Photosynthesis and Long-term Carbon Sequestration in Field Grown *Jatropha* under Elevated Carbon Dioxide

Thesis Submitted To the University Of Hyderabad for the Degree of

Doctor of Philosophy in Plant Sciences

By

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DECLARATION

I, SumitKumar, hereby declare that this thesis entitled "**Photosynthesis and long-term carbon sequestration in field grown *Jatropha* under elevated carbon dioxide"** submitted by me under the guidance and supervision of Professor Attipalli R. Reddy 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 Institution for the award of any degree or diploma.

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   1. Physiologia Plantarum (2014) 152: 501 –519 [DOI: 10.1111/ppl.12195; ISSN: 1399-3054].
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1. **Kumar S, Chaitanya BSK, Ghatty S, Reddy AR (2014).** Growth, reproductive phenology and yield responses of a potential biofuel plant, *Jatropha curcas* grown under projected 2050 levels of elevated CO_2. *Physiologia Plantarum*, 152: 501 – 519. ■ **3%**

2. **Kumar S, Sreeharsha RV, Mudalkar S, Sarashetti PM, Reddy AR (2017)**. Molecular insights into photosynthesis and carbohydrate metabolism in *Jatropha curcas* grown under elevated CO_2 using transcriptome sequencing and assembly. *Scientific Reports*, 7: 11066. ■ **8%**

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indicate that the values are significantly different between each treatment at $P < 0.01$. [A – Ambient; E – Elevated]

Abbreviations

A	Net CO_2 assimilation rate
A_{Sat}	Light saturated photosynthetic rates
A_{max}	Light and CO_2 saturated maximum photosynthetic rate
ANOVA	Analysis of variance
AQE	Apparent quantum efficiency
BTP	1,3-bis[tris(hydroxymethyl) methylamino]propane
C	Carbon
CA	Carbonic anhydrase
CCS	Carbon capture and storage
C_i	Intercellular concentration of CO_2
C_t	Threshold cycle
CUE	Carbon use efficiency
DAP	Days after plantation
DAT	Days after treatment
DEG	Differentially expressed gene
DGE	Differential gene expression
DTT	Dithiothreitol
E	Transpiration rate
EDTA	Ethylene diamine tetraacetic acid
EPA	Environmental protection agency
ETR	Photosynthetic electron transport rate of PSII
FACE	Free air CO_2 enrichment
FAD	Flavin adenine dinucleotide
FB	Cytosolic fructose 1,6-bisphosphatase
F_m	Maximum fluorescence of dark adapted leaves
F_o	Initial fluorescence of dark adapted leaves
F_v/F_m	Maximal photochemical efficiency of PSII
F_v/F_o	Efficiency of water splitting complex
F	Fluorescence yield of light-adapted sample
F_m'	Maximum light-adapted fluorescence yield
$\Delta F/F_m'$	Effective quantum yield of PSII
FDR	False discovery rate
FTA	F-type H^+-type transporting ATPase subunit beta
G6PD	Glucose-6-phosphate dehydrogenase, chloroplastic
GAP	Glyceraldehyde-3-PO_4 dehydrogenase
GBSS	Granule bound starch synthase
GD	Glutamate dehydrogenase
GHGs	Greenhouse gases
GO	Gene ontology
GPT	Glucose-6-phosphate/phosphate translocator, chloroplast
g_s	Stomatal conductance
GS	Glutamine synthetase
HXK	Hexokinase
IPCC	Intergovernmental panel on climate change
IRGA	Infra-red gas analyzer
J_{max}	Maximum electron transport rate
KEGG	Kyoto encyclopedia of genes and genomes

KAAS	KEGG automatic annotation server
KOG	Clusters of orthologous groups of proteins involving eukaryotes
LMA	Leaf mass per unit area
N	Nitrogen
N_A	N content per unit leaf area
NAD^+/NADH	Nicotinamide adenine dinucleotide
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate
NDHB	NAD(P)H-quinone oxidoreductase subunit 2, chloroplastic
NDHK	NAD(P)H-quinone oxidoreductase subunit K, chloroplastic
NDIR	Non dispersive infra-red
N_M	N content per unit leaf mass
NOAA-ESRL	National ocean and atmospheric administration-Earth system research laboratory
NPK	Nitrogen phosphorous potassium
NPP	Net primary productivity
NPQ	Non-photochemical quenching
NR	Nitrate reductase
OTC	Open top chambers
P	Phosphorous
PAR	Photosynthetically active radiation
PAM	Pulse amplitude modulated
PEG	Polyethylene glycol
PEP	Phosphoenol pyruvate
PFK	Phosphofructokinase
PGM	Phosphoglucomutase
Pg	Peta gram
P_n	Photosynthetic rate
PHGR	Plant height growth rate
PNUE	Photosynthetic nitrogen-use efficiency
PPFD	Photosynthetic photon flux density
PRK	Phosphoribulokinase
PSI	Photosystem I
PSII	Photosystem II
PsaA	Subunit of PSI
PsbA	PSII protein D1
PsbB	Subunit of PSII
PsbC	Subunit of PSII
PsbP	Photosystem II oxygen-evolving enhancer protein 2
PVPP	Polyvinylpolypyrrolidone
qP	Photochemical quenching
qRT-PCR	Quantitative real time polymerase chain reaction
RA	Rubisco activase
RI	Ribose-5-phosphate isomerase
R_d	Dark respiration rate
RL	Rubisco large subunit
RS	Rubisco small subunit
RuBP	Ribulose 1,5- bis phosphate

Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase
SCADA	Supervisory control and data acquisition
SB	Sedoheptulose 1,7-bisphosphatase
SP	Saturation pulse
SD	Standard deviation
SLA	Specific leaf area
SOM	Soil organic matter
SPS	Sucrose phosphate synthase
SRC	Short rotation coppice
TF	Transcription factor
TKL	Transketolase
TNC	Total non-structural carbohydrate
TOIL	Tree Oils India Limited
TPICH	Chloroplastic triose phosphate isomerase
TPICY	Cytosolic triose phosphate isomerase
TPT	Triose phosphate/phosphate translocator
UNEP	United nations environment programme
UNFCCC	United nations framework convention on climate change
V_{cmax}	Maximum carboxylation rate of Rubisco
VPD	Vapour pressure deficit
WUE	Water use efficiency
XTH	Xyloglucan endotransglucosylase/hydrolases

Chapter I

Rationale and Objectives

Chapter 1

Climate change is one of the pertinent issue of our time which needs global attention. Climate is not static but constantly changes in response to the variation in the factors that control it. One can define climate change as a long-term shift in weather conditions identified by changes in temperature, precipitation, wind, and other indicators. It is a natural phenomenon which has been occurring since the earth came into existence. However, the alarming pace with which this change is occurring in recent times is a matter of concern as it has started to show its ill impact on environment and biosphere. Hence, a concerted global action is needed in reducing the magnitude of climate change and to adapt to its impacts.

1.1 Causes of climate change

The earth's climate is regulated by the balance between energy entering and leaving the planet's system (Le Treut et al., 2007). Climate change can be enforced by any factor which can cause a sustained variation to this balance. There are plenty of evidences which suggest that the previous happenings of climate change on the Earth were the effects of four major factors (Hegerl et al., 2007). These factors also known as "climate drivers" may have resulted in either net warming or cooling and include:

- variations in the earth's orbital characteristics,
- atmospheric carbon dioxide variations,
- volcanic eruptions,

- variations in solar output.

Different factors including the above four have operated at different time periods ranging from few hundred years to hundreds of millions of years ago, but those factors that have been responsible for changes in earth's climate in the distant past are not relevant to present-time climate change. The factors relevant to contemporary climate change can be broadly divided into two categories - natural processes and human activities (**Fig. 1.1**). Among the natural causes, variations in solar output and volcanic eruptions have modulated the climate trends prior to industrial revolution by influencing the amount of incoming energy and earth's energy balance (Le Treut et al., 2007).

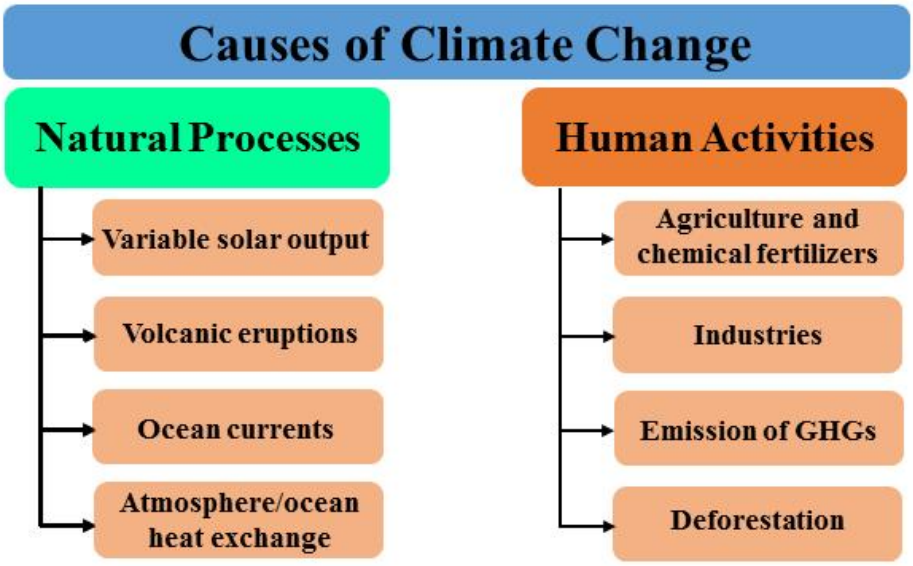


Fig. 1.1. Overview of events or factors causing climate change.

