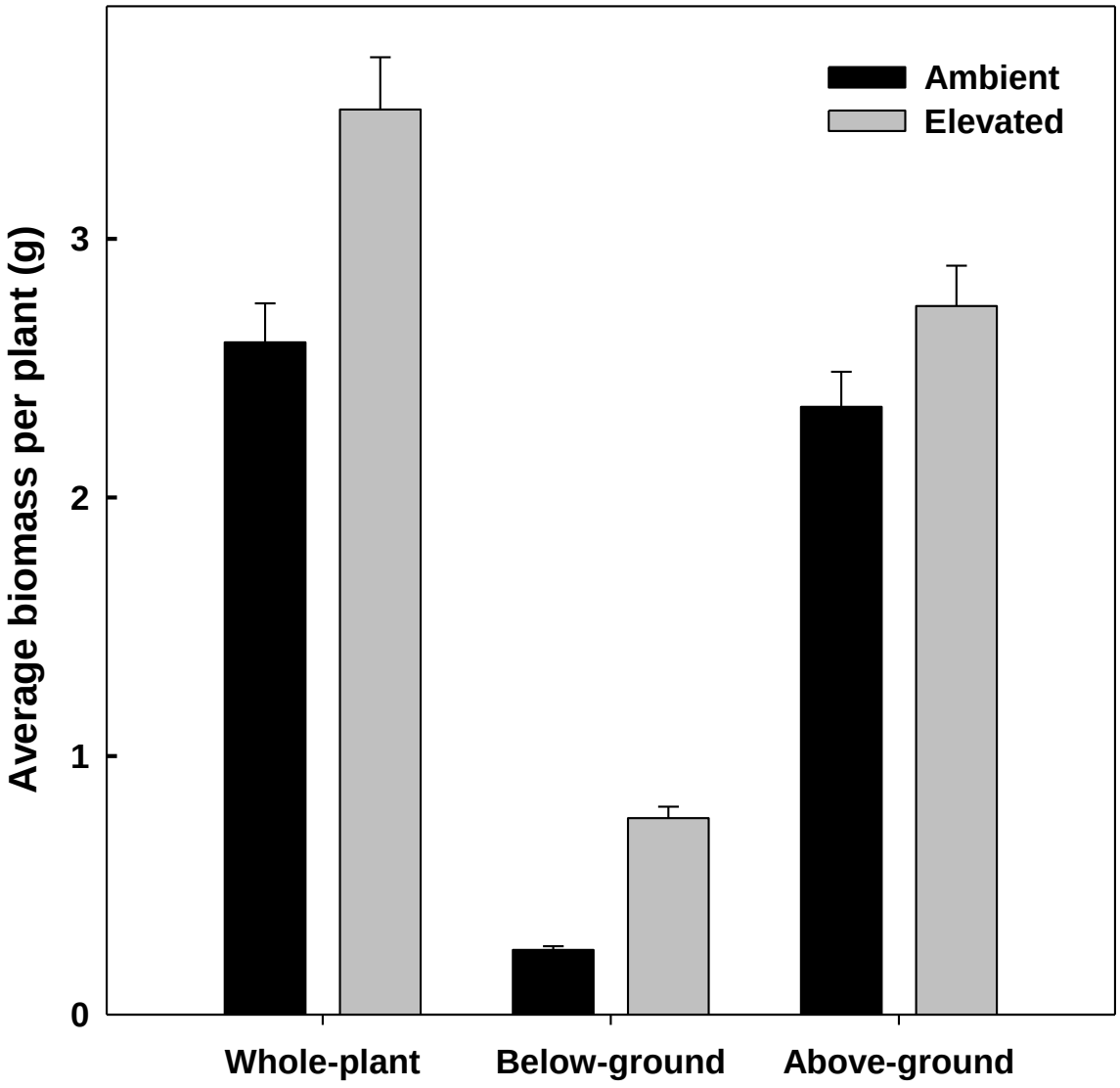

Fig. 4.2. Pattern of biomass harvested from pea plants, at the end of experimental treatment with elevated (550 ppm) or ambient (380 ppm) CO_2.

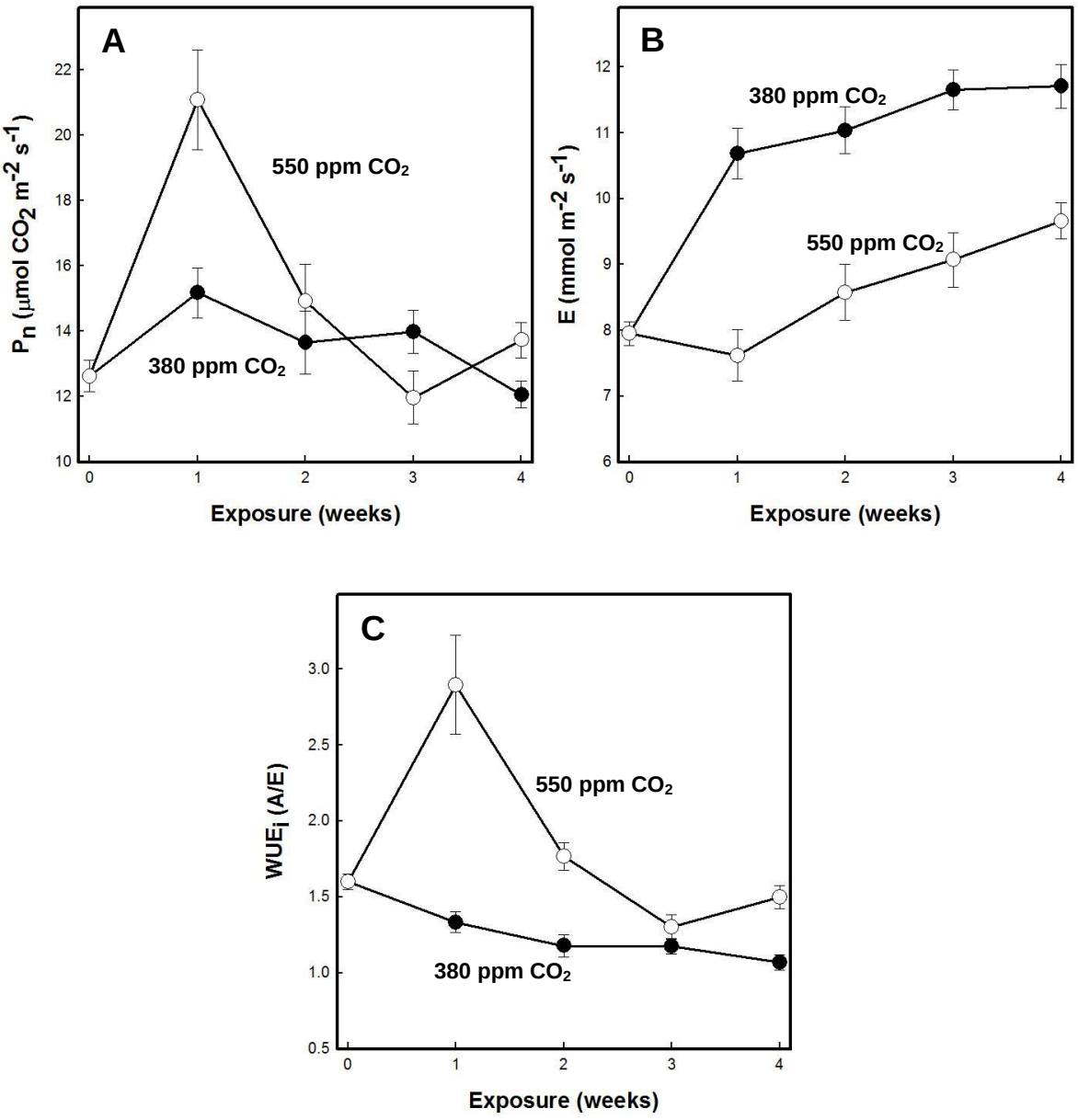

Fig. 4.3. Characteristics of plants grown in ambient CO_2 (380 ppm) or elevated CO_2 (550 ppm). The patterns of net photosynthetic rate (A), transpiration (B) and water use efficiency (C) were measured by Infra Red Gas Analyser (IRGA).

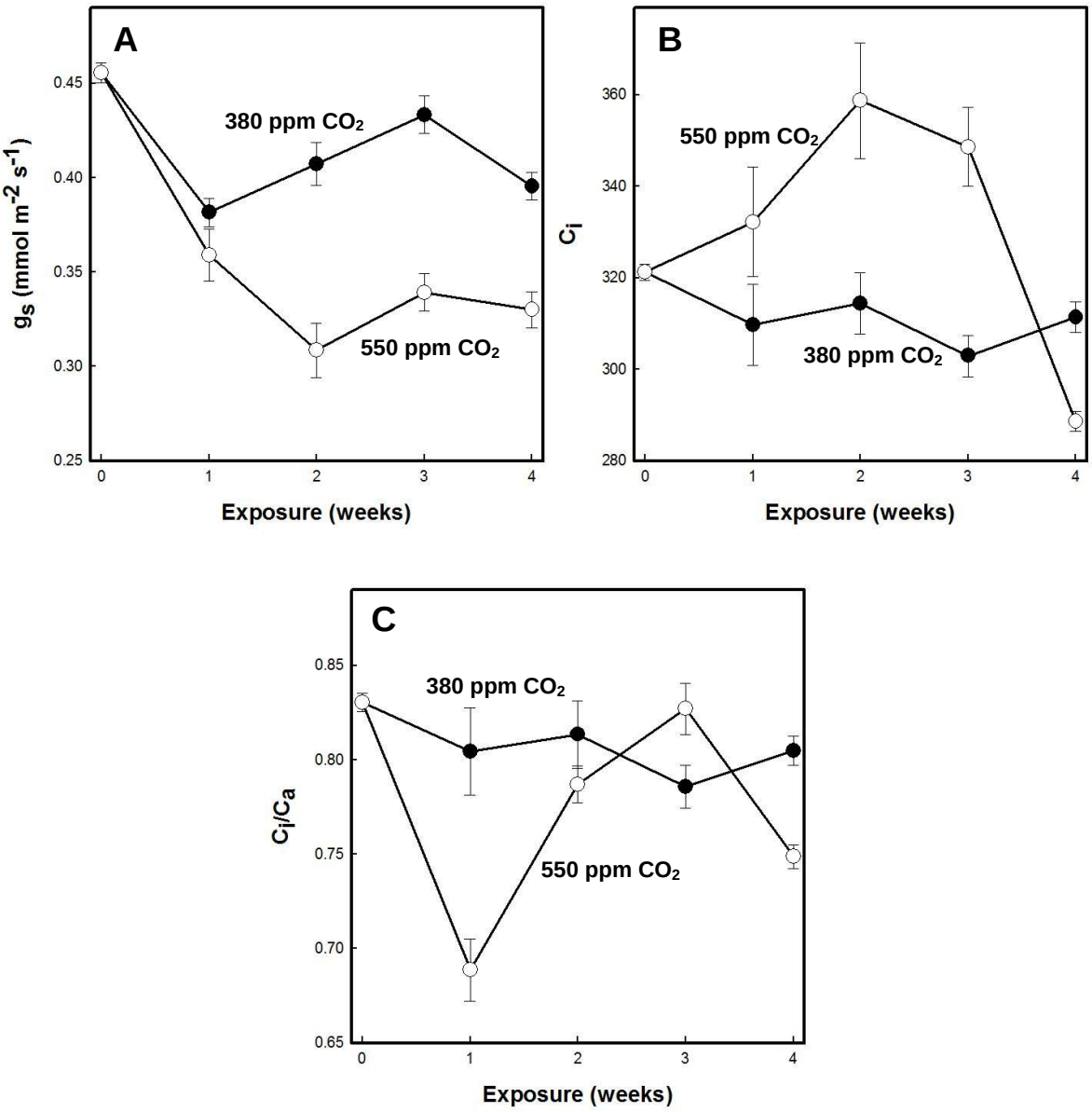

Fig. 4.4. Characteristics of plants grown in ambient CO_2 (380 ppm) or elevated CO_2 (550 ppm). The patterns of stomatal conductance (A), intercellular CO_2 concentration (B) and C_i/C_a (C) were measured by Infra Red Gas Analyser (IRGA).

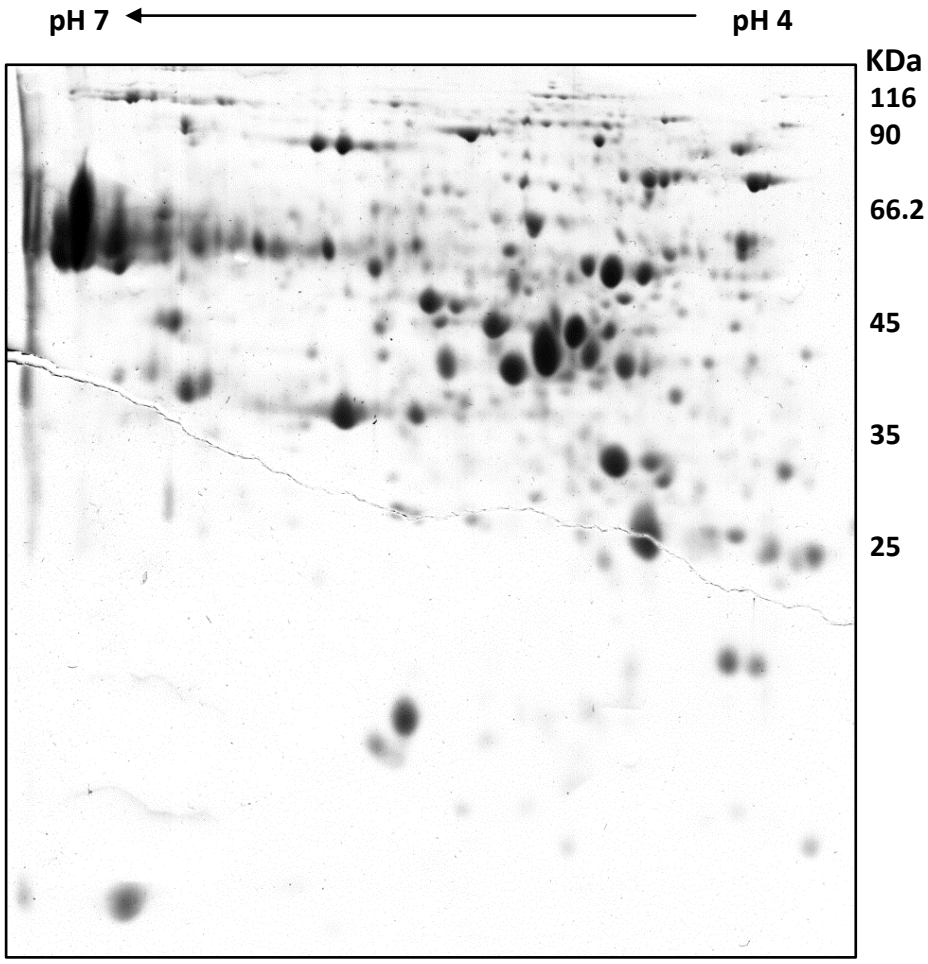

Fig. 4.5. The typical pattern of proteins from leaves of pea plants, separated by two-dimensional electrophoresis. The plants were taken at the start of the experiment. Proteins were separated in the first dimension on pH 4-7 immobilized pH gradient strips and in second dimension in 12% vertical slab gels. The relative molecular mass (116-25 KDa) of standard proteins is given on the right side of the gel.

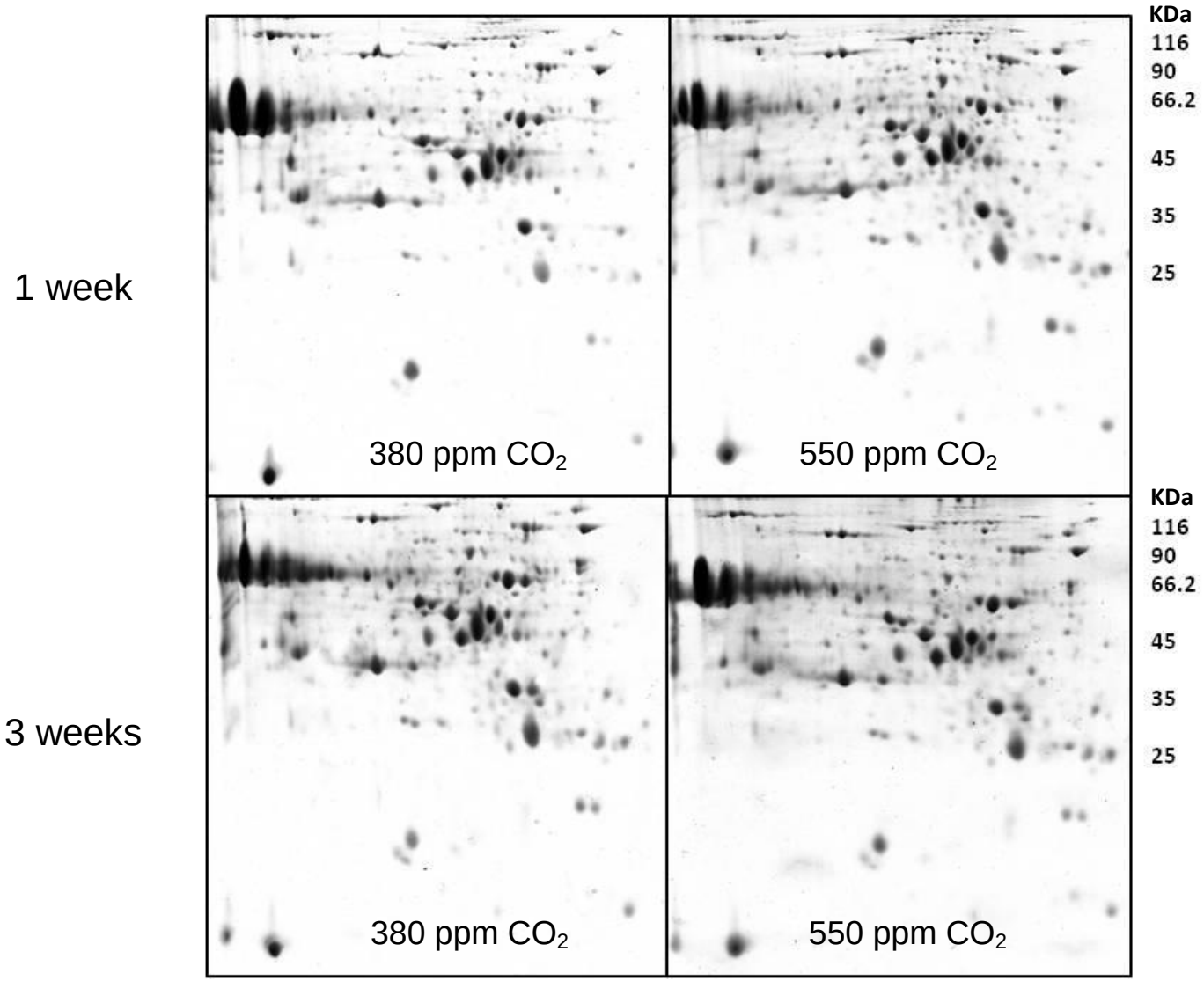

Fig. 4.6. The 2-DE gels of proteins from leaves of pea plants grown at different CO_2 treatments. Proteins were separated as described in Fig. 4.5.

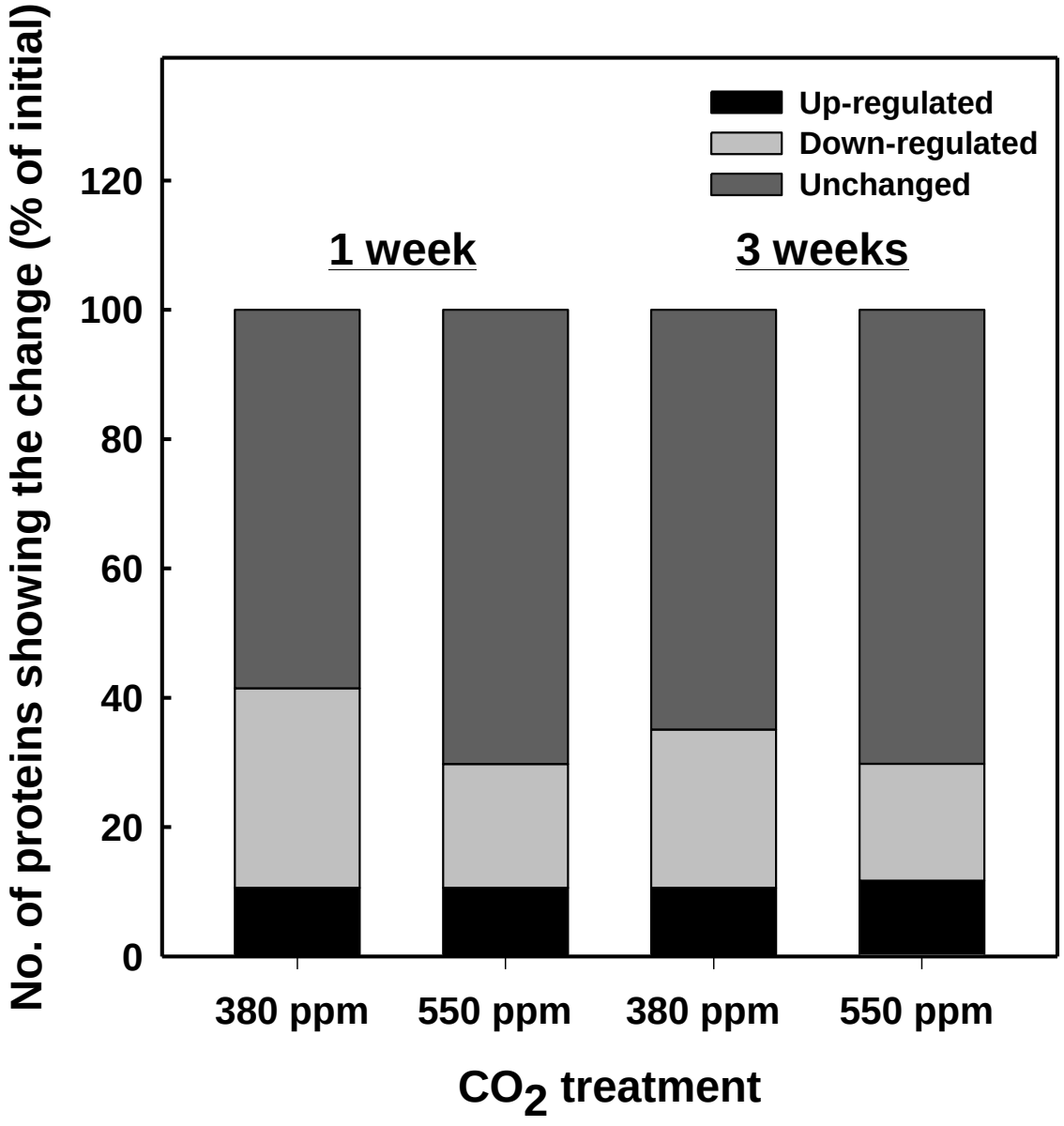

Fig. 4.7. Protein expression pattern represented as stacked bars in the ambient (380 ppm CO_2) and elevated (550 ppm CO_2) treatment conditions.