Comparative analysis of the relative contribution of natural processes and human activities induced climate change in recent past half century reveals that the increased accumulation of carbon from human sources is the largest climate change driver (**Fig. 1.2**). Since the industrial revolution, anthropogenic activities have contributed maximally in the addition of greenhouse gases (GHGs) to the atmosphere which has now become the major influence on earth's climate system. The primary GHGs in the Earth's atmosphere are water vapor, methane, nitrous oxide, carbon dioxide (CO_2) and ozone (O_3). Among these

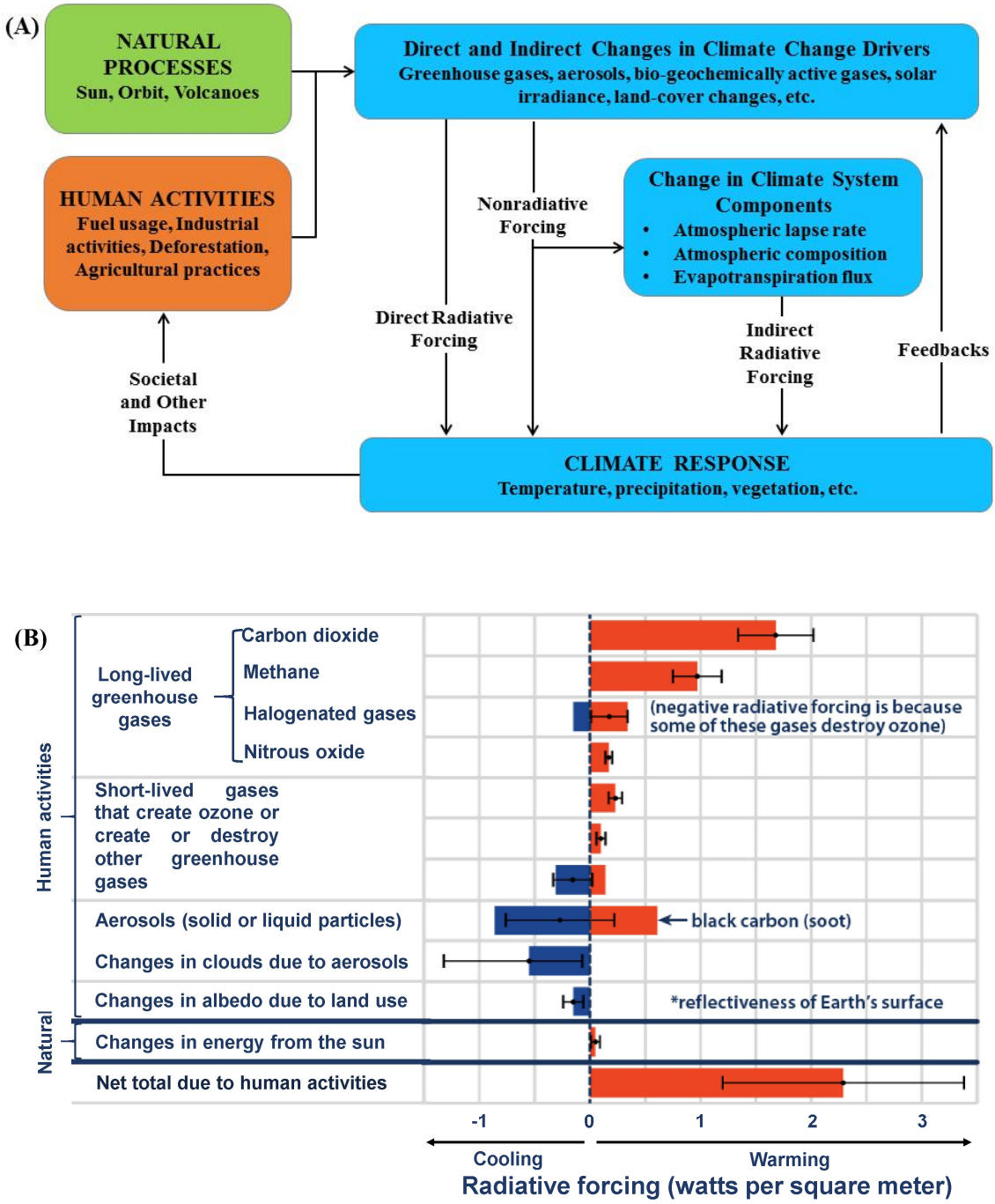


Fig. 1.2. Diagrammatic illustration of components of climate change process. (A) Conceptual framework of climate change network (adapted from: Radiative force of climate change, 2005) and (B) radiative forcing caused by human activities since 1750 [radiative forcing is the measurement of the capacity of different agents to affect the energy balance, thereby contributing to climate change; source: IPCC, 2013].