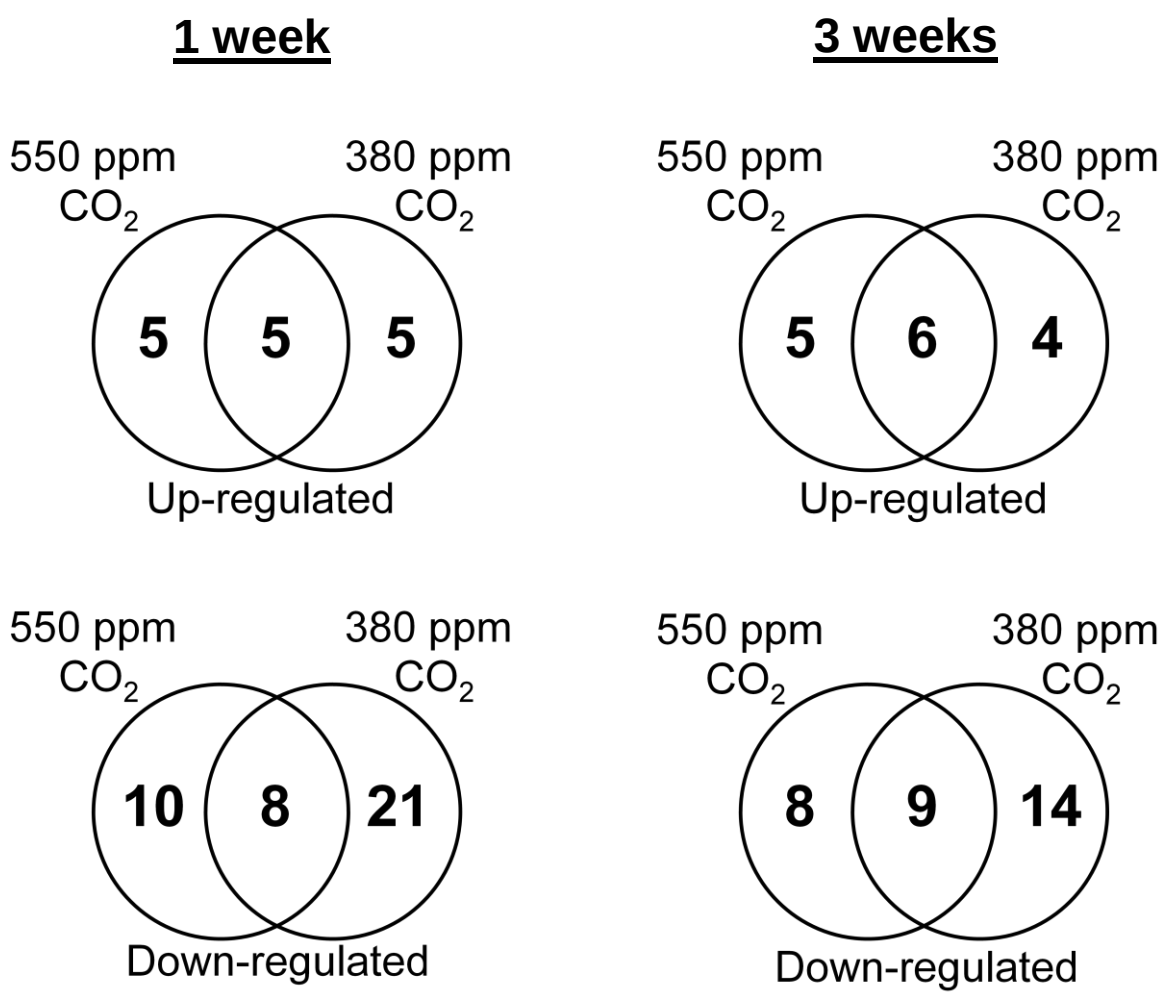

Fig. 4.8. Venn diagram analysis of up-regulated and down-regulated proteins at ambient (380 ppm CO_2) and elevated (550 ppm CO_2) treatment conditions. Numbers represent proteins up-regulated or down-regulated in the treatment conditions.

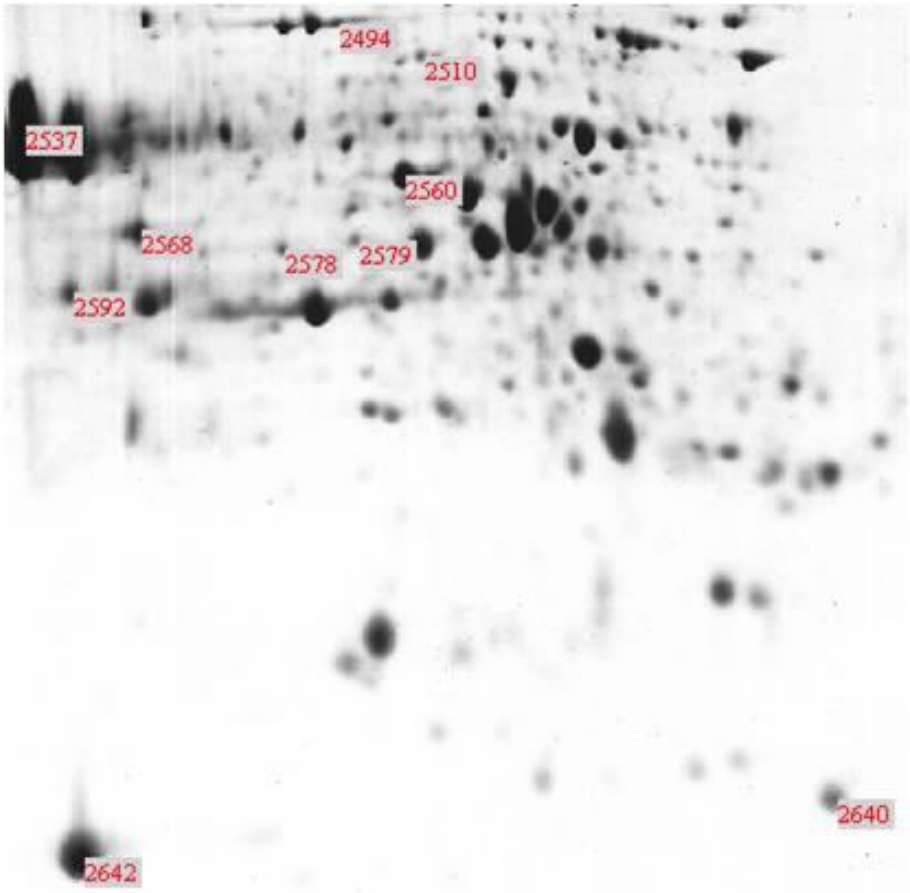

Fig. 4.9. A typical 2-DE gel showing up the identity of some of the prominent protein spots. The details of two-dimensional electrophoresis are as in Fig. 4.5. The names of proteins are listed with corresponding numbers in Tables 4.1 and 4.2 The protein spots were excised from the master gel, subjected to in-gel digestion and identified by MALDI-TOF and MASCOT software. Further details are in Tables 4.1 and 4.2.

Table. 4.1. Protein spots on 2-DE gel represented in Fig. 4.9., whose peptide sequences matched with known proteins from pea (*Pisum sativum*) in the databases of Swiss-Prot, NCBI and Plant EST.

Spot No	Protein	Peptide mass	Peptide sequences matched	Accession number	% sequence covered	M_r/pH	MS/MS Score
2568	Glyceraldehyde -3-phosphate dehydrogenase	2598.4463	R.KAAEGPLKGILDVCDVPLVSVDFR.C	gi|120663	8	48/7.6	188
		2470.3426	K.AAEGPLKGILDVCDVPLVSVDFR.C				
		1803.9436	K.GILDVCDVPLVSVDFR.C				
		1772.8121	K.VVAWYDNEWGYSQR.V				
2537	RuBisCo large chain	2395.2629	K.LTYYTPDYQTKDTDILAAFR.V	gi|399659508	53	53/6.6	108
		1021.5163	K.DTDILAAFR.V				
		3855.0271	R.VTPQPGVPPEEAGAAVAAESSTGTWTTVWTDGLTSLDR.Y				
		985.5647	R.ALRLEDLR.I				
		1479.8003	R.LEDLRIPYAYVK.T				
		853.4613	R.IPYAYVK.T				
		1465.7538	K.TFQGPPHGIQVER.D				
		1502.8510	K.YGRPLLGCTIKPK.L				
		910.4333	R.AVYECLR.G				
		2186.0680	R.GGLDFTKDDENVNSQPFMR.W + Oxidation (M)				
		2187.9215	R.GGLDFTKDDENVNSQPFMR.W + Oxidation (M)				
		1466.7132	K.DDENVNSQPFMR.W + Oxidation (M)				
		1532.7644	R.DRFLFCAEAIYK.S				
		1261.6086	R.FLFCAEAIYK.S				
		2910.7356	K.SQAETGEIKGHYLNATAGTCEEMLKR.A + Oxidation (M)				
		3052.6569	R.ELGVPIVMHDYLTGGFTANTTLSHYCR.D				
		3069.1696	R.ELGVPIVMHDYLTGGFTANTTLSHYCR.D + Oxidation (M)				
		1187.6470	R.DNGLLLHIHR.A				
		1170.6185	R.QKNHGMHFR.V + Oxidation (M)				
		1860.0329	K.LEGEREITLGFVDLLR.D				
		2494.3662	K.LEGEREITLGFVDLLRDDYIK.K				
		1275.7108	R.EITLGFVDLLR.D				
		1910.0367	R.EITLGFVDLLRDDYIK.K				
		2038.1287	R.EITLGFVDLLRDDYIKK.D				

		1116.5658	R.VALEACVQAR.N				
		1546.7514	K.WSPELAAACEVWK.E				
2592	Fructose bisphosphate aldolase	2220.4337	K.YIATPGKGILAADESTGTIGKR.L	gi|1168408	24	38/6.4	84
		2282.3840	K.GVVELAGTDGETTTQGFDSLGAR.C				
		2123.3128	K.IGPNEPSELSIQQNAQGLAR.Y				
		1647.7974	K.VSPEVIGEYTVNALR.R				
		1803.9967	K.VSPEVIGEYTVNALRR.T				
		920.0184	K.AQDVFLAR.C				
2578	Glutamine synthetase	2542.6368	R.GNNILVICDVYTPAGEPLPTNKR.Y	gi|121333	10	39/5.9	83
		1611.5903	R.HKEHIAAYGEGNER.R				
2510	Phosphoglucomutase	1803.8784	R.IQTTPFDGQKPGTSGLR.K	gi|12585296	2	64/5.5	53
2560	Elongation factor Tu	1141.7343	K.VGDVVDLVGLR.E	gi|6015084	5	53/6.6	51
		1920.0564	R.HSPFFAGYRPQFYMR.T + Oxidation (M)				
2642	RuBisCo small chain	2258.5085	R.KGWVPCLEFELEKGFVYR.E	gi|132097	14	20/9.2	38
		934.0574	R.IIGFDNVR.Q				
2640	Glycine cleavage system H protein	1698.8319	K.IKPTSPDELESLLGAK.E	gi|121080	9	18/5.2	26

Table. 4.2. Protein spots on 2-DE gels represented in Fig. 4.9. matched with sequences other than pea and had more than one possible hit by using databases.

Spot No	Protein	Peptide mass	Peptide sequences matched	Accession number	% sequence covered	M_r/pH	MS/MS Score
2494	Probable caffeine synthase	3303.6134	K.GDECDGPNTMDLLEMAINDLVAEGRLGEEK.L + Oxidation (M)	gi|75168238	7	44/5.7	42
2494	L-ascorbate oxidase	3303.6134	K.ISGVFRVGSISDRPTGGGIYLDTSVLQADYR.S	gi|502131162	5	61/9.2	41
2579	K-box region and MADS-box transcription factor family protein	1762.0251	R.LHQNHQVNLLHHGGR.F	gi|508699035	6	27/9.6	33
2579	Respiratory burst oxidase	2541.4477	K.KTTWMYLAVPVVLYVSERILR.L		2	102/9.3	27
2494	Stilbene synthase	3776.1317	R.KVQRAEGPATVLAIGTANPPNCVDQSTYADYYFR.V		8	43/6.1	0
2494	ATP synthase subunit beta	3776.1317	K.NAAGQDIDVTCEVQQLLGDNKVRAVAMSATDGLMR.G + 2 Oxidation (M)		7	53/5	0

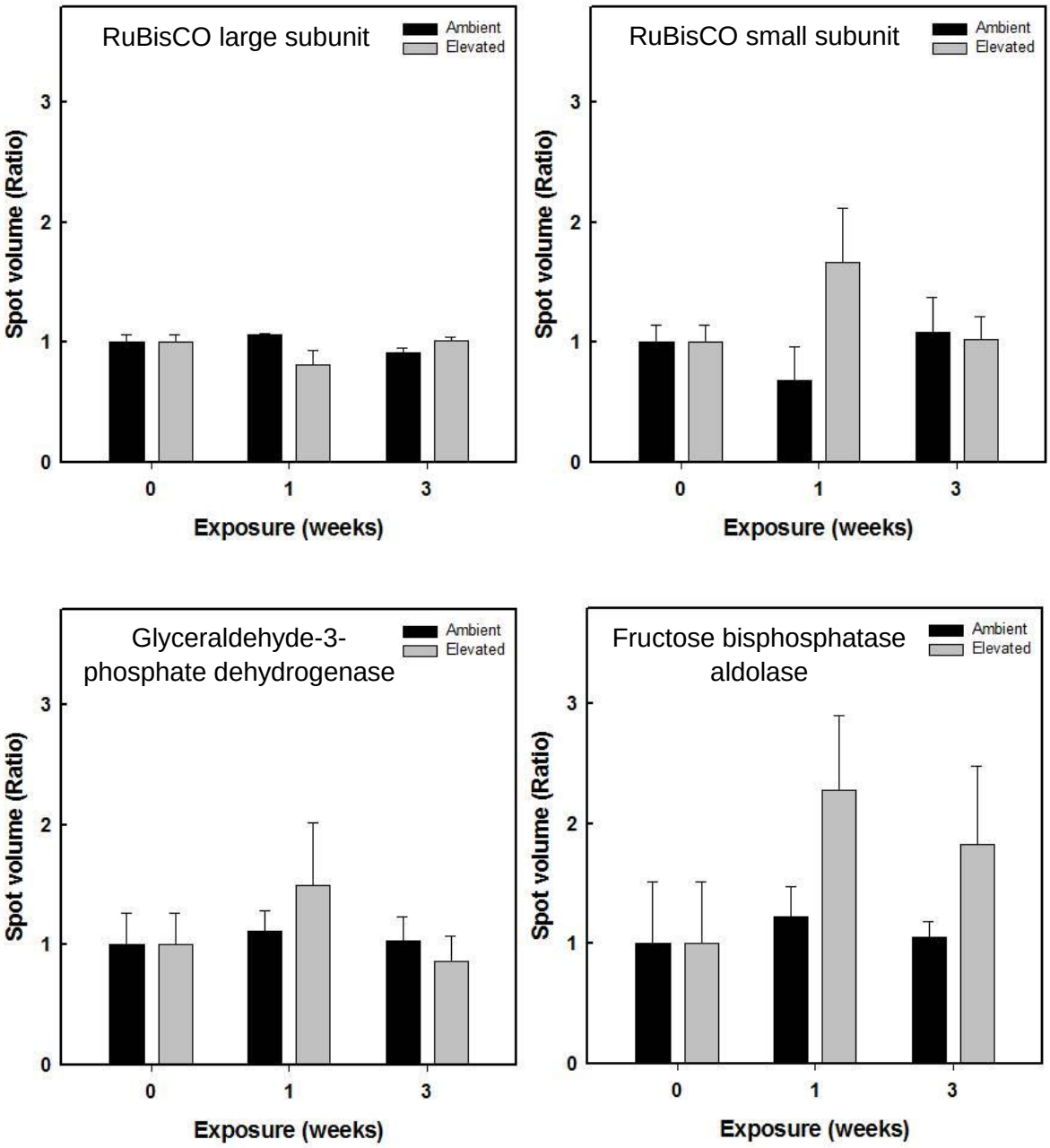

Fig. 4.10. Relative spot volumes (as ratios) represented as levels at either 'ambient' or 'elevated' in relation to those at that of control, (i.e. at the 'start' of the experiment). Each spot was analysed by MS/MS and their fragmentation was checked with available databases for matching peptide sequences (See Table. 4.1). Proteins represented here belong to enzymes of Calvin cycle metabolism. The differential regulation of proteins at ambient (380 ppm CO_2) and elevated (550 ppm CO_2) during the exposure is obvious.

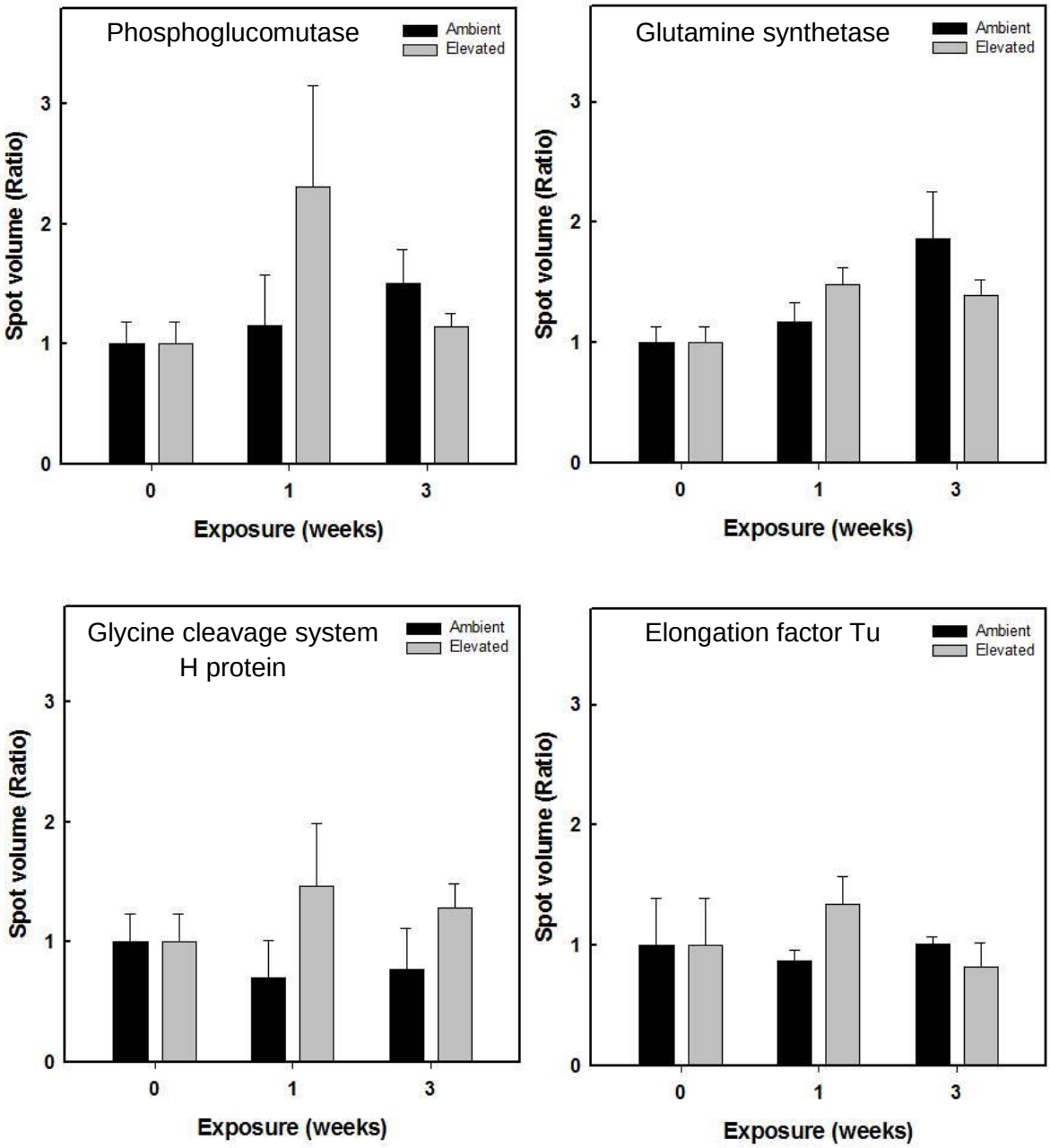

Fig. 4.11. Relative spot volumes (as ratios) represented as levels at either 'ambient' or 'elevated' in relation to those at that of control, (i.e. at the 'start' of the experiment). Each spot was analysed by MS/MS and their fragmentation was checked with available databases for matching peptide sequences (See Table. 4.1). Proteins represented here belong to sucrose/starch synthesis, nitrogen metabolism, photorespiratory metabolism and protein synthesis. The differential regulation at ambient (380 ppm CO_2) and elevated (550 ppm CO_2) during the exposure is obvious.

Discussion

Photosynthetic response to field plants grown at elevated CO_2

Marked physiological changes were recorded in pea plants, when grown at elevated CO_2. Along with an increase in the rate of photosynthesis, plants exhibited greater plant growth at elevated CO_2 (Fig. 4.3A, 4.2). This tendency of biomass enhancement appeared to be mainly due to increase of whole-plant photosynthesis. Carbon gained during growth was invested into biomass. Though there are species-specific variations, the nitrogen fixing plants respond strongly to the elevated CO_2 (Poorter and Navas, 2003). These evidences correlate to the observed changes in morphology and physiology of the plants grown at elevated CO_2 (Fig. 4.1, 4.3A). It is also possible that biomass increase or plant growth is facilitated by also the retention of water within the leaves due to the decrease in stomatal conductance/water loss.

Stomatal conductance at elevated CO_2 and ambient CO_2 treatments

The stomatal conductance is often less at elevated CO_2 concentrations than that in ambient levels (Ainsworth and Rogers, 2007). It is known that mesophyll cells in leaves have an increased response, compared to the isolated epidermis and this is dependent on intercellular CO_2 rather than external concentrations of CO_2 (Mott, 2009). Responses obtained from the measurements of stomatal conductance and intercellular CO_2 indicate the corresponding relation between the changes of decreasing stomatal conductance and increasing internal CO_2. Such decrease in stomatal conductance could be due to either direct effect of elevated CO_2 on guard cells or indirectly by decreased photosynthesis. It is therefore not surprising that stomatal conductance in leaves of pea were markedly lowered at 550 ppm CO_2 (Fig.

4.4 A). The sensing of high levels of CO_2 is possibly achieved by the changes in supply of carbon skeletons to malate synthesis and ion channels which lead to the change in turgor of guard cells (Vavasseur and Raghavendra, 2005). The lowering of stomatal conductance in elevated CO_2 also led to decrease of transpiration and improved water use efficiency. Such responses can benefit the plants grown in future climatic scenario which are water limited (Wullschleger et al., 2002).

Use of proteomics for studies on elevated CO_2 effects

Proteins are not only important components of biochemical pathways but also play a key role in signaling. Protein levels are modified by the changes in the expression pattern during growth, development and responses to environmental factors. Proteomic analysis gives specific information about the metabolic status of the plant experiencing the varied condition, compared to genomics and transcriptomics (Chen and Harmon, 2006).

Proteomic analysis involves a series of steps, like sample preparation, protein separation and protein identification. The protein fraction is separated from the other cellular components by either of the methods: (i) TCA/acetone precipitation and (ii) phenol extraction methanol/ammonium acetate precipitation. The latter precipitation method yields less number of protein spots with small mass (<25 kDa), but outperforms the TCA/acetone precipitation in other aspects. The resultant 2-DE gels from the phenol extraction methanol/ammonium acetate precipitation have been devoid of streaks (Carpentier et al., 2005). Our 2-DE gels obtained in present study (Fig. 4.5, 4.6), were good and reproducible, indicating the efficacy of the extraction by phenol-methanol/ammonium acetate, as well as separation.

The availability of wide range of IPG strips and the technique Isoelectric focussing is applied in the first dimension to allow the detailed analysis of the proteome with greater resolution. Following this, the proteins segregated to their specific isoelectric point (*pI*) are subjected to run down the gel based on their molecular sizes by applying electric charge (Chen and Harmon, 2006). This technique gives well resolved gels with separated proteins which can be visualized by coomassie or silver staining and analysed further with software packages for gel screening and spot detection. In our case, employing this isolation procedure has yielded an estimate of 94 reproducible spots in all the gels throughout the treatment variations.

The commonly used mass analysers are with matrix-assisted laser desorption/ionization (MALDI) and electron spray ionization (ESI). The protein spots are usually excised and digested to reveal the peptide mass finger print by MALDI-TOF MS and the determination substantial amino-acid sequences of the peptide fragment by ESI-MS/MS (Pandey and Mann, 2000). Analysis by this method yielded confirmed identification of eight protein spots out of ten spots, which were picked and excised from the master gel and undergone trypsin digestion (Table 4.1).

Proteins up-regulated at elevated CO_2: Metabolic significance

Observations made by previous workers at elevated CO_2, with rice they showed decreased levels of Calvin cycle enzymes which correlated to decreased photosynthesis (Bokhari et al., 2007). Improved salinity tolerance is observed in *Aster tripolium* by providing more energy at elevated CO_2 (Giessler et al., 2010). Apart from these, in wheat, development of ears after 2 weeks of anthesis did not contribute for the increasing the sink strength at elevated CO_2. Proteomic

characterization showed up increased phosphoglycerate mutase, suggesting a possible channelling of carbon into respiratory pathway (Aranjuelo et al., 2011). Calvin cycle consists of three different phases they are carboxylation, reduction and regeneration. Carboxylation is mediated by RuBisCO, reduction phase involves enzymes like phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) regeneration phase includes enzymes like aldolases, transketolases, phosphatases isomerases and kinases. Most of these proteins are abundant and found in the chloroplast proteome except ribulose 5-phosphate epimerase (Kleffmann et al., 2004).

Our analysis revealed the upregulation of RuBisCO small subunit after the first week of exposure to elevated CO_2, which is known to be involved in the carboxylation. In contrast to the small subunit response the large subunit of RuBisCO showed no perceptible changes in the protein regulation, possibly due to the abundance of the availability of RuBisCO in the plant (Fig. 4.10). RuBisCO content in the plants depend on the biosynthesis and degradation, RuBisCO biosynthesis is higher in the phase of leaf elongation with *rbc* gene expression. This response was decreased in the later phases (Irving and Robinson, 2006). The absence of decrease in RuBisCO levels is possibly due to the symbiotic N fixation, a contrast to the N limited scenario, where the N has to be remobilized to attain the plant survival (Hirel and Gallais, 2006).

In the enzymes related to reduction phase of Calvin cycle, GAPDH was up-regulated after first week of exposure to elevated CO_2 (Fig. 4.10). The enzyme GAPDH catalyses the reduction of 1, 3-bisphosphoglycerate by consuming NADPH, and is also known to be involved in redox regulation (Sparla et al., 2005). GAPDH

was modulated by the thioredoxin or glutathiolation for inactivation under oxidized conditions and dark (Fermani et al., 2007). Fructose 1,6-bisphosphate aldolase (FBP aldolase), belongs to the regeneration phase of Calvin cycle and is found to be up-regulated in our experiments at elevated CO_2 (Fig. 4.10). Overexpression of *Arabidopsis* FBP aldolase in tobacco plants increased the enzyme activity by more than 1.4-fold. Transgenic plants grown under high CO_2 (700 ppm) showed an increase in biomass up to 2.2-fold along with increased photosynthetic rate by 1.5-fold (Uematsu et al., 2012). Apart from the phosphorylation and acetylation of enzymes for regulation, recent evidences show that FBP aldolase undergoes methylation at lysyl residue by lysine methyltransferase, which might determine their fate in carbon metabolism (Mininno et al., 2012).

The responses of GAPDH and FBP aldolase to elevated CO_2 point out the possibility of changes in energy generation mechanism and lowering the oxidative status of the plant (Fernie et al., 2004). Exposing the plants to elevated CO_2 for one week might increase respiratory carbon metabolism and also up-regulated phosphoglucomutase (PGM) (Fig. 4.11), an enzyme responsible for the interconversion glucose 6-phosphate (G6P) to glucose 1-phosphate (G1P) (Davies et al., 2003). Over expression of PGM as found in work (Fig. 4.11) reveal the importance of carbon flow into starch synthesis by formation of G1P or G6P (Lytovchenko et al., 2005, Fettke et al., 2010).

Photorespiration is an unavoidable event in oxygenic environment. When RuBisCO forms glycerate and glycolate, the later (glycolate) is converted to glycerate by series of enzymatic conversions located in peroxisomes, mitochondria and chloroplasts (Maurino and Peterhansel, 2010). Glycine decarboxylase (GDC), a

multi protein complex in mitochondria, is made of P, T, L and H proteins (Bauwe et al., 2010). The up-regulation of glycine cleavage system H protein at elevated CO_2 system emphasize the importance the GDC function and photorespiration under these conditions (Fig. 4.11). These could also establish the link between balance of redox and nitrogen metabolism (Voss et al., 2013, Sunil et al., 2013).

The link between the responses of elevated CO_2 and nitrogen metabolism is further emphasized by the up-regulation of glutamine synthtase (GS) after the one week of exposure to elevated CO_2 (Fig. 4.11). GS is an important enzyme in nitrogen assimilation pathway, facilitating the incorporation of NH_3 to glutamate (Miflin and Habash, 2002). Transgenic plants with higher plastidic GS had improved photorespiration and protection from photoinhibition (Kozaki and Takeba, 1996). The up-regulation of GDC and GS proteins in elevated CO_2 conditions provide evidence that carbon and nitrogen metabolisms are interlinked and photorespiration and N assimilation occur at tandem. Photorespiratory ammonia released is assimilated back by the enzymes glutamine synthtase and glutamine 2-oxoglutarate amino transferase (Keys, 2006).

Elongation factor Tu protein (EF-Tu) is involved in protein synthesis (Ursin et al., 1993). In pea, the levels of EF-Tu protein were differentially regulated in response to various abiotic stresses: salinity and low temperature, plant hormones: ABA and salycilic acid (Singh et al., 2004). EF-Tu exhibits chaperone activity, heat tolerant lines of maize had higher expression of EF-Tu under heat stress (Rao et al., 2004; Momcilovic and Ristic, 2007). This protein is also known to undergoing oxido-reduction by thioredoxins along with other proteins of Calvin cycle (Balmer et al., 2003). Our experiments showed up slightly increased EF-Tu levels after the first

week of exposure to elevated CO_2, that indicate action of protein synthesis (Fig. 4.11). Our observations collectively indicate that under these elevated CO_2 conditions, plants are increasing their metabolic activity and also coping with the stressful scenario.

Conclusions

1. Photosynthetic rate was increased in plants grown at elevated CO_2, increase in biomass was sustained in these plants towards the end of experiment.
2. Plants exhibited a decrease in stomatal conductance when grown in elevated CO_2, leading to improved water use efficiency under these conditions.
3. There was a differential regulation of proteins under these conditions, the fraction of down-regulated proteins were lower in elevated CO_2 treatments compared to the ambient CO_2.
4. The up-regulation pattern was not same for the analysed proteins, elevated CO_2 treatment effected the regulation of each of the proteins based on the prolongation of time.
5. The 2-DE separation followed by MS/MS analysis indicated that the up-regulated proteins under elevated CO_2 treatments belong to C and N metabolism. Most of the up-regulated proteins belonged to Calvin cycle and starch synthesis. Similarly, proteins of GDC, GS and EF-Tu related to nitrogen metabolism were also up-regulated in elevated CO_2 treatments.

Chapter 5

Responses of Photosynthetic O_2 Evolution and Calvin Cycle Metabolites in Mesophyll Protoplasts to Varied Bicarbonate (mimicking high CO_2) and Inorganic Phosphate

Introduction

The responses of increased photosynthesis to elevated CO_2, as described in Chapter 4 are consistent with the available literature. The increase in the photosynthesis leads to high growth, when there are no other limitations, on nutrient availability or sink capacity (Kirschbaum, 2011). The most important nutrients that affect the plant performance at high CO_2 are nitrogen and phosphorus. The sink capacity is dependent on the transport of assimilates out of chloroplasts and their translocation. The availability of phosphate influences the formation of end products, namely starch and sugars. Further, photosynthetically derived sugars can regulate, in turn, other plant processes, by modulating gene expression. Phosphate deficiency also causes changes in the levels and expression of photosynthesis related proteins (Hermans, 2006).

Chloroplast envelope has important translocators for exchange of metabolites from chloroplasts to cytoplasm, so as to achieve an optimal functioning of chloroplasts. The assimilation of carbon into starch or sugars, was greatly influenced by the presence of chloroplast phosphate translocator (Facchinelli and Weber, 2011). In photosynthetic processes, several metabolites synthesized (i.e. trioses/hexoses) are sent to cytosol and other compartments for different metabolic functions. Triose phosphate translocator (TPT), Xylulose phosphate translocator (XPT) and Glucose phosphate translocator (GPT) present on the chloroplastic

membrane can render exchange of these respective sugar phosphates with inorganic phosphate (Pi) (Weber and Linka, 2011).

Since elevated CO_2 can increase photosynthesis, thereby carbohydrates, a parallel interaction with phosphate status and sugar content is expected. Pi facilitates the exchange of metabolites like triose phosphate (triose-P) by carbon fixed in photosynthesis (Flugge et al., 2011). We therefore studied the events occurring at the cellular level by using isolated mesophyll cell protoplasts (MCP). The photosynthetic response of mesophyll protoplasts to the increased CO_2 concentration in the medium (by varying the bicarbonate) was examined in relation to varying inorganic phosphate, so as to establish the possible relationship between the increased CO_2 levels interacting with altered levels of Pi.

Results

Response of mesophyll cell protoplasts to varying bicarbonate

The rate of photosynthesis by mesophyll cell protoplasts (MCP) at 1 mM rised as the bicarbonate was more than that at no added bicarbonate (Fig. 5.1A). The photosynthetic rate of MCP then slightly decreased, as the bicarbonate level was raised to 10 mM (Fig. 5.1B). The response of MCP photosynthesis to varying bicarbonate was similar at the three mentioned light intensities. In all the light variations, the highest photosynthetic rate was at 1 mM bicarbonate (Fig. 5.1C).

Since bicarbonate may change the pH of the medium, the pH of the medium was tested. The pH of the medium rised with increasing bicarbonate concentration, when 10 mM HEPES-buffer was used. However, with 50 mM HEPES-buffer (increased buffer strength), the pH of the medium did not alter, as bicarbonate

concentration was raised (Fig. 5.2A). The photosynthetic rate of MCP was higher at 1 mM bicarbonate than that at no added bicarbonate in both 10 and 50 mM HEPES (Fig. 5.2B).

Changes in photosynthesis and Calvin cycle metabolites in response to varying bicarbonate

The rate of the photosynthesis was depended on bicarbonate concentration. Maximum photosynthesis was at 1 mM bicarbonate, while the rate decreased at 10 mM bicarbonate (Fig. 5.3A). Similarly, there were changes in the concentrations of important metabolites like phosphoglyceric acid (PGA), triose phosphate (triose-P) and ribulose bisphosphate (RuBP). The levels of PGA were highest at 1 mM bicarbonate. Triose-P levels increased, while RuBP levels did not alter much with varying bicarbonate (Fig. 5.3B). Changes in the ratios of three metabolites, in response to varying bicarbonate were quite prominent. The ratio of PGA to RuBP was maximum at 1 mM bicarbonate. The ratio of triose-P to PGA also increased at higher bicarbonate. However, the ratio of the RuBP to triose-P decreased markedly with increasing bicarbonate (Fig. 5.3C).

Photosynthetic response of mesophyll protoplasts to varying Pi

The rate of oxygen evolution by MCP was dependent on not only added bicarbonate but also on the concentration of Pi in the reaction medium. In the absence of bicarbonate, photosynthesis reached its peak at 0.5 mM Pi, the rate then decreased with increasing the Pi (Fig. 5.4A). The stimulation by Pi was maximal when MCP were provided with 1 mM bicarbonate.

Maximal photosynthesis occurred at 1 mM bicarbonate, irrespective of Pi. Similarly, the optimal photosynthesis was at 1 mM Pi (Fig. 5.4B). The role of Pi was further studied by using mannose (Pi sequester) to assess the photosynthetic functioning of MCP. Treatment with 20 mM mannose decreased markedly the rate of photosynthesis. As a result, the response to bicarbonate was also dampened (Fig. 5.4C).

Modulation of Calvin cycle metabolites and ratios in mesophyll cell protoplasts at varying Pi and bicarbonate

The levels of the important intermediates of Calvin cycle namely PGA, triose-P and RuBP were determined. In the presence of 0.5 mM Pi, the PGA levels were highest at 1 mM bicarbonate. RuBP levels were highest at 10 mM bicarbonate, while there were only marginal changes in levels of triose-P (Fig. 5.5A). In contrast, at 10 mM Pi, irrespective of bicarbonate concentration, all the three metabolites, namely PGA, triose-P and RuBP, changed marginally, with a small peak at 5 mM bicarbonate (Fig. 5.5B).

At 0.5 mM Pi, the ratios of triose-P/PGA did not chage much (Fig. 5.6A). In the concentrations of high Pi (10 mM) and higher bicarbonate, the ratio of triose-P/PGA was higher (Fig. 5.6B). However, the ratio of RuBP/triose-P reached maximum at 10 mM bicarbonate. In contrast, at 10 mM Pi, the ratio of triose-P/PGA reached maximum at 1 mM bicarbonate and kept steady (Fig. 5.6A,B). The plots of metabolite ratios in relation to Pi reaffirmed the trend. Rise in Pi from 0.5 mM to 10 mM increased the ratios of triose-P/PGA, while decreasing the ratios of RuBP/triose-P. Increased bicarbonate buffered these changes.

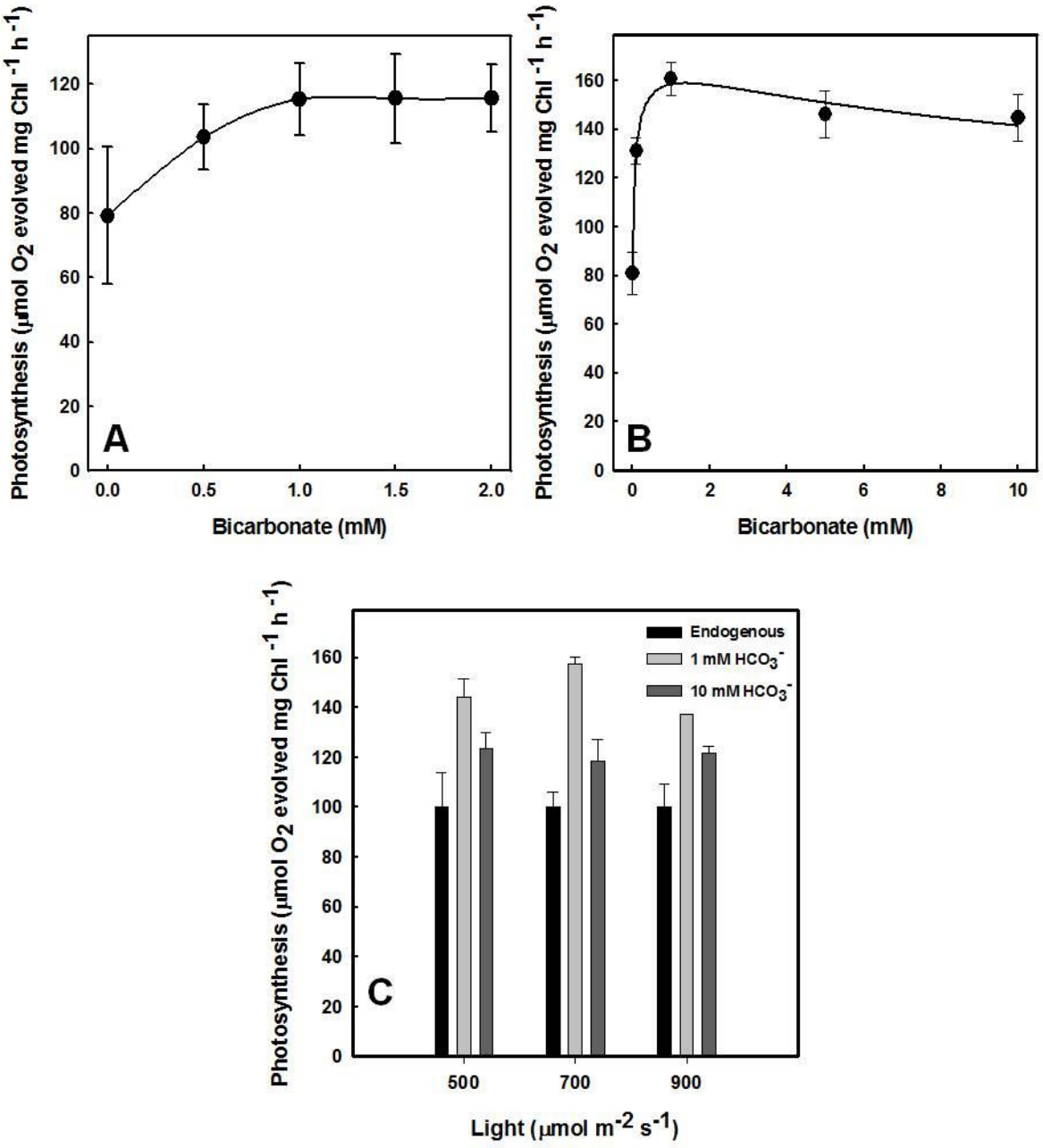

Fig. 5.1. Response of photosynthesis to bicarbonate, either in the range of 0 to 2 mM (A) or 0 to 10 mM (B). C: Response of photosynthesis to bicarbonate at different light intensities. Photosynthetic oxygen evolution of mesophyll cell protoplasts isolated from pea leaves was assayed at 25 °C and 700 µmol m^{-2} s^{-1}, if not mentioned otherwise.