GHGs, CO_2 is the major gas which has been emitted due to human activities (IPCC 1995) (**Fig. 1.3A**). It is a very long-lived gas, which means it continues to affect the climate system during its long residence time in the atmosphere. The concentration of CO_2 in the atmosphere has risen continuously from pre-industrial levels of ~280 parts per million (ppm) to the current CO_2 levels of ~405 ppm [National Ocean and Atmospheric Administration-Earth System Research Laboratory (NOAA-ESRL), USA] (**Fig. 1.3B**). The quest for technological advancement by developed and developing countries has led the world to a path where human fingerprints in the form of industries, land use, deforestation and burning of fossil fuels is increasing the carbon overload on the atmosphere. Apart from CO_2, anthropogenic activities also emit other substances that also act as climate forcers. Some, such as nitrous oxide, also long-lived as CO_2, contribute to long-term climate change (**Fig. 1.3A**). Other substances have shorter atmospheric lifetimes because they are removed fairly quickly from the atmosphere. Therefore, their effect on the climate system is similarly short-lived. These include methane and tropospheric ozone (both GHGs), black carbon, a small solid particle formed from the incomplete combustion of carbon-based fuels (coal, oil and wood for example) and sulphate aerosols, formed by the combination of fossil fuel combustion emitted sulphur dioxide and water vapour in the atmosphere (Blanco et al., 2014) (**Fig. 1.3A**).

1.2 Impacts of climate change

Climate change is already having a significant impact on human society and the natural environment in many ways. The most prominent effect on earth's climate due to human activities has been the rise of average surface temperature of Earth with each passing year (**Fig. 1.4)**. This phenomenon has been termed as global warming which has been used synonymously with climate change as all other effects are linked with this phenomenon. These effects include rising sea levels, variation in precipitation patterns and expansion of deserts in the subtropics. Warming is expected to be greater over land than over the oceans and greatest in the Arctic, with the continuing retreat of glaciers, permafrost and sea ice. Other likely changes include more frequent extreme weather events including heat

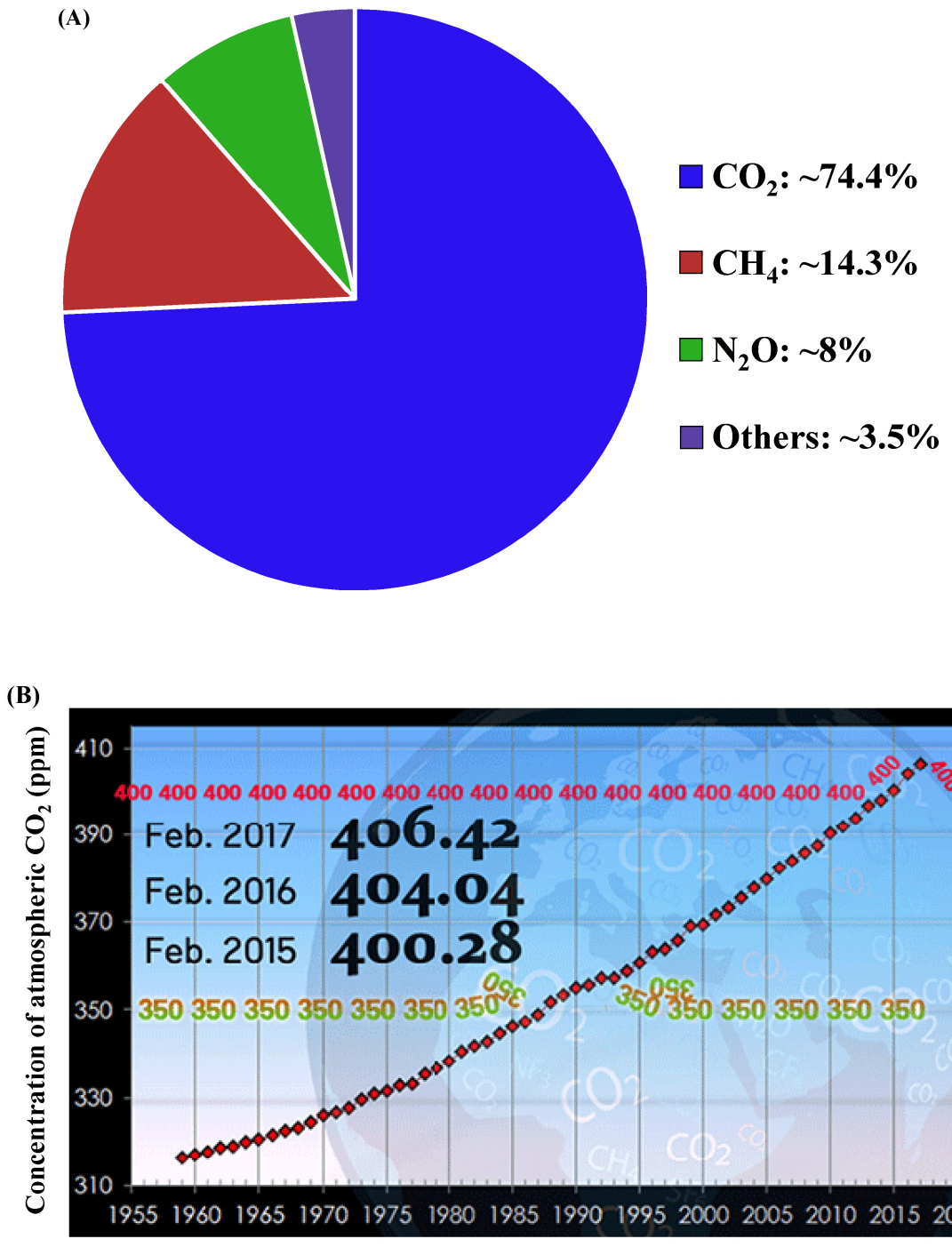


Fig 1.3. (A) Relative proportion of GHGs (CO_2, N_2O, CH_4 and O_3) and other molecules (black carbon and sulphated aerosols) emitted in the atmosphere due to anthropogenic activities (source: IPCC 2014). (B) Graph showing the rise in concentration of CO_2 measured on Earth's atmosphere during the past 70 years (adapted from: NOAA-ESRL 2017).