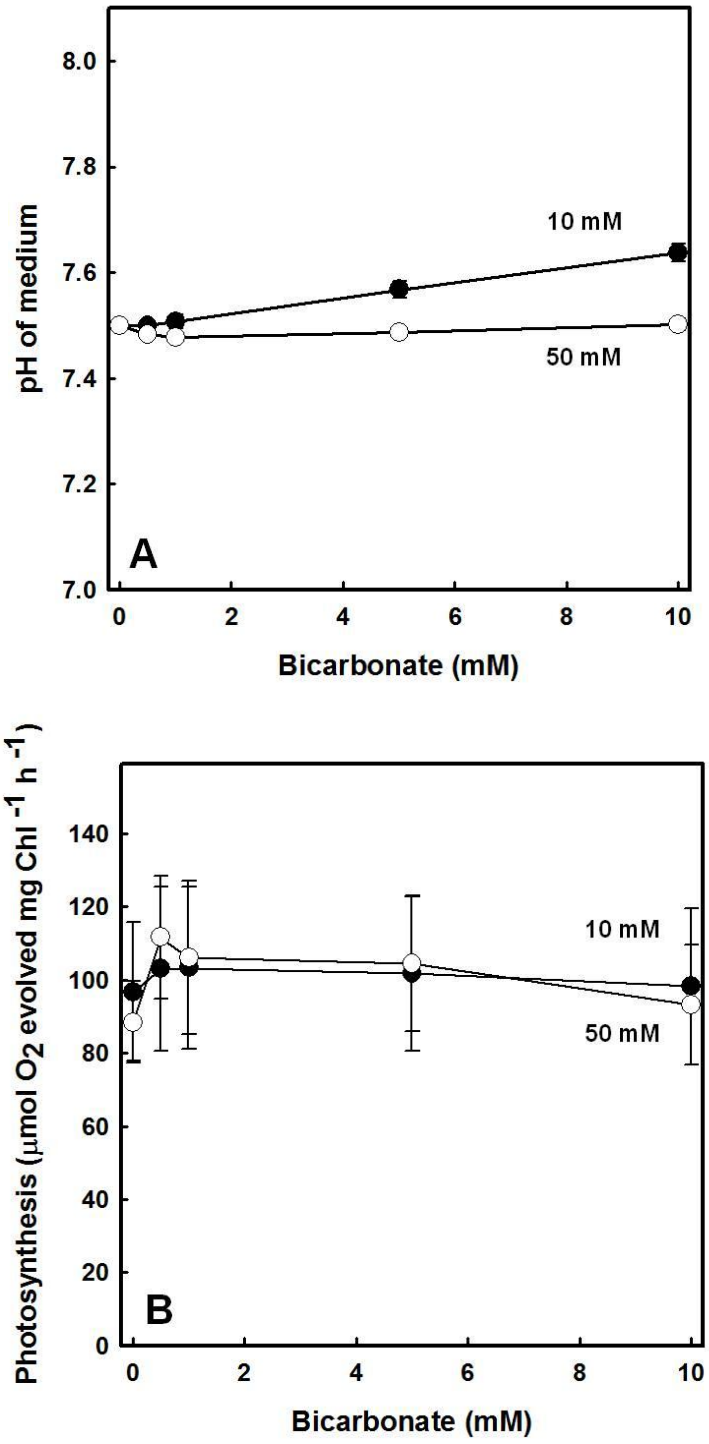

Fig. 5.2. A: The pattern of reaction medium pH in relation to added bicarbonate, at two different buffer (Hepes-KOH) strengths. B: Response of protoplast photosynthesis to varying bicarbonate at varied buffer strength.

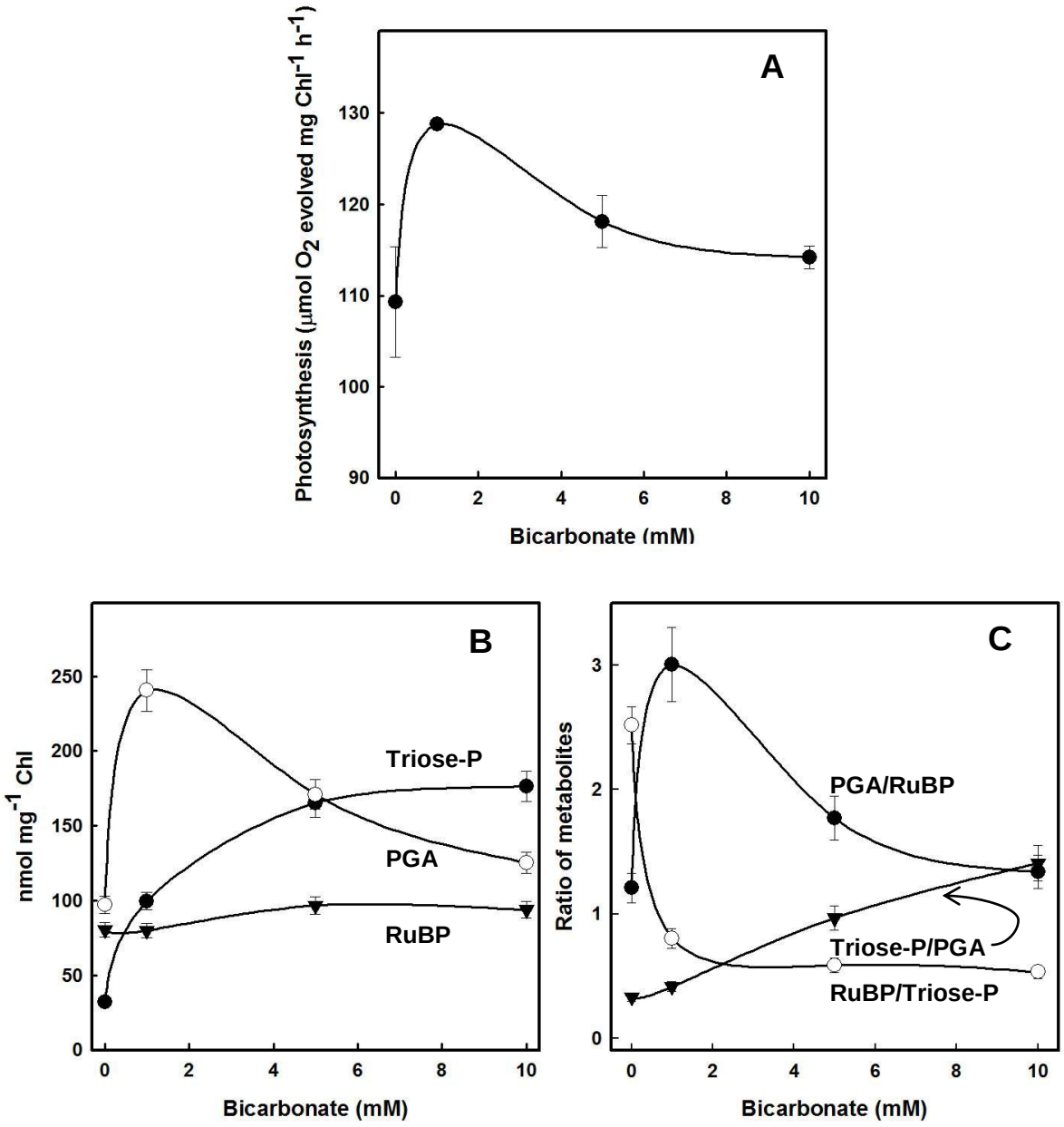

Fig. 5.3. Patterns of photosynthesis, including the metabolite variations by mesophyll protoplasts in relation to varying bicarbonate in reaction medium. A: Photosynthetic rates; B: Metabolite changes; C: Ratio of key metabolites calculated from the panel B. The treatment conditions provided were 25 ° C, pH 7.5 and light intensity of 700 µmol m^{-2} s^{-1}.

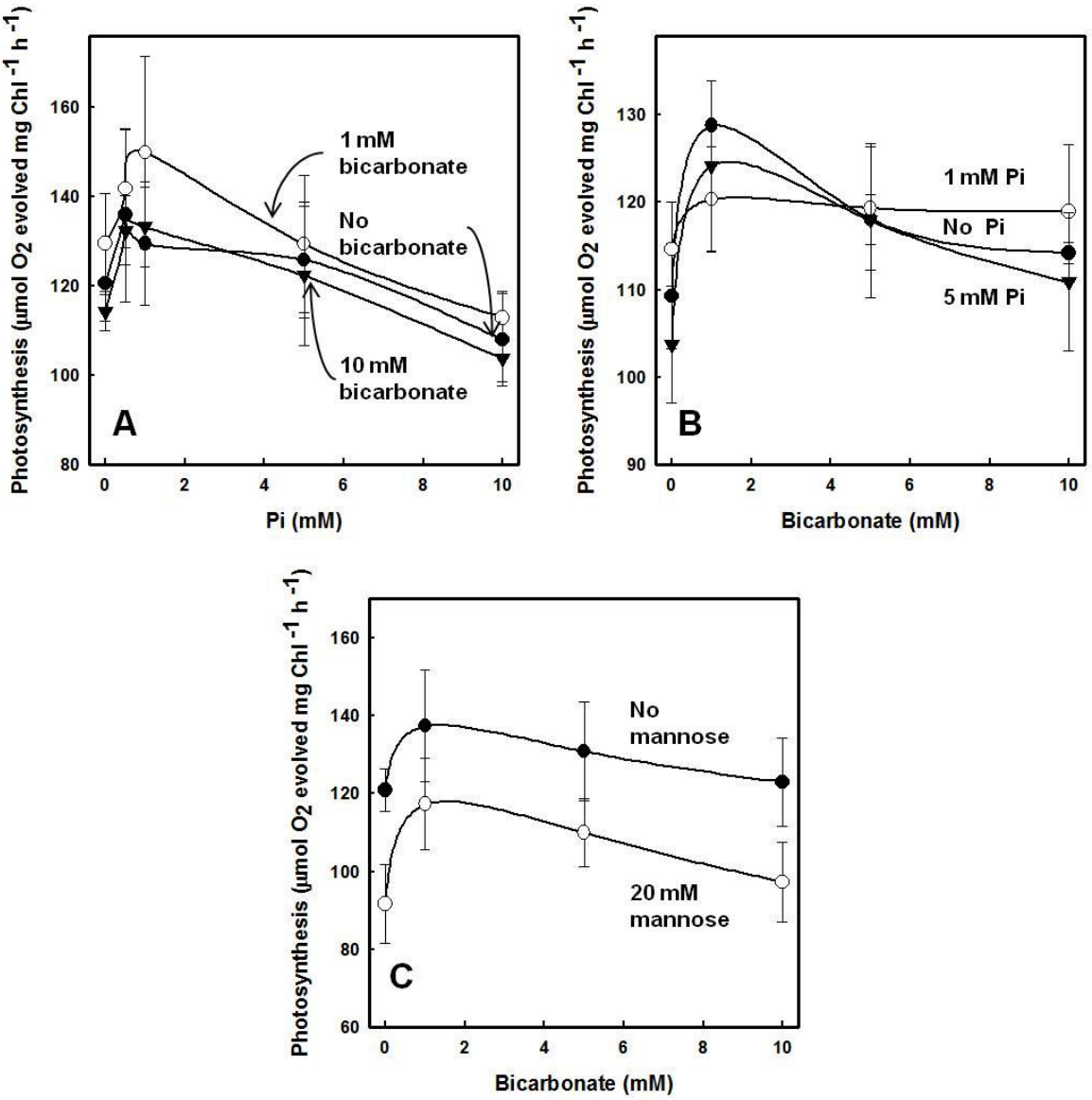

Fig. 5.4. Modulation of photosynthesis by either inorganic Pi (A) or bicarbonate (B) Effect of mannose on photosynthesis in relation to external bicarbonate (C). Unless otherwise mentioned, photosynthesis was monitored at 25 ° C and light intensity of 700 µmol m^{-2} s^{-1}. The pH of 50 mM Hepes buffer was pH 7.5 and Pi buffer was pH 7.5.

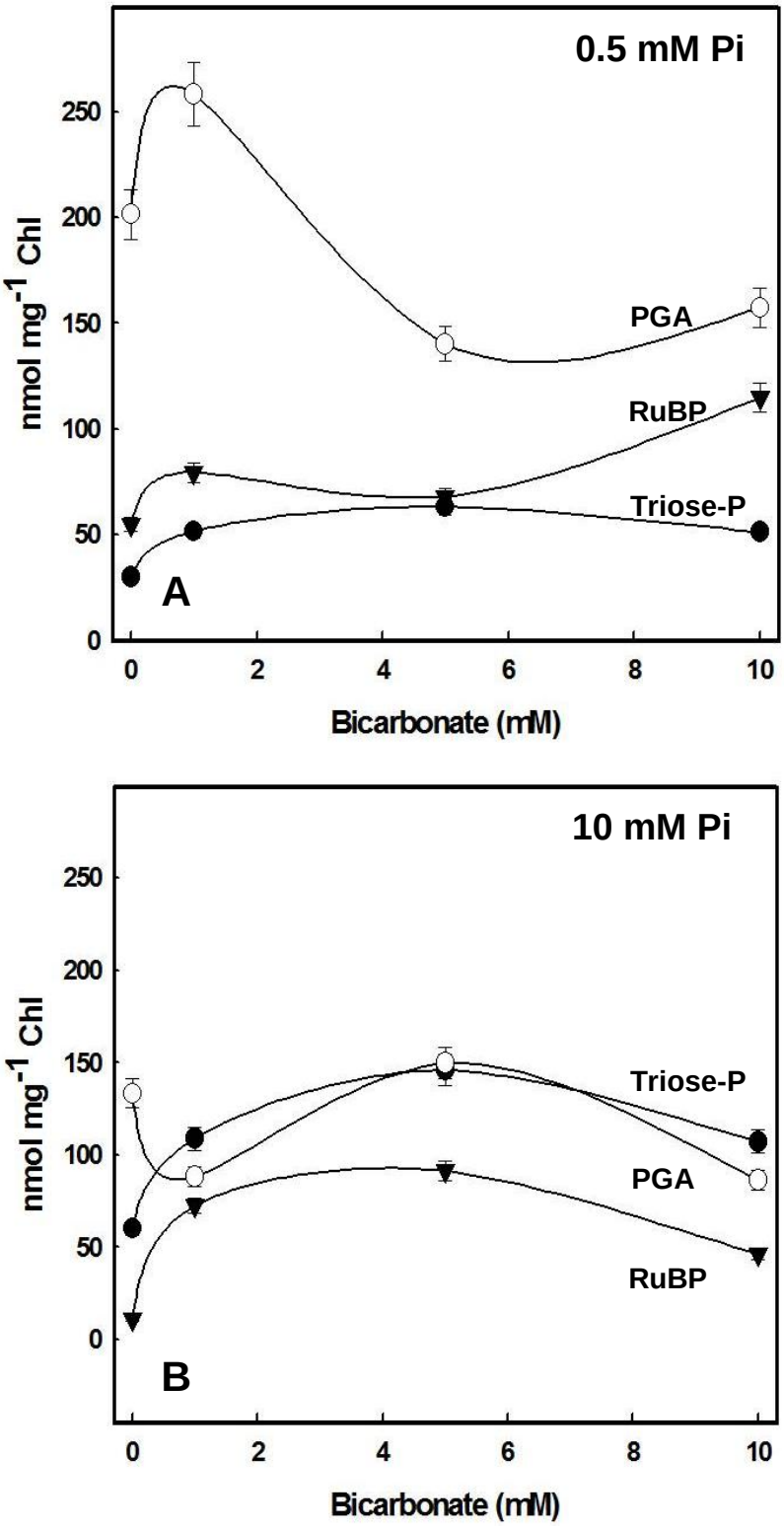

Fig. 5.5. Levels of Calvin cycle metabolites in mesophyll protoplasts: Triose-P (●), PGA (○) and RuBP (▼) at varied inorganic phosphate (A: 0.5 mM and B: 10 mM) during photosynthesis at different levels of bicarbonate. These patterns can be compared with responses in the absence of Pi represented in Fig. 5.3B.

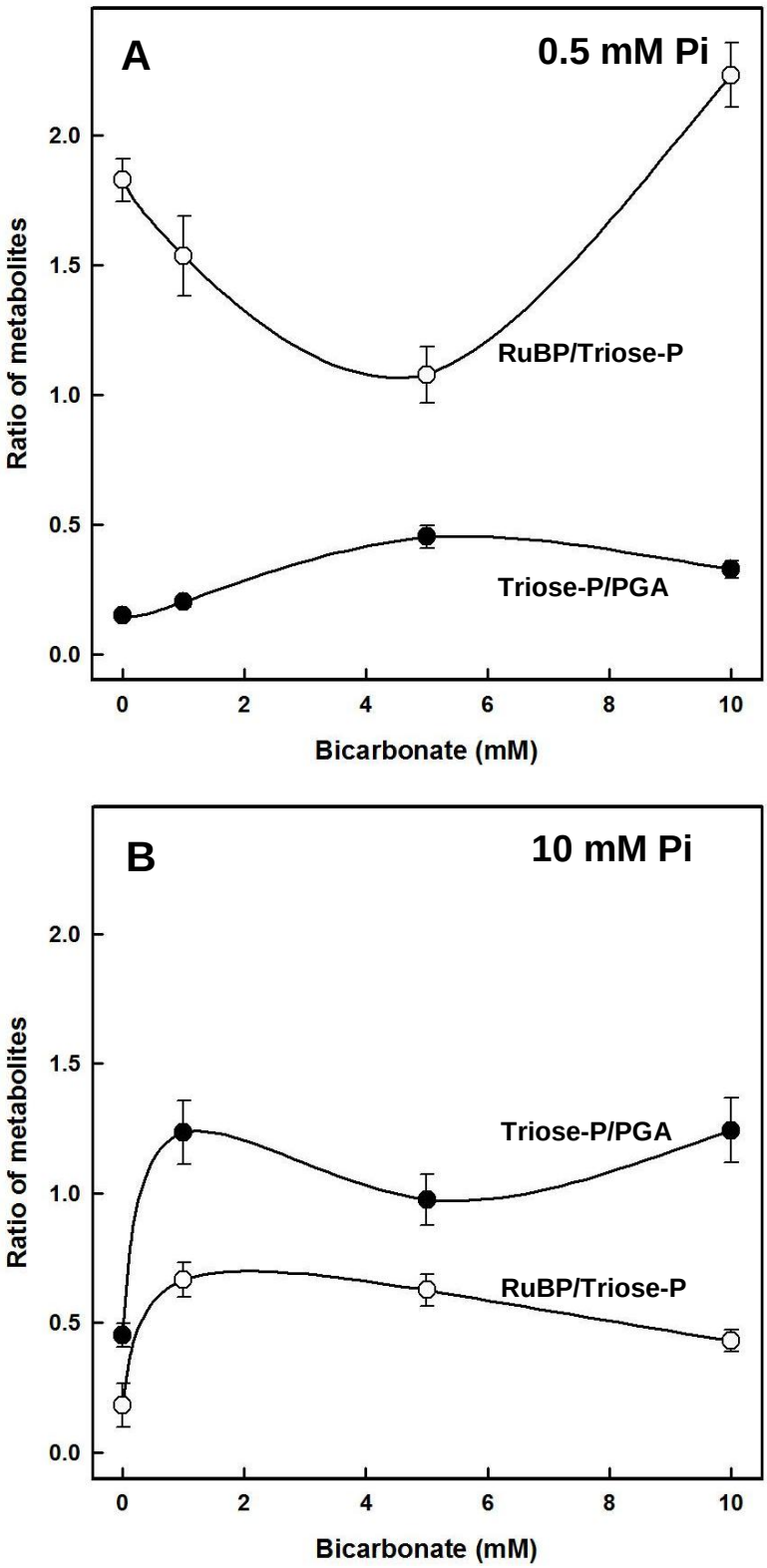

Fig. 5.6. Ratios of Triose-P/PGA and RuBP/Triose-P in mesophyll protoplasts at varied inorganic phosphate (A: 0.5 mM and B: 10 mM) during photosynthesis at different levels of bicarbonate. These ratios may be compared with those at 'no added Pi', represented in Fig. 5.3C.

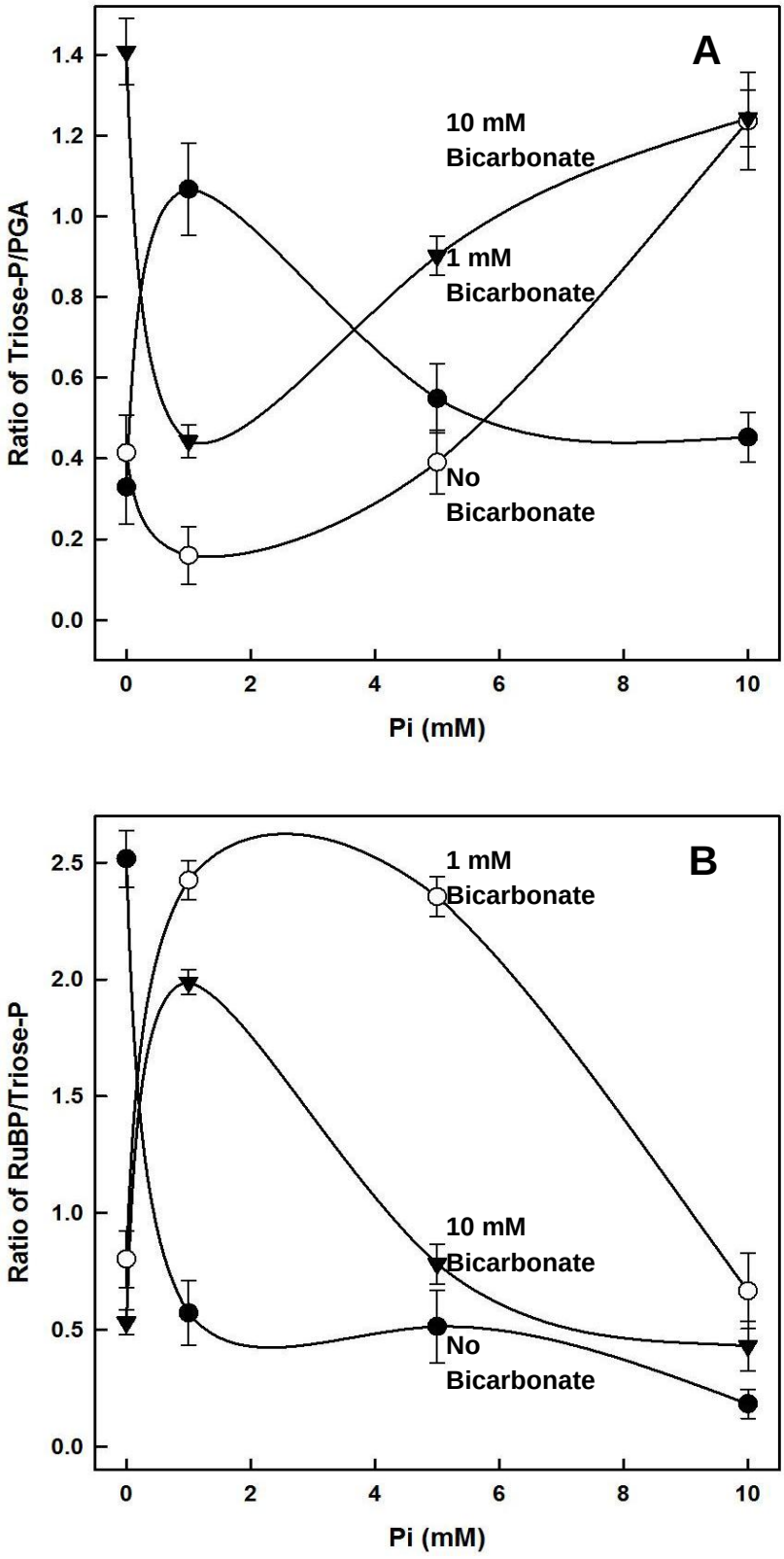

Fig. 5.7. A: Ratio of Triose-P/PGA; B: Ratio of RuBP/Triose-P in mesophyll protoplasts at varied bicarbonate and in relation to Pi.

Discussion

Decrease in photosynthesis by mesophyll protoplasts at higher bicarbonate

Activity of RuBisCO and suboptimal stomatal closure (decreased mesophyll conductance) are considered to be the limiting factors on photosynthesis at elevated CO_2 concentrations (Tcherez et al., 2006; Ainsworth and Rogers, 2007; Flexas et al., 2008). It is therefore not surprising that photosynthesis by MCP increased with externally added bicarbonate (Fig. 5.1A) Bicarbonate dependent oxygen evolution monitored by Leegood and Walker (1979), from the mesophyll cell protoplasts of pea, also indicated an increase in photosynthesis at higher concentrations of inorganic carbon. Similar responses were observed with protoplasts of barley and pea, when added sodium bicarbonate resulted in the high rates of photosynthetic oxygen evolution (Kromer et al., 1993; Riazunnisa et al., 2006).

At a higher concentration of bicarbonate, there was always a downward trend in photosynthesis of mesophyll protoplasts (Fig. 5.1B). Although this was surprising such decrease in photosynthesis at higher bicarbonate was noticed in protoplasts of pea (Raizunnisa et al., 2006) and spinach (Kaiser and Heber, 1983). Such decrease may be due to the limitation of Pi, as photosynthetic rate was quite stable in presence of added Pi (Fig. 5.4B).

Modulation by Pi of photosynthesis at optimal and high bicarbonate

Phosphorus is one of the most important minerals and is needed for everal metabolic reactions. Reactions of photosynthetic carbon reduction (PCR) cycle are enzyme based and they utilize the ATP generated in the light reactions (Noctor and Foyer, 2000). The increased utilization of Pi and consumption of ATP in PCR cycle

at elevated CO_2 levels might deplete the levels of Pi. Naturally a decrease in Pi availability imposes a serious limitation of photosynthesis.

Besides variation in photosynthetic rates at different bicarbonate levels, inorganic phosphate also modulated the photosynthetic rates of protoplasts (Fig. 5.4). Similarly, in spinach chloroplasts when Pi is limiting the decrease in the photosynthesis of spinach chloroplasts was explained to be lowering of available ATP levels and restriction of kinase action, inhibition associated with regeneration phase at limiting Pi. The addition of Pi and changed in the levels of metabolites and resulted in photosynthetic rate (Giersch and Robinson, 1987).

Photosynthesis appeared to require a sharp optimal level of Pi. Photosynthetic rate reached maximum at 1 mM of Pi and it decreased further at all bicarbonate concentrations above 1 mM (Fig. 5.4A). Similar response was found in wheat chloroplasts, where the increase of photosynthesis at optimal Pi concentration was linked to the movement of triose-P out of the chloroplasts, possibly to be assimilated into sucrose (Leegood and Walker, 1979). Unlike the response in the absence of Pi (Fig. 5.1), the photosynthetic rate did not decrease when 1 mM Pi was present, and was sustainable even at high bicarbonate (10 mM) (Fig. 5.4B). In earlier report any decrease or increase away from optimal Pi (≅ 1 mM) restricts photosynthesis (Brautigam and Weber, 2011).

Mannose can sequester Pi from the cells and was used to assess the role of Pi in mediating the response of photosynthesis to varying levels of bicarbonate (Herold et al., 1976). Presence of mannose in the reaction medium lowered markedly the photosynthetic rate in all irrespective of presence/concentration of bicarbonate (Fig. 5.4C), demonstrating importance of Pi. Similar observations were

made with corn and wheat leaves fed with mannose, which lead to decreased photosynthetic rate (Morison and Batten, 1986; Harris et al., 1986). Our results with externally added Pi as well as mannose emphasize that Pi is a key regulator and needs to be available at optimal concentration for optimal photosynthetic rate.

The patterns of key metabolites in relation to bicarbonate and Pi

Phosphate regulates the export of fixed carbon out of the chloroplasts, as triose-P is exchanged for Pi by a chloroplastic TPT (Flugge et al., 2011). Such exchange modulates the concentrations of other key metabolites present in the chloroplasts, participating in Calvin cycle. Under Pi deficiency, decrease in photosynthesis is complimented with altered partitioning in the photoassimilates, i.e. increased starch accumulation in chloroplasts and decreased levels of sugar in cytosol. Pi starved plants had lower levels of phosphorylated carbohydrates (Hernandez and Munne-Bosch, 2015). Besides the data on metabolite levels of PGA, triose-P and RuBP, their ratios provided clear explanation. The levels of these three metabolites, namely PGA, triose-P and RuBP were monitored in the presence of varying bicarbonate and varying Pi.

The first stable product of carboxylation reaction by RuBisCO was PGA (Bassham, 2003). Triose-P formed from PGA is exported out of chloroplasts in-exchange of Pi during carbon fixation, to be further metabolized as sucrose and transported across the phloem (Flugge et al., 2011). Finally, RuBP needed for carboxylation, was also regenerated in the sequential steps of PCR cycle (Bassham, 2003). Triose-P levels are higher at 10 mM bicarbonate in absence Pi but reduced when 0.5 mM Pi is supplied but started to build up at higher concentrations of Pi (Fig. 5.3B, Fig.5.5). At high CO_2, supply of Pi to Pi deficient leaves of barley and spinach

showed stimulation of photosynthesis and was correlated with increased accumulation of triose-P and PGA (Dietz and Foyer, 1986). Here, in the protoplasts, the PGA levels were stimulated at high bicarbonate with inclusion of 0.5 mM Pi which might facilitate the conversion of assimilated carbon to other phosphorylated metabolites.

RuBP levels remained lower at high bicarbonate in the absence of Pi but raised at 0.5 mM Pi (Fig. 5.3B, Fig. 5.5A). Altering the Pi levels, by increasing the Pi concentration to 0.5 mM might have favoured the regeneration of RuBP which is essential for CO_2 fixation. Rao and Terry (1995) also found similar response with sugar beet, the photosynthetic rate was increased when a low Pi grown plants were supplied with Pi. Resupply of Pi also resulted increased accumulation of sugar phosphates in the leaves which lead to the increase of RuBP levels and concluded to be controlling the photosynthesis.

In summary, optimal photosynthesis is achieved, when optimal bicarbonate as well as optimal Pi are available. Beyond these levels, for e.g. above 1 mM bicarbonate or 1 mM Pi there is a significant decrease in the rate. The decrease in photosynthesis at 5 or 10 mM bicarbonate appears to be limitation on RuBP (Fig. 5.3C). Similarly the triose-P conversion to PGA and regeneration of RuBP is possibly affected at supraoptimal Pi (Fig. 5.7).

Conclusions

1. Maximum photosynthesis by mesophyll cell protoplasts occurred at 1 mM bicarbonate, but decreased at higher bicarbonate concentrations.

2. Varying Pi modulated markedly the photosynthesis by mesophyll protoplasts, with the response dependent on relative levels of both bicarbonate and Pi. Presence of Pi at 0.5 mM or 1.0 mM stimulated the photosynthesis, irrespective of bicarbonate concentrations. However, when 1 mM or higher Pi is included in the medium, photosynthesis decreased at high bicarbonate.
3. The levels of the key Calvin cycle metabolites altered during the photosynthesis in response to varied bicarbonate and Pi. The changes were prominent when ratios of three key metabolites were calculated.
4. The decrease in photosynthesis at 10 mM bicarbonate appears to be due to a limitation of RuBP as indicated by the steep decrease in RuBP/triose-P ratio. In contrast, the ratio of triose-P/PGA was maximum at 10 mM bicarbonate, suggesting that conversion of PGA to triose-P kept pace with photosynthesis.
5. An increase in external Pi made protoplasts to sustain higher photosynthesis at even 10 mM bicarbonate at an optimal Pi (≅ 1 mM). Similarly, the extent of triose-P formation from PGA was facilitated by high Pi (≅ 10 mM) but not that of RuBP regeneration.

Chapter 6

Effect of Varying Bicarbonate (mimicking CO_2) on Nitrogen Assimilation, Reactive Oxygen Species, and Antioxidant Enzymes in Mesophyll Protoplasts

Introduction

Nitrogen is an essential nutrient and is required for plant metabolism as well as growth/development. Nitrogen is taken up from soil and assimilated into plants. In agriculture, nitrogen has to be supplemented as chemical fertilizer to boost the production. Increasing the availability of nitrogen enhances further the ability of plants to assimilate CO_2 (Gruber and Galloway, 2008), as photosynthesis is strongly correlated with leaf nitrogen content. Similarly, the yield and total biomass of the plants were correlated with crop nitrogen content (Makino, 2011). During the photosynthetic process, the reductants generated during the Z scheme are utilized for carbon assimilation. However, nitrogen assimilation also utilizes the available reductants. Further, the nitrate assimilation by chloroplasts, can even promote the electron flow and this process is termed as nitrate-dependent O_2 evolution (Torre et al., 1991).

Prolonged exposure of plants to elevated CO_2 would help us to understand the carbon sequestering mechanisms and the physiological feature of plants grown in high CO_2. Such studies emphasized the necessity of enriched nitrogen supply, as the biomass of the plant tends to decrease at elevated CO_2, mainly due to the limitation in plant nitrogen (Reich and Hobbie, 2013). Nitrate reductase (NR), nitrite reductase (NiR), glutamine synthase (GS) and ferredoxin-dependent glutamine: oxoglutarate amino transferase (Fd GOGAT) are among the important enzymes of

nitrogen metabolism. NR and NiR are involved in the step-wise conversion nitrate to ammonium. Ammonium is assimilated into amino acid by GS/GOGAT cycle (Xu et al., 2012). Change in N metabolism are usually reflected in the activities of these enzymes

Plants are vulnerable to abiotic and biotic stresses and need to adapt quickly as they cannot move away. Abiotic stress is often caused by unfavourable changes in environmental conditions, such as light, temperature, radiation salinity, nutrients, H_2O, O_2 and CO_2 (Atkinson and Urwin, 2012; Hirayama and Shinozaki 2010). ROS generation is inevitable under such stressful conditions. The concentration of ROS or the type of ROS present in the cell is determined by the efficiency of ROS removal mechanisms in relation to ROS generating reactions (Moller et al., 2007). Among the different forms of ROS, H_2O_2 is the most stable and predominant form that accumulates in stress conditions and was involved in signalling (Quan et al., 2008).

Elevated CO_2 is also considered to be a form of stress to plants. For e.g., Soybean plants grown at elevated CO_2, had greater antioxidant capacity and increased levels of antioxidant enzymes (Gillespie et al., 2011). The elevated CO_2 itself could be a form of abiotic stress. It is therefore not surprising that there are changes in ROS levels and antioxidant systems in plants when exposed to elevated CO_2. Arabidopsis and soybean grown in elevated CO_2 exhibited symptoms of oxidative stress, as leaves had increased protein carbonylation (Qiu et al., 2008). The decreased photorespiration due to elevated CO_2 could cause altered redox status. The change in the redox in turn can lead to oxidative stress and altered antioxidant metabolism (Munne-Bosch et al., 2013). Catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) are the prominent antioxidant

enzymes that respond to oxidative stress. CAT degrades H_2O_2 to water and oxygen, APX and GR are the components of Foyer-Halliwell-Asada pathway involved in step-wise scavenging of ROS (Foyer and Noctor, 2011).

The present chapter is an attempt to examine the changes in plant cells at elevated CO_2 in relation to nitrogen assimilation. Experiments were designed to assess the ROS levels and the antioxidant systems in plant cells on exposure to elevated CO_2. The system of MCP was used to examine changes in photosynthesis, nitrogen metabolism and antioxidant metabolism to understand the modulation of plant cells by elevated CO_2.

Results

Changes in nitrate-dependent O_2 evolution by MCP at varied bicarbonate

Nitrate-dependent O_2 evolution by MCP was maximum at 2.5 mM of nitrate (Fig 6.1). Inclusion of bicarbonate in the reaction medium along with nitrate decreased slightly the rates of oxygen evolution by MCP. The rates of oxygen evolution decreased markedly when 10 mM bicarbonate was present in the reaction media (Fig.6.1)

Response of enzymes involved in nitrogen metabolism to varied bicarbonate

Increased concentration of bicarbonate decreased the activity of NR, in terms of the both total activity (in presense of EDTA) and actual activity (in presence of $MgCl_2$). NiR activity at 1 mM bicarbonate was higher than that to other bicarbonate concentrations. GS activity was almost similar in all the bicarbonate concentration. Fd GOGAT activity was also higher at 1 mM bicarbonate quite similar to NiR (Fig. 6.2).

Change in the levels of ROS in MCP at varying bicarbonate

The levels of ROS are in MCP were modulated at varying bicarbonate levels. The ROS levels were reduced at lower concentrations of bicarbonate. However, at high bicarbonate of 10 mM, the ROS levels were higher by about 20% when estimated with H_2DCFDA fluorescence and DAB. Maximum photosynthetic rate was at 1 mM bicarbonate. There was no clear correlation between rate of photosynthesis and ROS levels (Table 6.1).

Response of antioxidant enzymes to varying bicarbonate in the MCP of pea

Compared to the sample with no added bicarbonate, the activity of CAT was less till 5 mM bicarbonate and then increased at 10 mM bicarbonate. The APX activity also decreased with increasing concentration of bicarbonate. In contrast, the activity of GR did not alter much varying bicarbonate (Fig. 6.3).

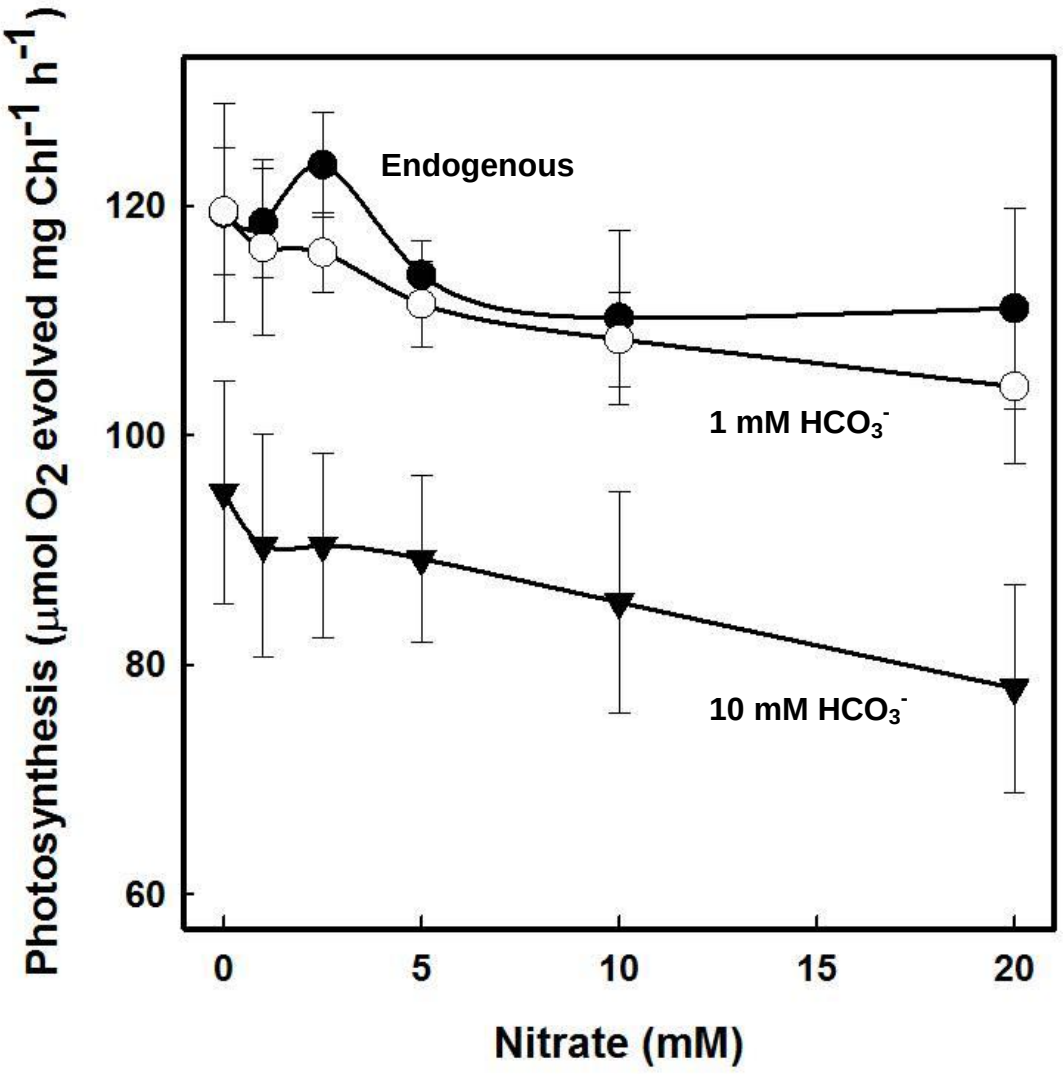

Fig. 6.1. Response of O_2 evolution in the mesophyll cell protoplasts in presence of nitrate in the reaction media at varying concentrations of bicarbonate.