waves, periods of drought, heavy rainfall with floods and heavy snowfall, ocean acidification and species extinctions and migrations due to shifting temperature regimes (IPCC 2007). Humans are also significantly affected with temperature modulations which include the threat to food security due to decreasing crop yields, irrigation demand for agriculture sector, abandonment of populated areas due to rising sea levels, and social and economic issues such as food supply, health, industry, transportation and ecosystem integrity (IPCC 2007).

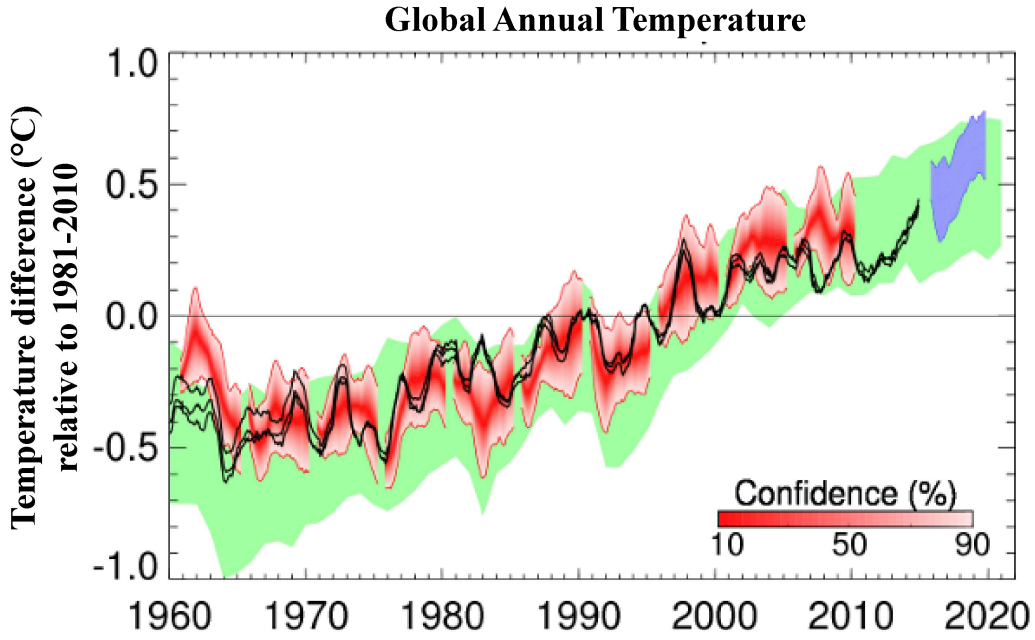


Fig. 1.4. Earth's mean surface-temperature changes (black line) from 1960 to 2016 (source: NOAA and NASA, 2016).

However, some short-lived climate forcers have climate cooling effects, most notably sulphate aerosols. Sulphate aerosols remain in the atmosphere for only a few days (washing out in what is referred to as acid rain), and so do not have the same long-term effect as GHGs. The cooling from sulphate aerosols in the atmosphere has, however, offset some of the warming from GHGs and other substances. That is, the warming we have experienced to date would have been even larger had it not been for elevated levels of sulphate aerosols in the atmosphere (Hansen et al., 2005).

1.3 Elevated levels of CO_2 as a climate forcer

The natural greenhouse effect has effectively warmed the atmosphere and surface of Earth, thereby maintaining Earth's temperature [~33 °C (59 °F) warmer] which is critical to support life on Earth. The greenhouse effect is the process by which absorption and emission of infrared radiation by gases in a planet's atmosphere warm its lower atmosphere and surface. In the absence of this natural greenhouse effect, the Earth's average temperature would be well below the freezing temperature of water. The major GHGs involved are water vapour, which causes about 36–70% of the greenhouse effect; CO_2, which causes 9–26%; methane, which causes 4–9%; and O_3, which causes 3–7% (Kiehl and Trenberth, 1997). The GHGs will stay in the atmosphere for a long time and their effects will not only exist for decades or centuries, but will persist for tens of thousands of years. However, anthropogenic activities – particularly burning fossil fuels (coal, oil and natural gas) and deforestation – are increasing the concentrations of GHGs. This is the enhanced greenhouse effect, which is contributing to warming of the Earth.

CO_2 is the main GHG largely responsible for most of the additional average warming over the past several decades and, fossil fuel emissions comprise its largest source (Forster et al., 2007). Further, this increased levels of CO_2 in the atmosphere has significantly disturbed the natural carbon cycle. Along with the nitrogen cycle and the water cycle, the carbon cycle comprises a sequence of events that are key to make the Earth capable of sustaining life; it describes the movement of carbon as it is recycled and reused throughout the biosphere, including carbon sinks. The global carbon cycle operates through a variety of response and feedback mechanisms. The net effect of land and ocean climate feedbacks is an increase in atmospheric CO_2 concentrations. A number of processes in the carbon cycle can produce feedbacks. The increased atmospheric CO_2 has both positive and negative feedbacks, and it is hard to discern which would prevail globally. For example, an increase in atmospheric CO_2 that leads to an increase in the earth's surface temperature may be a positive feedback in some regions because warmer temperatures increase soil respiration, thereby further increasing atmospheric CO_2. However, warmer temperatures can also extend the growing season of plants, and thus the length of time over which they

draw CO_2 out of the atmosphere. This effect would reduce the amount of atmospheric CO_2, which then could reduce the amount of warming. However, the uptake of anthropogenic CO_2 is primarily a physically and chemically controlled process superimposed on a biologically driven carbon cycle (IPCC, 2001). In the present scenario, the mechanism of CO_2 uptake by natural sinks is insufficient to offset the accelerating pace of emissions in the atmosphere (**Fig. 1.5**).

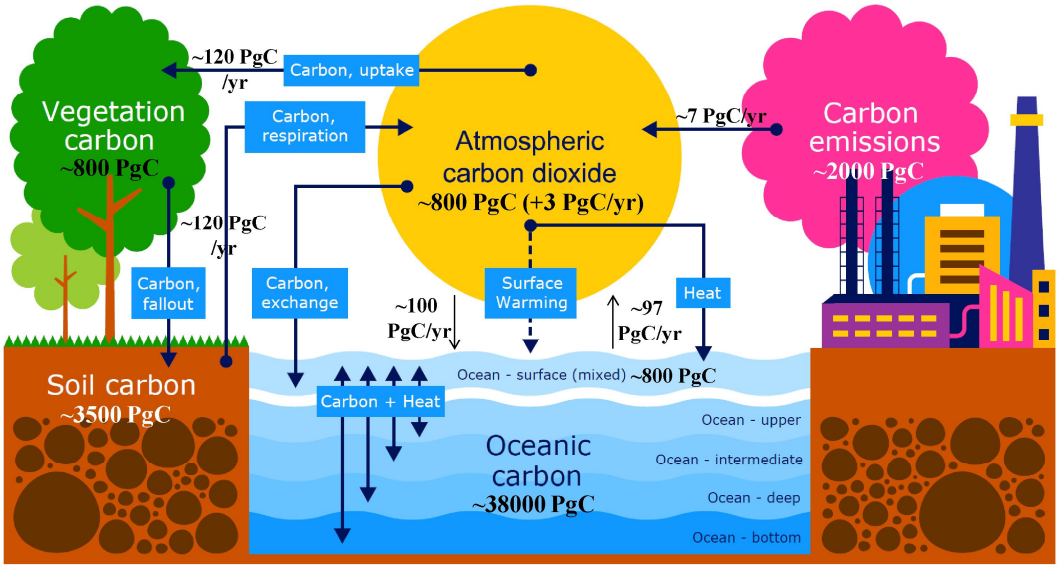


Fig. 1.5. Carbon flow between the atmosphere, land and ocean indicating the amount of carbon stored and exchanged in events participating in carbon input and output on an annual basis [PgC – Petagram carbon; modified from: IPCC 2014].

The presence of this extra CO_2 has effected the variations in temperature, precipitation patterns, rise in sea-levels and geographical distribution of different vegetation.

1.4 Mitigation of excess CO_2 by sequestration

In spite of the efforts to reduce CO_2 emissions, the world appears to be on a path that is likely to lead to a [CO_2] that exceeds the highest Intergovernmental Panel on Climate Change (IPCC) predicted emissions scenario. The IPCC is the leading international body

for the assessment of climate change. It was established by the United Nations Environment Programme (UNEP) and the World Meteorological Organization (WMO) in 1988 to provide the world with a clear scientific view on the current state of knowledge in climate change and its potential environmental and socio-economic impacts. There is a need for concerted action on a global level to develop and implement strategies for mitigation of excess CO_2 from the atmosphere. The developed and developing countries under the umbrella of United Nations Framework Convention on Climate Change (UNFCCC) regularly meet, frame plan of action and undertake ambitious efforts to combat excess CO_2 and adapt to its effects. There are three primary methods for reducing the amount of carbon dioxide in the atmosphere advocated by UNFCCC:

(A) employing energy efficiency and conservation practices,

(B) using carbon-free or reduced-carbon energy resources, and

(C) capturing and storing carbon either from fossil fuels or from the atmosphere.