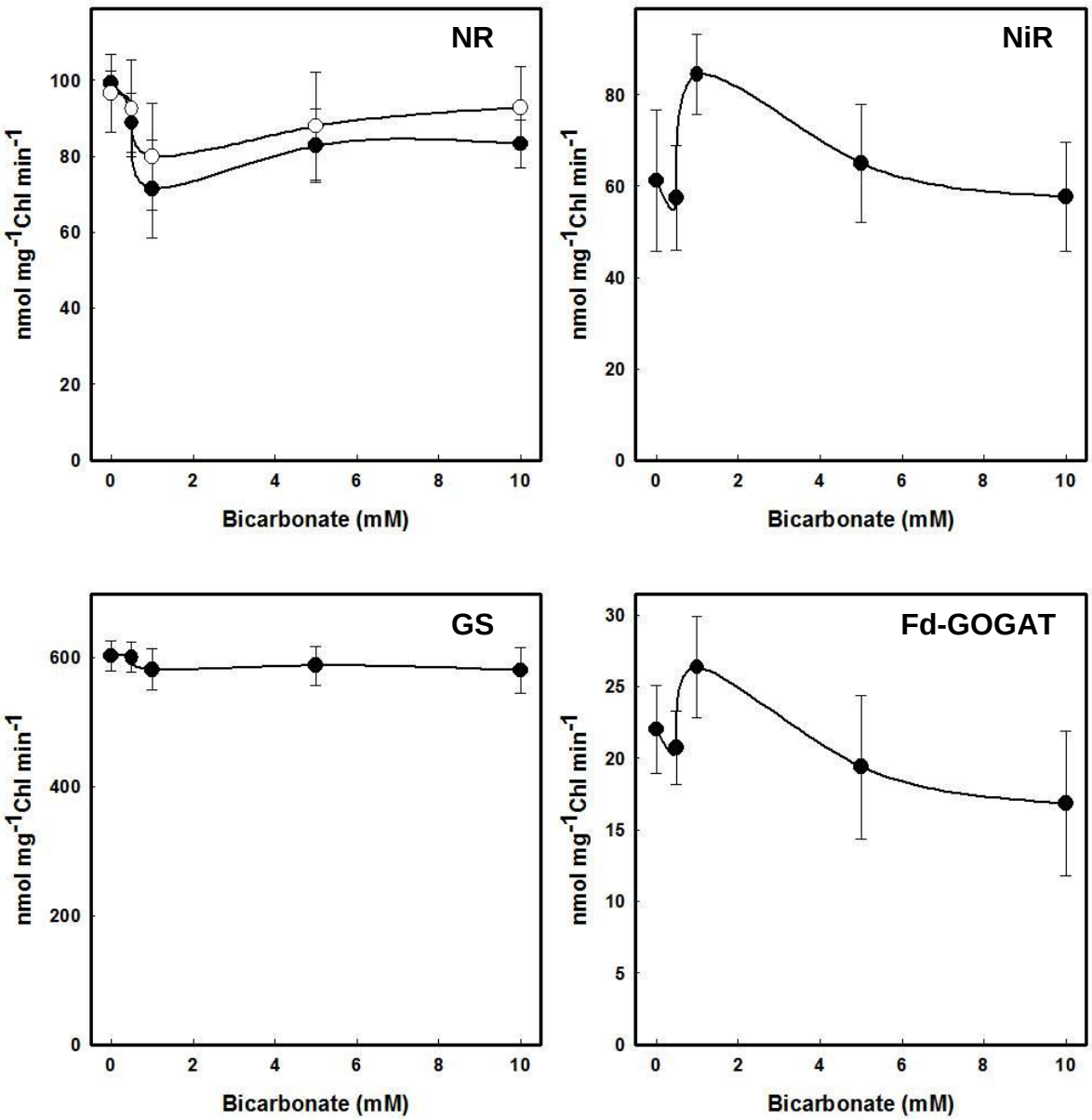

Fig. 6.2. Modulation of N-fixing enzymes at different bicarbonate concentrations. The activities of nitrate reductase (NR), nitrite reductase (NiR), glutamate synthase (GS) and ferredoxin-dependent glutamate oxoglutarate amino tranferase (Fd-GOGAT) in different bicarbonate concentrations at light intensity of 700 µmol m^{-2} s^{-1} for 10 min duration. Protoplasts equivalent to 100 µg Chl are used for assays.

Table 6.1. Estimation of reactive oxygen species (ROS) in mesophyll cell protoplasts of pea by H_2DCF-DA fluorescence and DAB absorbance read at 450 nm at varying bicarbonate with corresponding photosynthetic response.

	Photosynthesis (% control)	Reactive oxygen species by H_2DCF-DA (% control)	Reactive oxygen species by DAB (% control)
Endogenous	**100 ± 11**	**100 ± 11**	**100 ± 15**
0.5 mM HCO_3^-	**173 ± 3**	**90 ± 8**	**72 ± 9**
1 mM HCO_3^-	**199 ± 4**	**71 ± 20**	**81 ± 17**
5 mM HCO_3^-	**181 ± 7**	**85 ± 14**	**89 ± 14**
10 mM HCO_3^-	**179 ± 7**	**118 ± 9**	**120 ± 4**

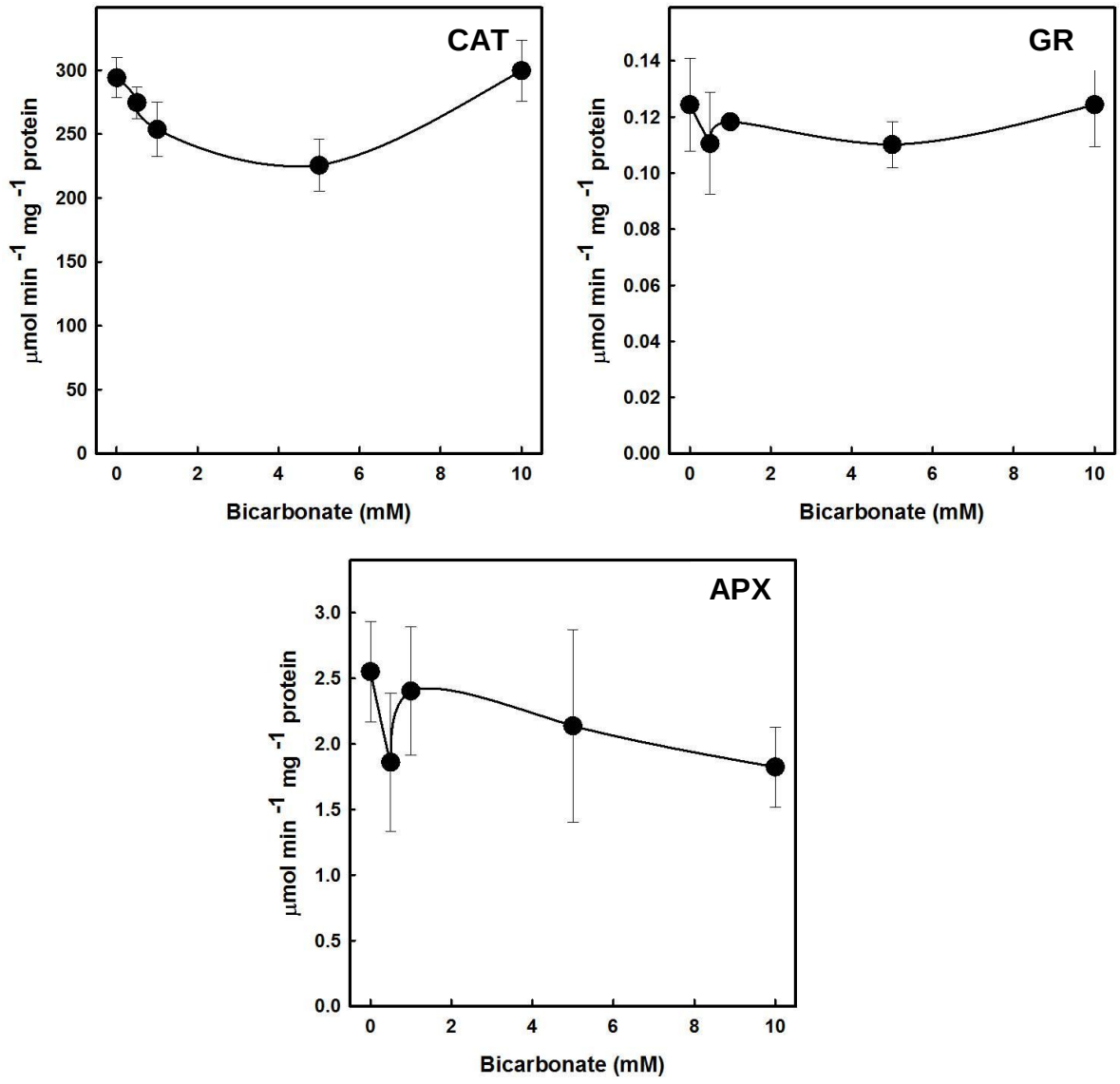

Fig. 6.3. Modulation of antioxidant enzymes at different bicarbonate concentrations. The activities of catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) in different bicarbonate concentrations at light intensity of 700 µmol m^{-2} s^{-1} for 10 min duration. Protoplasts equivalent to 100 µg Chl are used for assays.

Discussion

Photosynthesis by MCP at varied bicarbonate in relation to nitrate

Reducing equivalents are generated in the bioenergetic reactions of both chloroplasts and mitochondria. These reductants are utilized in various metabolic processes of cell including the nitrogen metabolism (Taniguchi and Miyake, 2012). Thus, photosynthetic carbon metabolism and nitrogen metabolism can be complementary.

Nitrate-dependent O_2 evolution was stimulated when bicarbonate was not added to the medium (Fig. 6.1), indicating the availability of N can help photosynthesis. In an earlier report, photosynthetic O_2 evolution rate was found to be higher in the plants fed with NO_3 compared to those fed with NH_4 (Eichelmann et al., 2011). However at 10 mM bicarbonate, photosynthesis decreased as NO_3 concentration was increased (Fig. 6.1). This indicates that at increased CO_2 the nitrogen assimilation is restricted, possibly due to competition for photosynthetically generated reductant.

Modulation by CO_2 of NR and GS, but not NiR and Fd GOGAT

We found varied modulation of N-metabolizing enzymes on exposure to elevated CO_2. The activities of enzymes NiR and Fd GOGAT increased at optimal bicarbonate (1 mM). The photosynthetic rate was also maximal at 1 mM bicarbonate (Fig. 6.2). Both these two enzymes utilize the reductants generated from the photosynthetic electron transport. In contrast, the enzyme NR is dependent on NADH as a source of reductant, from the mitochondrial metabolism (Foyer et al., 2011; Nunes-Nesi et al., 2013). The activities of NR and GS did not respond to

bicarbonate, possibly, due to the relative independence on reductants (Fig. 6.2). Similar observations were made in the barley plants exposed to elevated CO_2 in the activities of NR and GS (Wang et al., 2013).

The response of nitrate-dependent oxygen evolution and the activity of NiR and Fd-GOGAT to varied bicarbonate indicates the possibility of a link between N metabolism and C metabolism, mediated by the availability of reductants (Bloom et al., 2010).

ROS is an integral part in the energy transfer reaction and is counteracted by antioxidant defence

ROS generation is unavoidable during electron transfer pathways of photosynthesis and respiration. Transcriptomic analysis by Queval et al. (2012) shows that CO_2 and H_2O_2 interact with each other, due to the changes caused by environmental stimuli and carbon status. Though the experimental procedures of estimating the ROS by H_2DCF-DA and DAB differed in the values, the levels of ROS in the MCP were very high at 10 mM bicarbonate (Table 6.1). Similar situation of high ROS accumulation and changes in the antioxidant enzymes was observed in seedlings of pea and soyabean at high CO_2 (Ershova et al., 2011). In another study with *Phaseolus vulgaris*, plants treated with elevated CO_2 also had higher amounts of H_2O_2 and MDA which indicate oxidative damage (Lambreva et al., 2006).

Differential response of antioxidant enzymes to varied bicarbonate

Catalase activity was found to be increased when H_2O_2 was supplied externally to yeast and callus of *Nitraria tangutorum* (Martins and English, 2014; Yang et al., 2012). Our results with varied bicarbonate also indicate that catalase

activity was higher when the ROS levels were higher, particularly at no added bicarbonate and high bicarbonate concentration (Fig. 6.3). CAT activity was found to be increased in the treatments of elevated CO_2 in soybean along with O_3 and well irrigated grapevine (Gillespie et al., 2012; Salazar-Parra et al., 2012). Photorespiration decreases under elevated CO_2 conditions which decreases the H_2O_2 production and CAT activity (Maurino and Peterhansel, 2010). Even in our study, the lowering of ROS decreased the CAT activity till 5 mM of bicarbonate but CAT activity was found to be stimulated due to presence of higher levels of ROS at 10 mM bicarbonate which hinders photosynthesis (Fig. 6.3, Table. 6.1).

Among the other two important antioxidant enzymes, GR did not respond to the varying bicarbonate, this response was similar to the response of GR in arabidopisis treated with elevated CO_2 (Zinta et al., 2014). APX activity was decreased with increasing concentrations of bicarbonate this response is similar to the observations of Ershova et al. (2011) as exposure of high CO_2 decreased the APX activity in the seedlings of pea. Varied response of antioxidant components is known in elevated CO_2 treatments (Toppi et al., 2002; Booker and Fiscus 2005). In our experiments, it is shown that catalase is the main enzyme which responds to the existent H_2O_2 content when bicarbonate was varied.

Conclusions

1. The respose of nitrogen assimilating enzymes, particularly the increased ativities of NiR and Fd-GOGAT and nitrate-dependent oxygen evolution indicate the coordination of C metabolism and N metabolism *via* reductants.

2. The modulation of ROS levels imply the occurrence of oxidative stress at high concentrations of bicarbonate. Hence elevated CO_2 could be detrimental to physiology and metabolism of plant.
3. The varied response of antioxidant enzymes is earlier known at elevated CO_2. Our results point out the active participation of catalase in scavenging the ROS generated due to elevated bicarbonate levels.

Chapter 7

Effect of Varying Bicarbonate on Chlorophyll Fluorescence in Mesophyll Protoplasts

Introduction

Responses of MCP to varying bicarbonate (as described in the previous chapters) indicated that high bicarbonate levels would cause stressful conditions by increasing ROS and thereby decrease photosynthesis. When the conditions are unfavourable for optimal performance of the plants, their metabolism will be altered. The altered metabolic status can be assessed by various physiological measurements (Furbank and Tester, 2011; Dhondt et al., 2013). Chlorophyll *a* (Chl) fluorescence analysis is such a robust technique to assess and understand the photosynthetic performance of plant. It is a measure of absorption, as well as emission of light by PSII and can indicate the extent of utilized light during the photochemical conversions (Murchie and Lawson, 2013). Chl fluorescence technique is non-invasive and also facilitates rapid analysis.

Chl fluorescence analysis is done by observing either continuous/direct fluorescence or modulated fluorescence. Continuous fluorescence monitoring technique induces typical transients of fluorescence rise (Fig. 1.1 in Chapter 1), which gives structural and functional information, such as PSII activity, antenna size and electron transport (Strasser et al., 2000). Pulse amplitude modulated (PAM) fluorescence technique induces several fluorescence peaks (Fig. 1.2 in Chapter 1) by saturated intensities of light. The induction pattern reveals the information about the quenching mechanisms and differentiates photochemical quenching and non-photochemical quenching (NPQ) (Baker, 2008).

Mechanism of regulation of photosynthesis is often studied by Chl fluorescence kinetics and related to changes, if any, in photochemical events. The fluorescence transients are well correlated with PSII efficiency under stress factors, such as salinity or drought or temperature (Naumann et al., 2007; Dwyer et al., 2007). For e.g. in the event of heat stress the most affected was PSII and thereby photosynthetic yield. The levels of ROS generated during these adjustments would also affect the repair mechanisms of the PSII (Allakhverdiev et al., 2008). The oxidative stress caused by herbicides is reflected in the changes of Chl fluorescence transients of treated cucumber seedlings suggesting the loss of PSII efficiency (Tripathy et al., 2007).

The fluorescence kinetics obtained during elevated CO_2 treatment of populus trees showed higher levels of photosynthetic electron transport rate (ETR) and net CO_2 exchange. ETR and net CO_2 exchange traces were varied in ambient CO_2 grown populus, leading to higher NPQ, when compared with elevated CO_2 (Ananyev et al., 2005). Exposure to elevated CO_2 of north china grassland showed a species-specific responses. Doubling of CO_2 caused an increase in biomass of all the examined species without significant changes of PSII efficiency. MDA levels which indicate peroxidation were enhanced in *Cleistogenes squarrosa* but not in other species (Xu et al., 2014). Suspension cultures of *Chlamydomonas acidophila* exposed to high CO_2 had a higher photosynthetic rate along with high PSII yield, than those to low CO_2 grown cells (Spijkerman, 2008).

The modulation of Chl fluorescence transients appeared to be a definite possibility in leaves or photosynthetic cells or protoplasts. The present chapter is therefore an attempt to examine the changes in Chl fluorescence kinetics of

protoplasts at high bicarbonate (mimicking elevated CO_2). Experiments were designed with MCP to understand the photochemical events in regard to PSII functioning and fluorescence quenching patterns. The basic measurements of Chl fluorescence were then analysed by different parameters listed in Table 7.1. Further details can be found in the Introduction (Chapter 1).

Results

Changes in fluorescence kinetics by MCP at varied bicarbonate

The polyphasic rise and decline of the Chl fluorescence was monitored with the help of Handy PEA. The dark adapted mesophyll protoplasts exhibited marked difference in the amplitudes of fluroscence kinetics, when treated with varying bicarbonate (Fig. 7.1A). The fluorescence transients (OJIP), normalized at F_o or F_m depicted the different amplitudes of transients. Highest fluorescence values were observed at optimal bicarbonate (1 mM), compared to other bicarbonate concentrations. The highest variable fluorescence (a measure of PSII efficiency) was also at 1 mM bicarbonate (Fig. 7.1B). Fluorescence transients normalized at P phase exhibited a lowering of fluorescence values at 10 mM bicarbonate which can be related to the flow of electrons away from PSII (Fig. 7.1C). To understand the underlying photochemical events, these transients were translated into JIP test parameters.

Specific fluxes calculated from fluorescent transients by using JIP test

The parameters of absorption (ABS), trapping (TR_o), dissipation (DI_o) and electron transfer (ET_o) per reaction centre were calculated by JIP test. These parameters were then converted into dynamic energy pipeline membrane model

(Fig. 7.2). The differences in the parameters like ABS/RC, TR_o/RC, DI_o/RC, ET_o/RC are visualized by the shape and size of the diagram. Rise in concentration of bicarbonate up to 1 mM, increased the parameters ABS/RC, TR_o/RC, ET_o/RC. The shape and size of the each variable was maximal at 1 mM bicarbonate. However, at 10 mM bicarbonate the shapes and sizes of these parameters were decreased (Fig. 7.3).

Variations in fluorescence kinetics and quenching patterns measured by pulse amplitude modulation (PAM)

Fluorescent transients induced by the strong pulses of light also varied in response to varying bicarbonate. High bicarbonate lowered the peaks of these fluorescent transients when compared to other bicarbonate concentrations (Fig. 7.4). The ϕ_{II} values increased with time in all the bicarbonate concentrations. The ϕ_{II} values decreased at high bicarbonate (i.e. 10 mM), when compared to other bicarbonate treatments which indicate the loss of PSII efficiency (Fig. 7.5A). In contrary, the ϕ_{NPQ} values increased at high bicarbonate (Fig. 7.5B). These observations indicated the loss of energy through NPQ mechanisms. Differences in ϕ_{NO} values were observed in instantaneously for a short duration with higher values at 5 and 10 mM bicarbonate (Fig. 7.5C).

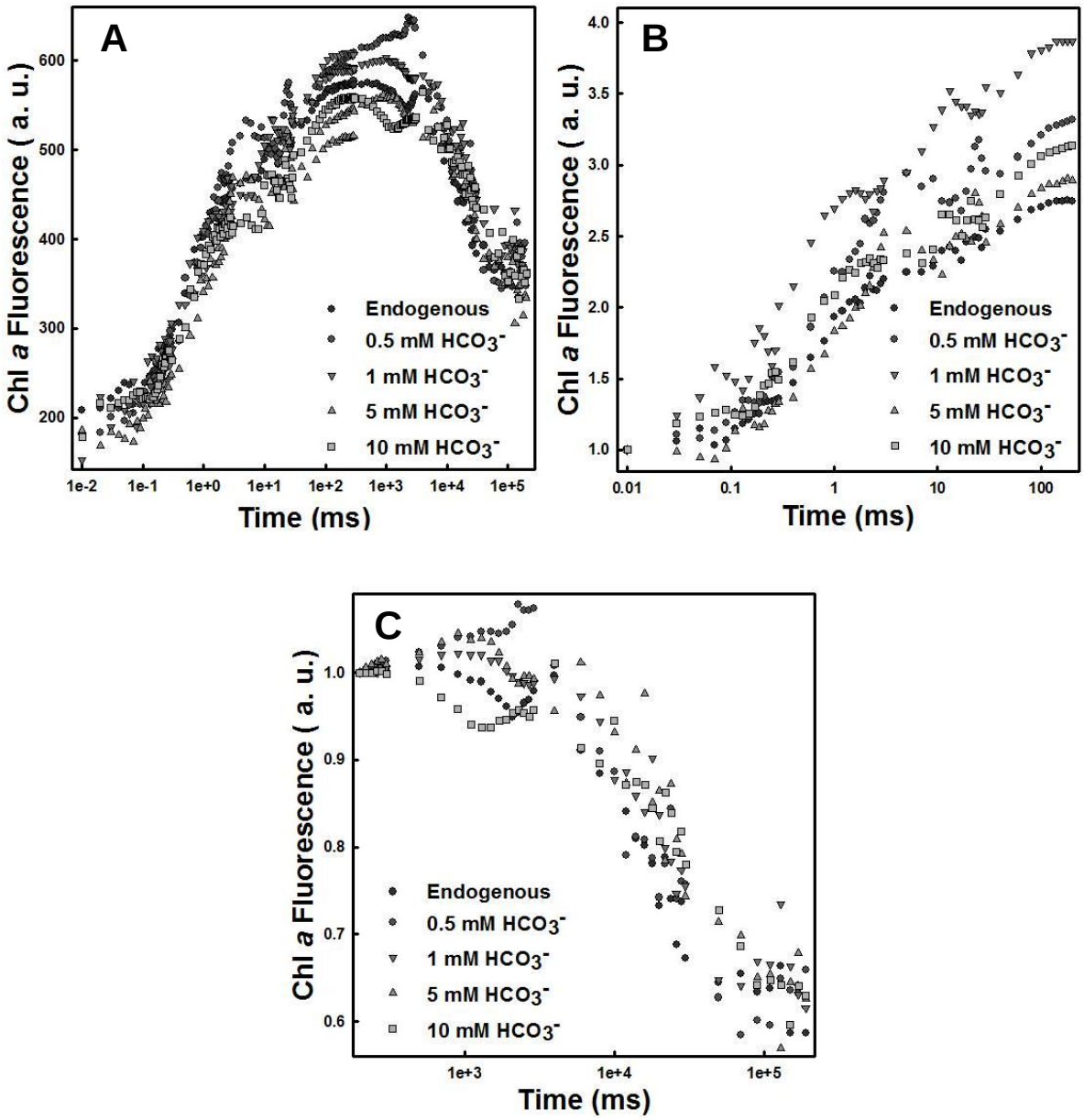

Fig. 7.1 Fluorescence transients of mesophyll protoplasts exposed to different bicarbonate concentrations as measured by Handy PEA (Hansatech, UK). A) Complete transient: O-T phase of Kautsky curve, B) Fluorescence transients of OJIP phase normalized at F_o, C) Fluorescence transients of PMST phase normalized at F_m. The description of these phases is given in Fig 1.1 of Chapter 1.