All these three strategies for controlling atmospheric CO_2 will require deliberate mitigation with an approach that combines reducing emissions and increasing storage. The third strategy listed above, also known as 'carbon capture and storage', has long been suggested to mitigate climate change (Marchetti, 1977). Carbon capture and storage [(CCS) or carbon capture and sequestration] is the process of capturing waste CO_2 from large point sources, such as fossil fuel power plants, transporting it to a storage site, and depositing it where it will not enter the atmosphere. The storage of carbon or "carbon sequestration" is used to describe both natural and deliberate processes by which CO_2 is either removed from the atmosphere or diverted from emission sources and stored in the ocean, terrestrial environments (vegetation, soils, and sediments), and geologic formations. The natural sinks of CO_2 are ocean and land (**Fig. 1.6A**). Terrestrial carbon sequestration includes plants which fix CO_2 into their biomass, peat bogs by accumulation of partially decayed biomass that would otherwise continue to decay completely and soil which stores CO_2 as soil organic carbon released through plants. Broadly, the four major modes of sequestration (**Fig. 1.6B**), both natural and deliberate can be summarized as follows:

(A) *Oceanic sequestration*: Ocean-atmosphere gas exchange naturally removes large amounts of carbon from the atmosphere at shallow depths but the increase in

anthropogenic emissions has resulted in greater rate of absorption of CO_2 in oceans. In fact, the oceans have absorbed about $1/3^{rd}$ of the CO_2 produced from human activities since 1800 and about half of the CO_2 produced by burning fossil fuels (Sabine et al., 2004). The oceans absorb large quantities of CO_2 from the atmosphere principally because CO_2 is a weakly acidic gas, and minerals dissolved in sea water have created a mildly alkaline ocean. However, this has resulted in a decrease of pH or increase in acidity at the ocean surface as anthropogenic CO_2 resides primarily in the upper ocean and virtually no change in pH deep in the oceans. This puts a stress on carbonate levels of the ocean surface and affects marine organisms. Many marine organisms and ecosystems depend on the formation of carbonate skeletons and sediments that are vulnerable to dissolution in acidic waters. One of the prominent example is coral reefs which cannot absorb the calcium carbonate they need to maintain their skeletons and the stony skeletons that support corals and reefs will dissolve. A proposed solution in this regard is deliberate injection of captured CO_2 into the ocean at great depth, where most of it would remain isolated from the atmosphere for centuries. There has been small-scale field experiments and modelling studies happening at present but intentional ocean storage of CO_2 has not yet been deployed or extensively tested as long-term effects on deep sea ecosystem has to be characterised before approval of this approach (Caldeira et al., 2005).

(B) *Chemical/Mineral sequestration*: The reactive nature of CO_2 forms the basis of chemical sequestration technologies. The carbon can be stored in the form of carbonate minerals form in coal beds and rocks. However, this can cause the coal and carbonaceous sediment to swell reducing its porosity and permeability (Sheps et al., 2009). Further, by taking advantage of the chemical reactivity of CO_2, it can be used to prepare value-added organic compounds. One such effort describes electrocatalytic CO_2 conversion to oxalate by a copper complex (Angamuthu et al., 2010). Many other efforts are being made which enhances natural mineralisation of CO_2.

(C) *Geological sequestration*: Geological sequestration involves collecting and placing CO_2, as either a liquid or a critical fluid, into suitable subsurface formations including aquifers, oil and gas reservoirs where suitable porosity and permeability exist. Geological sequestration has also been applied to rocks and sediments in marine continental shelf and