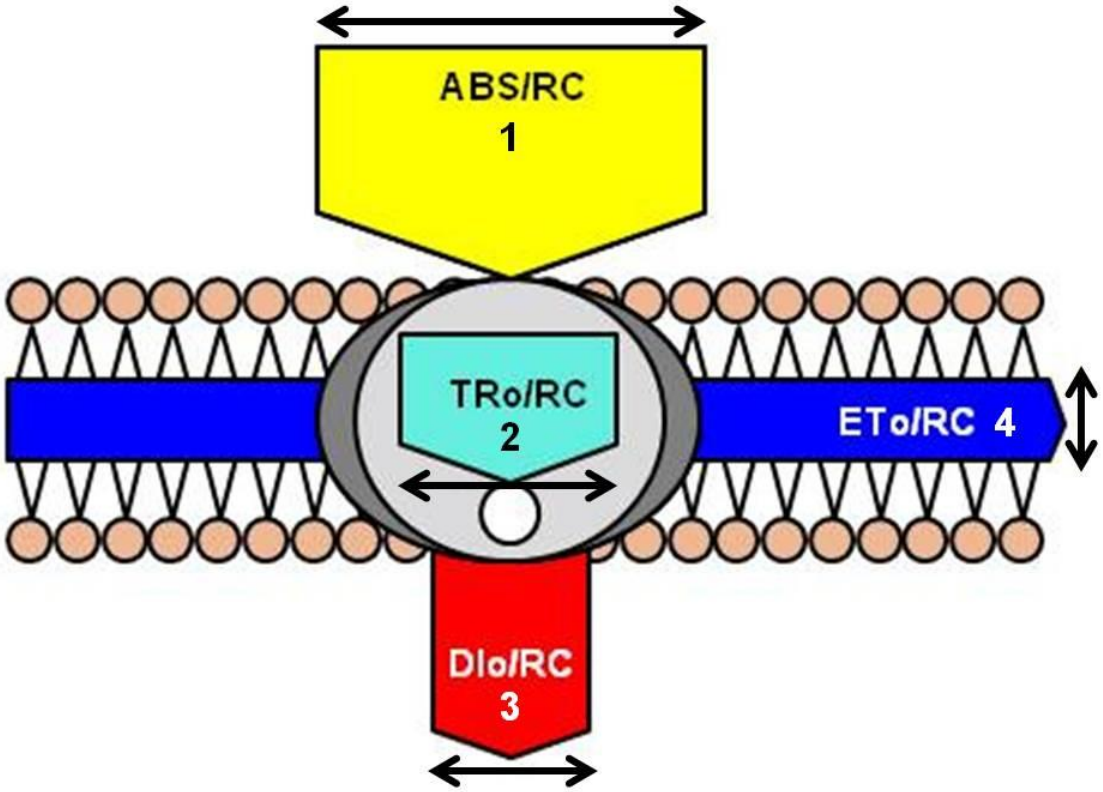

Fig. 7.2 Fluorescence transients obtained from Fig 7.1 are translated to JIP-test parameters by Biolyzer software (Strasser et al., 2000). These calculations are represented diagrammatically in terms of 1) Absorption (ABS), 2) trapping (TR_o), 3) dissipation (DI_o) and 4) electron transport (ET_o) in relation to the unit of reaction centre (RC) were shown in yellow, cyan, red and blue colours respectively. The thickness (width) of each arrow (↔) represents the extent of parameter. A decrease/increase in arrow is a measure of decrease/increase of said parameter. Size and shapes of each parameter indicates the increased/ decreased involvement of each parameter in energy distribution. Additional information is provided in Table 7.1.

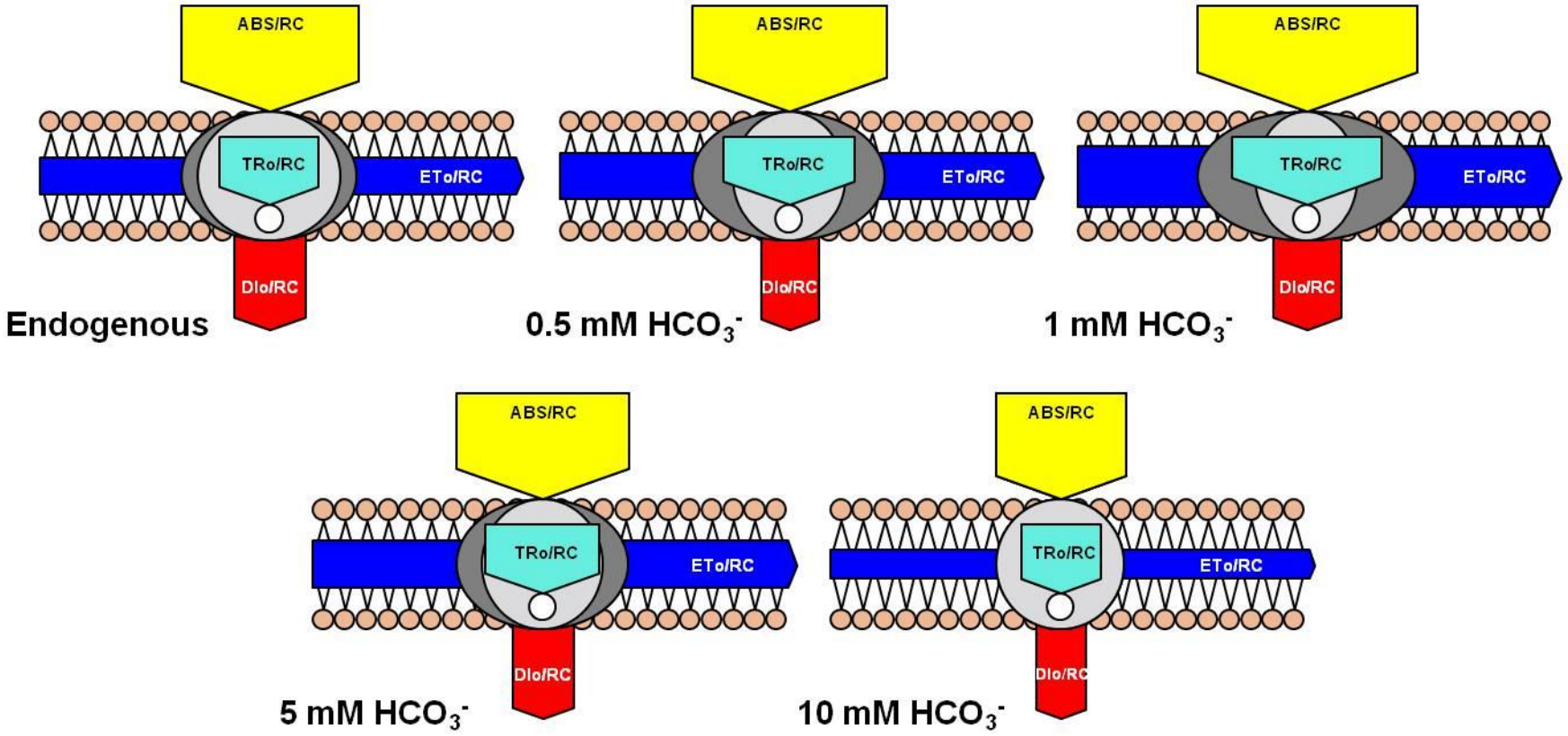

Fig. 7.3 Specific flux (per reaction centre) parameters of energy partitioning in MCP exposed to different bicarbonate concentrations. These were derived from the Fig. 7.1 and as mentioned in Fig. 7.2. Dark adapted protoplasts treated with different bicarbonate concentrations and 700 μmol m^{-2} s^{-1} light were measured by Handy PEA (Hansatech) which yielded energy pipelines (membrane model) with varying shapes and sizes (Strasser et al., 2000). Additional information is provided in Table 7.

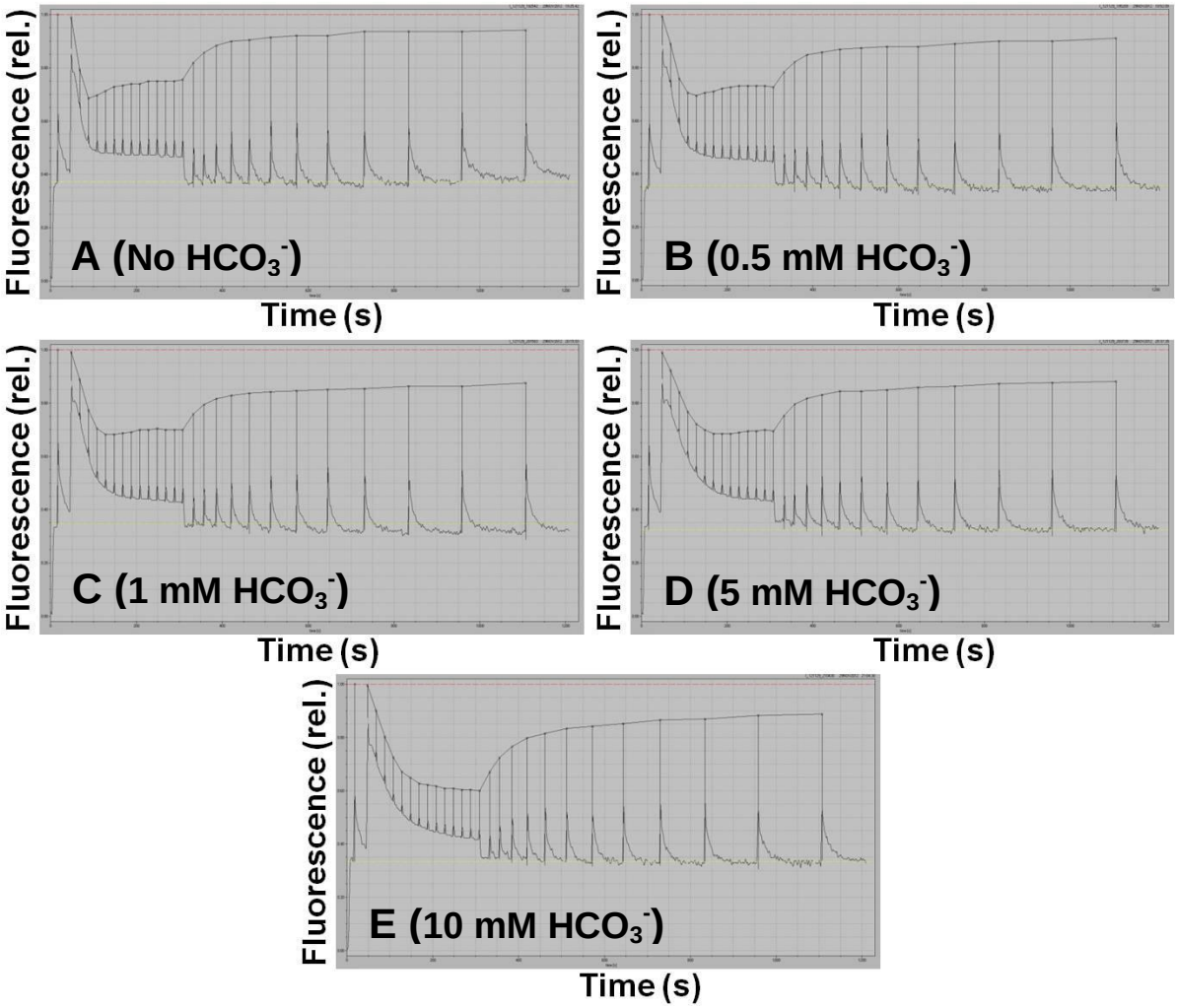

Fig. 7.4 Kinetics of modulated chlorophyll fluorescence monitored by PAM fluorometer (Portable PAM-WALZ). Isolated protoplasts were dark adapted for 30 min with respective bicarbonate (A: no added bicarbonate, B: 0.5 mM, C: 1.0 mM, D: 5.0 mM and E: 10 mM) in the media and exposed to pulses of irradiance. The actinic light is switched on before the second saturating pulse. Light is turned off after 5 min to check the recovery of fluorescence. The details of PAM kinetics were mentioned in Fig. 1.2 of Chapter 1.

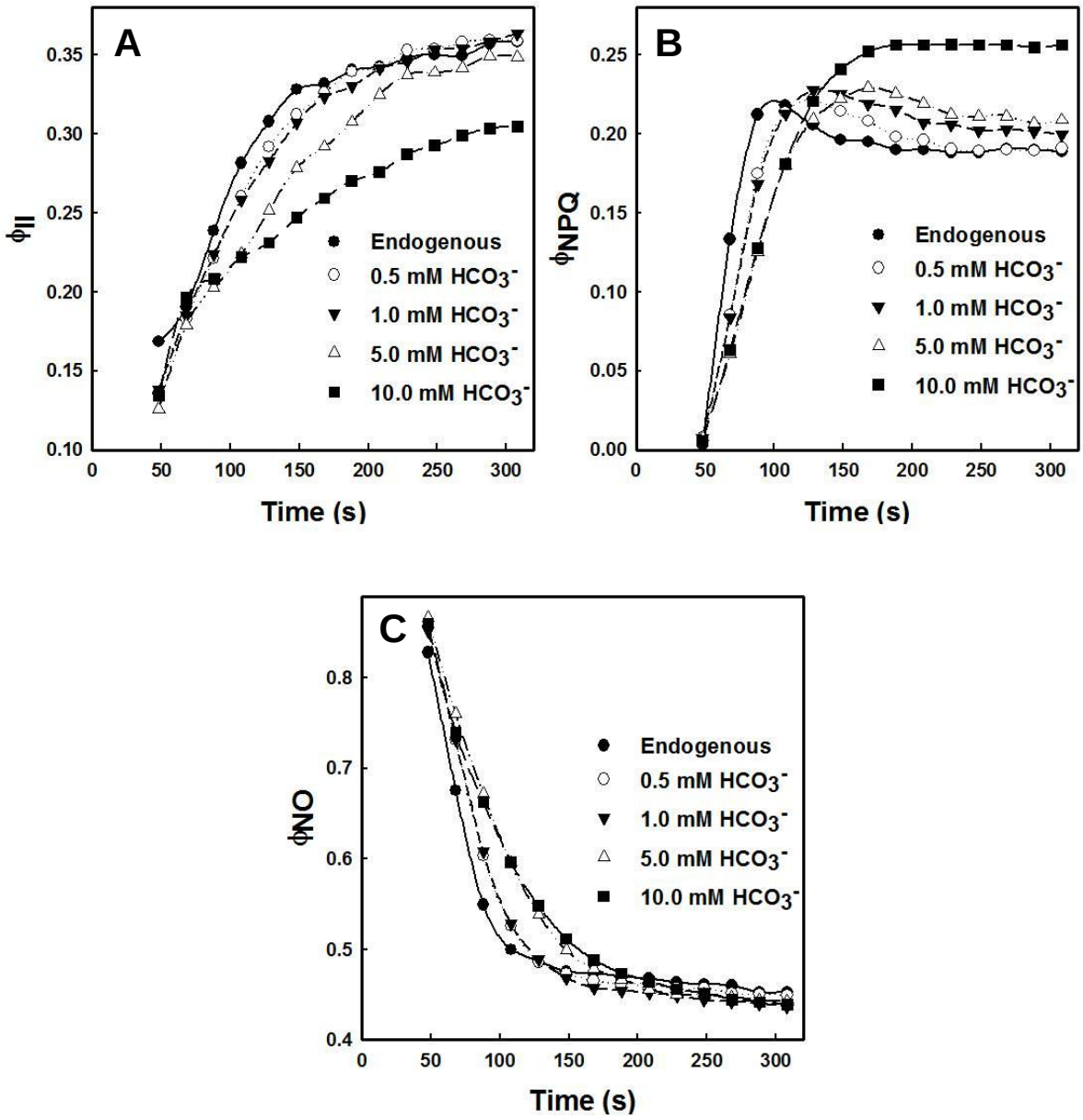

Fig. 7.5 Fluorescence quenching patterns observed in the mesophyll protoplasts treated with different concentrations of bicarbonate. Mean of the yields ϕ_{II}, ϕ_{NPQ} and ϕ_{NO} calculated from the kinetics obtained from Fig. 7.4. The details of these are mentioned in Table 7.1 and also are described in the chapter of Introduction. (Kramer et al., 2004; Renger and Schreiber,1986)

Table 7.1. Parameters derived from the Chl *a* fluorescence kinetics using two different instruments

Parameters analysed by instruments	Abbreviation	Expansion of abbreviation	Significance of the parameter
Specific fluxes obtained from Handy PEA (Hansatech) & Biolyzer	ABS/RC	Absorption per reaction centre	Amount of energy absorbed by the pigments, mainly chlorophylls
	TR_o/RC	Trapping per open reaction centre	Fraction of energy trapped in the reaction centre leading to Q_A reduction
	DI_o/RC	Dissipation per open reaction centre	Dissipated energy by other systems as heat or fluorescence from the reaction centre
	ET_o/RC	Electron transport per open reaction centre	Flux of energy carried beyond Q_A^- in electron transport chain
Quenching mechanisms assessed by PAM fluorometer (Walz)	ϕ_{II}	Photochemical yield of PSII	Energy utilized by primary photochemistry of PSII also know as photochemical quenching
	ϕ_{NPQ}	Yield of non-photochemical quenching dissipation by means other than PSII/PSI	Down-regulatory processes that quench energy in light, high lumen (H^+) and xanthophyll cycle
	ϕ_{NO}	Yield of other non-photochemical losses	Non-light induced quenching processes, basal intrinsic non-radiative decay or thermal loss

Further description is given in Introduction Chapter - Section on “Use of Chl fluorescence”. These details are available in the reviews of Strasser et al. (2000) and Kramer et al. (2004).

Table 7.2. Response of Chl *a* fluorescence parameters in MCP on exposure to varying bicarbonate concentrations

Parameter			Varying bicarbonate		
			No bicarbonate	Optimal (1 mM) bicarbonate	High (10 mM) bicarbonate
PAM	ϕ_{II}	Photochemical yield of PSII	No change	No change	Decrease
	ϕ_{NPQ}	Non-photochemical quenching	No change	No change	Increased
	ϕ_{NO}	Other non-photochemical losses	No change	No change	Increased
JIP test	ABS/RC	Absorption per reaction centre	No change	Increase	Decrease
	TR_o/RC	Trapping per open reaction centre	No change	Increase	Decrease
	DI_o/RC	Dissipation per open reaction centre	No change	Increase	Decrease
	ET_o/RC	Electron transport per open reaction centre	No change	Increase	Decrease

Discussion

Efficiency of PSII is affected along with the photosynthetic rate at high bicarbonate

The fluorescence kinetics revealed interesting information on the functioning of PSII. These changes could be related to the changes in photosynthetic rate described in Chapter 5. The maximum variation in the fluorescence values was observed at the end of the P phase (Fig. 7.1B). The high variable fluorescence, suggests that PSII was very efficient at 1 mM bicarbonate. Thus the maximum variable fluorescence matched the peak rates of photosynthesis. Such relationship of photosynthetic rate and PSII efficiency has been known (Mehta et al., 2010; Stirbet and Govindjee 2011). Bicarbonate ion was known to promote electron transport to PQ pool. Depletion of bicarbonate in medium decreased the fluorescence transients and the rate of photosynthetic oxygen evolution (Blubaugh and Govindjee 1984; Stemler et al., 1974). However, at the supersaturating concentration of 10 mM bicarbonate, the efficiency of PSII decreased. Such decline in the fluorescence from the P-T refers to the gradual re-oxidation of Q_A might be due to carbon assimilatory pathway, which was decreased at high bicarbonate (Papageorgiou et al., 2007).

Distribution of energy fluxes is also impaired at high bicarbonate

The functioning of the PSII can be further elaborated by JIP test, so as to indicate the distribution of energy fluxes at the PSII reaction centre (Yin et al., 2010; Strasser et al., 2010). All the parameters of ABS/RC, TR_o/RC, DI_o/RC and ET_o/RC were high at 1 mM bicarbonate but decreased at further bicarbonate, e.g. 10 mM (Fig. 7.3). ABC/RC represents fraction of total number of photons absorbed per total active reaction centres and DI_o/RC represents dissipation of untrapped energy with

respect to active reaction centres. TR_o/RC represents the rate at which the exciton is trapped in the reaction centre leading to the Q_A reduction and ET_o/RC represents the re-oxidation of Q_A through electron transport chain components in active reaction centre (Mathur et al., 2011). Our results indicated that the extent of energy entering the reaction centre was restricted, along with the restricted partitioning/distribution of energy fluxes, at high bicarbonate levels.

Predominant NPQ and non-regulated energy loss at high bicarbonate suggest activation of alternative electron sinks

NPQ occurs when the energy reaching the reaction centre is in surplus. When the light energy is in excess, the unused energy in the photochemical events is dissipated as heat (Muller et al., 2001). Change in the thylakoid ΔpH, xanthophyll pool, PsbS protein and LHCII content, all cause variation in NPQ (Horton and Ruban, 2004). Oxidative stress caused by salt and osmotic changes is countered by an increase in NPQ and over-accumulation of zeaxanthin in *Physcomitrella patens* (Azzabi et al., 2012). Singlet oxygen, a form of ROS, mostly generated in the chloroplasts during energy reaction was also quenched by NPQ and could prevent the PSII photo-inhibition under stressful conditions (Triantaphylides and Havaux, 2009).

Fluorescence kinetics through PAM revealed the details of the quenching mechanisms. At high bicarbonate levels, ϕ_{NO} and ϕ_{NPQ} were higher where as ϕ_{II} was lower than the values at optimal bicarbonate (Fig. 7.5). The observations from previous chapter, about the increased ROS, altered antioxidant enzymes and nitrogen assimilation, along with an increase in NPQ (present chapter) indicated the operation of alternative sinks. In order to avoid the stress at high bicarbonate, MCP

increased NPQ, so that the excess energy was diverted to alternate electron sinks like water-water cycle, plastid terminal oxidase and xanthophylls pool (Miyake, 2010; Stepien and Johnson, 2009; Johnson et al., 2008). The excess energy can be diverted to also other metabolic bypasses like photorespiration, mitochondrial terminal oxidases, nitrogen assimilation (Wilhem and Selmar, 2011). The predicted advantages of high CO_2 and high light were negated in the species of marine phytoplankton with a loss in primary productivity and increased NPQ (Gao et al., 2012).

The overall observations from the Chl fluorescence studies indicated that MCP at high bicarbonate experience a state of excess energy. This might lead to oxidative stress. However, the excess energy in MCP was efficiently dissipated by NPQ to avoid the damage to PSII and its functioning.

Conclusions

1. Efficiency of PSII was impaired at high bicarbonate as indicated from Chl fluorescence kinetics. Both the reduction and re-oxidation of Q_A were affected. This could be the one of the reasons for down-regulation of photosynthetic rate at high bicarbonate (equivalent to elevated CO_2).
2. The energy specific fluxes from the JIP test, indicate that the extent of energy captured by the reaction centre was low and subsequently the flow of energy into the electron transport was limited, particularly at high bicarbonate.
3. Elevation of bicarbonate concentration increased the NPQ. Possibly the excess energy was diverted towards other energy requiring processes or dissipated.

Chapter 8

General Discussion and Major Conclusions

Elevated CO_2 has been persistent and poses a challenge for plants as well as animals. In case of plants, the elevated CO_2 can be beneficial for photosynthesis, but under certain circumstances. There has been a lot of interest in adaptive responses of plants to elevated CO_2, particularly in relation to CO_2 fixation and related metabolic aspects, such as P or N-nutrition. The biological sequestration of carbon is performed mostly by photosynthesis. Increased rates of photosynthesis are observed when plants are exposed to high CO_2. However, the responses of crop plants to the elevated CO_2 remain variable and depend on several external factors. Apart from source and sink balance the crop plants, the responses of plants to elevated CO_2 are dependent on water availability, temperature and mineral nutrition (Reddy et al., 2010).

The present thesis is an attempt to study the responses in a typical legume crop, *Pisum sativum* (pea) as the plant is capable of N-fixation and can be relatively independent of external N-source. Besides, the studies on photosynthesis, the proteomes of leaves from plants exposed to high CO_2 were analysed. The proteomics studies in relation to elevated CO_2 response are sparse. These limited reports point out the importance carbon assimilatory enzymes and occurrence of oxidative stress (Bokhari et al., 2007; Qiu et al., 2008). Proteomic studies on wheat plants, indicated increased levels of proteins belong to carbon storage and glycolysis (Aranjuelo et al., 2011). Our proteomic analysis highlight the importance of enzymes involved N- assimilation during the adaptation to high CO_2.

While the available literature on elevated CO_2 responses is mostly with whole plant responses, the adaptation at cellular level to high CO_2 are not much understood. For e.g. green algae exposed to high CO_2 show increased growth rate (Spijkerman, 2008), but the responses of cells in higher plants are unclear. We utilized MCP to assess the response of plant cells to high CO_2 and examined further the role of phosphate and nitrogen. Qiu et al. (2008) sound that oxidative stress develops at elevated CO_2. We therefore assessed the ROS levels and changes in antioxidant components in leaves on exposure to elevated CO_2. The results indicated the occurrence of oxidative stress under elevated CO_2 conditions. In the final set of experiments, efforts were made to examine the photochemical characteristics of energy absorption and dissipation. These features were followed by using Chl fluorescence as a non-invasive tool.

Physiological characteristics and proteomes of pea leaves from plants exposed to elevated CO_2 in open top chambers

The first part of this study emphasized the effects of elevated CO_2 on physiological parameters when the pea plants were grown in open top chambers, either at elevated CO_2 of 550 ppm or at ambient CO_2 (390 ppm). These studies suggested that the rates of photosynthesis increased during the first week of exposure to elevated CO_2 and thereafter declined in the following weeks (Fig. 4.3A). At the same time the decrease in stomatal conductance (Fig. 4.4A) suggested an increase in water use efficiency of plants under elevated CO_2. An initial stimulation of photosynthesis during short-term exposure to elevated CO_2 was seen in some but not all herbaceous species (Roumet et al., 2000; Poorter and Navas, 2003). Our observations on decrease in stomatal conductance and increase in photosynthetic

rate on exposure to elevated CO_2 were similar to those observed by Ainsworth and Rogers (2007). Although the stimulation of photosynthesis was short lived, growth of plants at the end of three weeks of exposure was enhanced (Fig. 4.2). Our observations indicate that legume plants such as pea could exploit the beneficial effects of elevated CO_2, unlike non-legumes. The ability of N-fixation of legumes is believed to help under elevated CO_2.

The changes in plant metabolism are frequently associated with changes in protein levels of related enzymes. Such changes can be are followed by proteomic analysis. Our studies on proteomic profiles of pea leaves exposed to elevated CO_2, revealed interesting changes. For e.g. the increased protein levels were of enzymes related to Calvin cycle, starch synthesis, photorespiration and nitrogen assimilation (Fig. 4.10, 4.11). Such alterations suggest the readjustment of whole metabolism to redox imbalance in organelles, as suggested by Dietz and Pfannschmidt (2011). Earlier studies on rice leaves at high CO_2, revealed an increase of enzyme proteins, related to carbon metabolism and energy pathways (Bokhari et al., 2007). We have found an up-regulation of not only Calvin cycle and starch synthesis but also of enzymes related to nitrogen metabolism, such as GDC, GS and EF-Tu (Fig. 4.11). Pea, being a legume plant capable of N-fixation, responded positively to elevated CO_2. As a result, the changes in protein levels belonging to carbon metabolism were associated with changes also in proteins/enzymes of nitrogen metabolism, thereby emphasizing the C-N coordination, in our test plant, namely pea (*Pisum sativum*).

Photosynthetic O_2 evolution and Calvin cycle metabolites in mesophyll protoplasts at varied bicarbonate (mimicking high CO_2) and inorganic phosphate

When plants were grown in field at elevated CO_2 their photosynthetic rates were altered. These changes might be due to modulation occurring at cellular level. The situation of elevated CO_2 can be mimicked *invitro* by raising the bicarbonate levels in reaction medium containing MCP. An examination of photosynthetic rates in MCP confirmed the modulation of photosynthesis by varying the bicarbonate concentration. While maximal photosynthetic rate was achieved at an optimal concentration of 1 mM, further increase of bicarbonate (beyond to 5 mM) decreased photosynthesis (Fig. 5.1B). This observation appears to be similar to the decrease in photosynthesis found after prolonged exposure to high CO_2 (Ainsworth and Long, 2005). Photosynthetic response is regulated by metabolites and enzymes involved in CO_2 fixation (Geiger and Serviates, 1994). Initial lag of the photosynthesis is suppressed when metabolites from Calvin cycle or mitochondrial respiration are abundant (Padmasree and Raghavendra, 1999b). It is possible that the modulation of photosynthesis by varying bicarbonate is due to changes in certain metabolites.

Elevation of CO_2 might have changed not only the metabolite levels in the cell but also essential minerals such as Pi, (known to be a powerful modulator of photosynthesis). Besides being a constituent of ATP, Pi is involved in several enzymatic reactions in light. Pi is also a component of several metabolites in Calvin cycle and plays an essential role also in translocation of metabolites across membranes (Brautigam and Weber, 2011). The decrease in photosynthesis at high bicarbonate can be partly due to altered Pi. This was checked by supplementing Pi

at different bicarbonate levels. It was not surprising that optimal Pi was required for sustaining the maximal photosynthetic rates (Fig. 5.4B). Earlier studies with chloroplasts indicated marked change in metabolites and increased photosynthesis when Pi was added externally (Leegood and Walker, 1979; Giersch and Robinson 1987).

High CO_2 promotes the synthesis of Calvin cycle metabolites and the levels of these metabolites depend on also the exchange by membrane translocators (Facchinelli and Weber, 2011). The levels of key metabolites were analysed by enzymatic estimations. Prominent metabolites of Calvin cycle, such as PGA, Triose-P and RuBP, were all altered due to the change in Pi as well as bicarbonate levels. At optimal Pi, there was an increase in RuBP as well as in the ratios of RuBP/Triose-P, both of which indicated the regulation by Pi (Fig. 5.5, 5.6). Similarly, at high CO_2 without Pi, the RuBP and the ratios of RuBP/Triose-P were low (Fig. 5.3). The data on metabolites suggested that RuBP was limiting at high CO_2 and supplementation with Pi could promote the formation of RuBP and help to sustain high photosynthetic rates. Similar observations on efficient recycling of Pi as well as metabolites of the Calvin cycle at high CO_2 were made by Paul and Foyer (2001) and Pieters et al. (2001).

Our results emphasize that the response of protoplast photosynthesis to elevated CO_2 could be a manifestation of the ability of CO_2 fixation in relation to other factors, such as Pi availability. The availability of key metabolites, such as RuBP, PGA and Triose-P, would ultimately regulate the rate. For e.g. a limitation of RuBP regeneration could be the major reason that restricts photosynthetic response at high CO_2.

Nitrogen assimilation, reactive oxygen species, and antioxidant enzymes in mesophyll protoplasts at varying bicarbonate (mimicking CO_2)

Any modulation of photosynthetic carbon metabolism depends on nitrogen metabolism as well. Coordination between N and C was evaluated by checking the responses in MCP at varying bicarbonate in relation to N assimilation. The photosynthetic response, to varied CO_2 in MCP was altered when supplemented with nitrate, confirming the interaction between C and N. Similarly, high CO_2, in turn affected the photosynthetic response of MCP to increase in nitrate (Fig. 6.1). Nitrate assimilation is known to use the reductants generated in photosynthesis (Torre et al., 1991) and high CO_2 could affect such interactions. Efforts were then extended further to study the changes in N-fixing enzyme activities at varying bicarbonate. Our results indicated that enzyme activities of NiR and Fd-GOGAT are modulated by bicarbonate (Fig. 6.2). Elevated activities of these two enzymes would ensure that optimal rates of photosynthesis are maintained at elevated CO_2. The increased demand for N and elevated activities of key enzymes of N-metabolism at high CO_2 were observed in barley (Wang et al., 2013).

Alterations in the antioxidant metabolism indicated the manifestation of stress. Antioxidative enzymes, APX and GR exhibited differential responses. For e.g. the activity of APX decreased at high bicarbonate and no marked changes occurred in GR activity with varying bicarbonate. In contrast, CAT being a prominent enzyme in detoxifying H_2O_2, seemed to play major role at high CO_2 (Fig. 6.3). High CO_2 might activate the CAT in countering the oxidative stress. There have been reports of

possible oxidative stress at high CO_2 as indicated by protein carbonylation and increased MDA levels (Qiu et al., 2008; Lambreva et al., 2006).

The present results emphasize that elevated CO_2 alters not only the carbon and nitrogen metabolism of the MCP but also the oxidative status of the cell, by creating oxidative stress. The MCP therefore appear to adjust to elevated CO_2 by modulating the key enzymes of nitrogen metabolism (e.g. NiR and Fd-GOGAT) and as well as anti-oxidative enzymes, such as CAT, APX and GR. The rise in ROS levels (Table 6.1) at elevated CO_2 is a demonstration of oxidative stress.

Chlorophyll fluorescence in mesophyll protoplasts at varying bicarbonate

The decreased in photosynthetic rates of MCP at supra-optimal bicarbonate was described in Chapter 5. The development of mild oxidative stress on exposure to elevated CO_2 or high bicarbonate was indicated in Chapter 6. The next attempt was to understand the patterns of photochemical events that were modulated by varying bicarbonate. Chl *a* fluorescence offers a non-invasive and rapid analytical tool to assess the physiological state of the leaf. Fluorescence induction kinetics of leaves were obtained by using two instruments: Handy PEA and Mini-PAM. The kinetic data were then analyzed by suitable software and fluorescence relaxation/quenching were converted to individual components.

As per Handy PEA measurements, the MCP exhibited highest F_m at optimal bicarbonate (1 mM) but not at supra-optimal bicarbonate (10 mM) (Fig. 7.1B). Maximal and variable fluorescence indicated a loss in the PSII efficiency at supra-optimal bicarbonate. It was known that the PSII efficiency was lost under stressful

situations like salinity, drought and temperature (Naumann et al., 2007; Allakhverdiev et al., 2008). Since we found similar response in PSII efficiency, along with increased ROS and the modulation of anti-oxidative enzymes, we conclude that oxidative stress develops at supra-optimal bicarbonate reflecting elevated CO_2.

JIP test parameters, that were observed, based on Chl *a* fluorescence were: absorption per RC (ABS/RC), trapped energy per RC (TR_o/RC), dissipated energy per RC (DI_o/RC) and electron transport per RC (ET_o/RC). All these values (indicating the functioning of photosynthetic apparatus) decreased at high bicarbonate (10 mM) compared to 1 mM bicarbonate (Fig. 7.3). The energy entering into the Chl pigments and RC was restricted. Trapping energy considered to be the efficiency of converting the primary electron acceptor Q_A to Q_A^-. Even the electron transport beyond the RC towards PSI was also decreased (Strasser et al., 2000). Our observations, suggest the energy entering into the RC is lowered at high bicarbonate and possibly diverted to other pathways.

Chl *a* fluorescence transients from PAM gave an overview of the photochemical events occurring in presence of light. At high bicarbonate (10 mM), in initial phase, the enegy was lost through ϕNO (non regulated/ non-radiative decay) which was not induced by light (Krammer et al., 2004). However, the ϕNPQ were increased with progression of time with a simultaneous decrease in ϕII. The rise in NPQ was evident under stressful scenarios (Dwyer et al., 2007; Azzabi et al., 2012). As per the data in Chapter 6, describing the modulation of nitrogen assimilation and antioxidant enzymes, the energy was diverted to other pathways or alternative electron sinks at supra-optimal bicarbonate. The higher NPQ (Fig. 7.5B) could be a

strategy to remove excess energy at high bicarbonate and counter the damage to photosynthetic machinery, as suggested by Wilhem and Selmar (2011).

The following are the major conclusions from the present study:

1. Among the physiological parameters studied, photosynthesis increased, along with biomass, while the stomatal conductance decreased in the plants grown at elevated CO_2.
2. Proteomic studies revealed that the protein levels of typical N-assimilatory enzymes (such as GS, GDC-H and EF-Tu), key Calvin cycle enzymes and starch synthesis increased, highlighting the C-N co-ordination at elevated CO_2.
3. The rates of photosynthesis by MCP were maximum at 1 mM bicarbonate and decreased at higher bicarbonate concentrations. Supplementation with Pi (0.5 mM or 1.0 mM) stimulated the photosynthesis, irrespective of bicarbonate concentrations. Thus, Pi (at 0.5 mM or 1.0 mM) relieved the inhibition of photosynthesis at high bicarbonate.
4. The patterns of Calvin cycle metabolites (PGA, triose-P and RuBP) and their ratios to varied bicarbonate and Pi, indicated that the decrease in photosynthesis at high levels of bicarbonate (10 mM) may be due to a limitation on RuBP regeneration.
5. The increased activities of NiR and Fd-GOGAT at optimal bicarbonate and decreased nitrate-dependent oxygen evolution at high bicarbonate, highlighted the coordination between C and N-metabolism.

6. The marked increase in the activity of CAT (anti-oxidative enzyme) and high levels of ROS at supra-optimal bicarbonate implied the occurrence of mild oxidative stress at high bicarbonate.
7. The Chl *a* fluorescence transients indicated that high bicarbonate (mimicking elevated CO_2) decreased the PSII efficiency, and impaired the Q_A reduction as well as Q_A re-oxidation. The energy flow between different photochemical components was also affected at high bicarbonate.
8. Chl *a* fluorescence measurements with PAM indicated a marked increase in NPQ at high bicarbonate. This could be a strategy to protect photosynthetic apparatus at elevated CO_2.

Chapter 9

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Appendix

Publications/Conference Presentations/Anti-plagiarism report

Paper published

Talla SK, Riazunnisa K, Padmavathi L, Sunil B, **Rajsheel P** and Raghavendra AS (2011) Ascorbic acid is a key participant during the cross-talk between chloroplasts and mitochondria to optimize photosynthesis at high light and protect against photoinhibition. *Journal of Bioscience* **36:** 163–173

Poster presented at a conference

Rajsheel P and Raghavendra AS (2011) Responses of photosynthesis, reactive oxygen species and antioxidant enzyme in mesophyll protoplasts of pea (*Pisum sativum*) to high bicarbonate simulating elevated CO_2. *International Conference on Plant Science in Post Genomic Era (ICPSPGE–2011)*; 17th-19th February 2011 Sambalpur University, Orissa, India.

Talla SK**,** Riazunnisa K, **Rajsheel P**, Kavya B and Raghavendra AS (2008) Role of ascorbate during optimization of photosynthesis and protection against photoinhibition by mitochondrial metabolism. *4th Asia-Oceanic conference on Photobiology;* 24th-26th Nov, 2008, BHU, Varanasi, India

Riazunnisa K, Talla SK, Sunil B, **Rajsheel P** and Raghavendra AS (2008) Role of ascorbate during optimization of photosynthesis and protection against photoinhibition by mitochondrial metabolism. *2nd International conference on Trends in Cellular and Molecular Biology;* 2nd-5th Jan 2008, JNU, New Delhi, India

Ascorbic acid is a key participant during the interactions between chloroplasts and mitochondria to optimize photosynthesis and protect against photoinhibition

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The possible role of L-ascorbate (AsA) as a biochemical signal during the interactions between photosynthesis and respiration was examined in leaf discs of *Arabidopsis thaliana*. AsA content was either decreased as in AsA-deficient *vtc1* mutants or increased by treatment with L-galactono-1, 4-lactone (L-GalL, a precursor of AsA; EC 1.3.2.3). In mutants, photosynthesis was extremely sensitive to both antimycin A (inhibitor of the cytochrome *c* oxidase pathway [COX pathway]) and salicylhydroxamic acid (SHAM, inhibitor of the alternative pathway [AOX pathway]), particularly at high light conditions. Mitochondrial inhibitors lowered the ratio of reduced AsA to total AsA, at high light, indicating oxidative stress in leaf discs. Elevation of AsA by L-GalL decreased the sensitivity of photosynthesis at high light to antimycin A or SHAM, sustained photosynthesis at supraoptimal light and relieved the extent of photoinhibition. High ratios of reduced AsA to total AsA in L-GalL-treated leaf discs suggests that L-GalL lowers oxidative stress. The protection by L-GalL of photosynthesis against the mitochondrial inhibitors and photoinhibition was quite pronounced in *vtc1* mutants. Our results suggest that the levels and redox state of AsA modify the pattern of modulation of photosynthesis by mitochondrial metabolism. The extent of the AOX pathway as a percentage of the total respiration in *Arabidopsis* mesophyll protoplasts was much higher in *vtc1* than in wild type. We suggest that the role of AsA becomes pronounced at high light and/or when the AOX pathway is inhibited. While acknowledging the importance of the COX pathway, we hypothesize that AsA and the AOX pathway may complement each other to protect photosynthesis against photoinhibition.

[Talla S, Riazunnisa K, Padmavathi L, Sunil B, Rajsheel P and Raghavendra AS 2011 Ascorbic acid is a key participant during the interactions between chloroplasts and mitochondria to optimize photosynthesis and protect against photoinhibition. *J. Biosci.* **36** 163–173] **DOI** 10.1007/s12038-011-9000-x

1. Introduction

Interorganelle interactions between chloroplasts and mitochondria through cytosol and peroxisomes are essential for the optimization of photosynthesis (Gardeström *et al.* 2002; Padmasree *et al.* 2002; Noctor *et al.* 2007; Nunes-Nesi *et al.* 2008). Such an interaction between chloroplasts and mitochondria is not surprising, but is intriguing. A well-known basis of interorganelle interactions is the exchange of metabolites between them. However, there could be other possible signals, such as reactive oxygen species (ROS), nitric oxide (NO), cytosolic pH and

Keywords. L-Ascorbate; L-galactone-1, 4-lactone; interorganelle interaction; *vtc1* mutant

Abbreviations used: AOX, alternative oxidase; AsA, ascorbate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COX, cytochrome c oxidase; L-GalL, L-galactono-1, 4-lactone; DHA, dehydroascorbate; L-GalLDH, L-galactono-1, 4-lactone dehydrogenase; L-GalLDH, L-galactono-1, 4-lactone dehydrogenase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid

Responses of pea plants to elevated

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