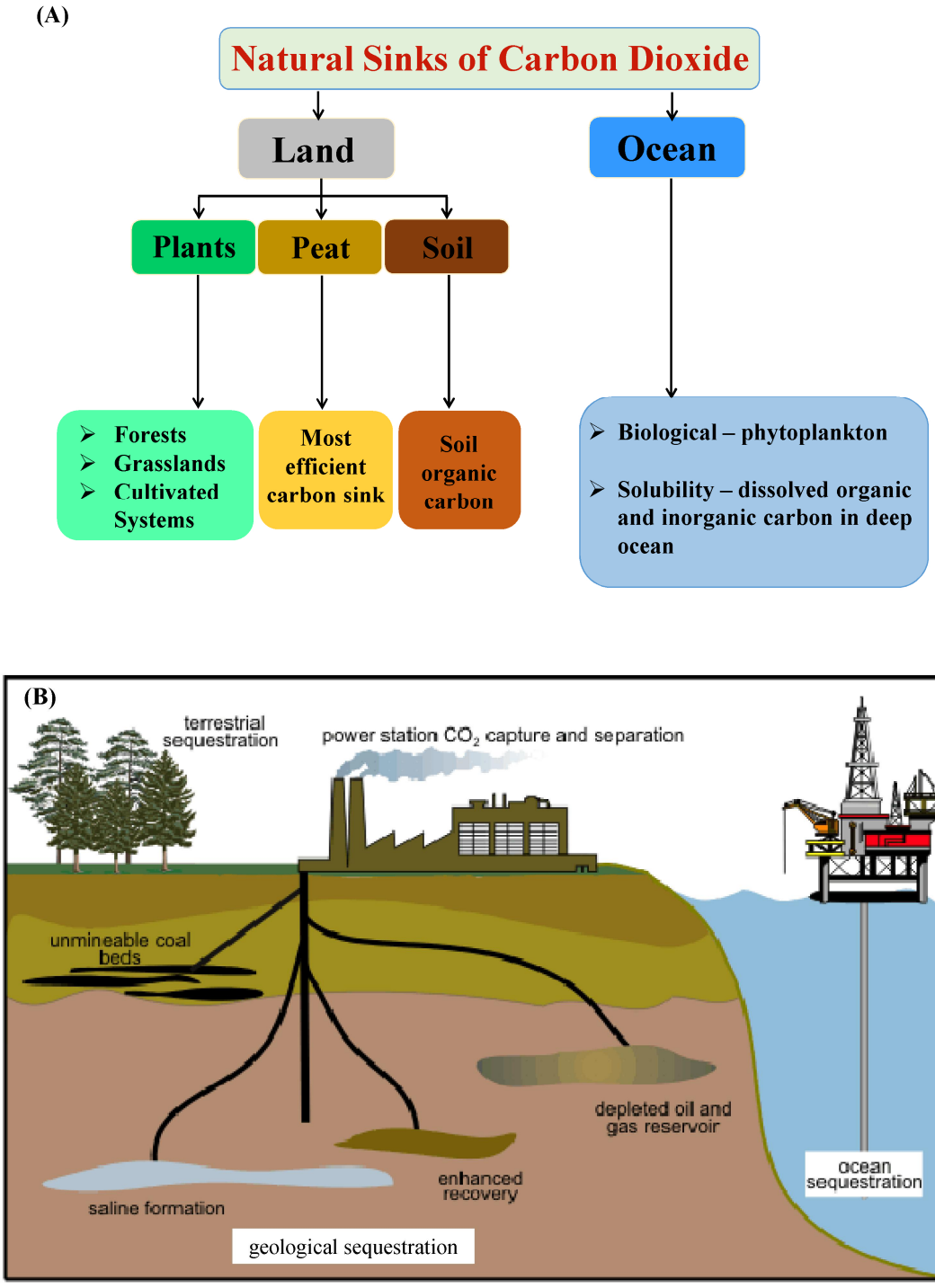


Fig. 1.6. (A) Natural sinks of carbon dioxide having the capacity to store CO_2. (B) Common modes of carbon sequestration (adapted from: CCS, climate change, United States Environmental Protection Agency, 2014).

slope areas. However, the durability of a geologic formation to be deemed suitable for placement and retention of CO_2 will depend on number of characteristics which varies for each geological and geographic site defining their storage potential. The success of any geological storage plan must be examined on a reservoir-by-reservoir basis (Sheps et al., 2009). Further, the geological formation should be deep enough, be covered with a formation that will prevent escaping of CO_2 to the surface and has to have adequate porosity and permeability to accept the injected carbon dioxide. The depth of injection would depend upon the depth of the formation being targeted for injection. Any modelling study to determine changes in equilibrium conditions in reservoirs due to CO_2 sequestration should also include a complete geochemical analysis (Wilson et al., 2008).

(D) *Biological sequestration*: Carbon sequestration through biological processes involve storage of carbon into biomass either dead or alive. This can be achieved in land, as stated earlier by peat production, plants and soil carbon enhancement or in oceans also by iron and urea fertilization. Peat bogs are very efficient carbon sinks due to the accumulation of partially decayed biomass that would otherwise continue to decay completely. There is a variance on how much the peatlands act as a carbon sink or carbon source that can be linked to varying climates in different areas of the world and different times of the year. Carbon is sequestered in peatlands so long as formation of new peat exceeds decay losses of all peat accumulated previously and the process can be enhanced by creating new bogs or enhancing the existing ones (Strack, 2008). Soil is the largest terrestrial reservoir of carbon [~3500 Pg; both organic and inorganic (**Fig. 1.5**)] which can act as source or sink of CO_2 depending on land use and management. Soil across the world has been a source of almost ~30% CO_2 and GHGs emissions, both direct and indirect which is contributed by agriculture. However, agroecosystems including croplands, grazing lands, drastically disturbed and degraded lands have a large sink capacity of soil organic carbon. It is important to create a positive carbon budget by improvising on the integrated approach of restorative land use and animal management. This can be aided by increasing the input of biomass carbon (above and below ground biomass, compost, manure) to offset the losses by erosion, mineralization and leaching. Strategies have been proposed to create a positive soil carbon budget which include conservation agriculture adopted in conjunction with

crop residue mulch, restoration of degraded soils and desertified ecosystems through afforestation, complex rotations along with agroforestry systems, integrated nutrient management, and water harvesting and recycling through micro-irrigation (Lal et al., 2015). The promotion of growth of CO_2 absorbing phytoplankton present in the oceans has been advocated by iron and urea fertilization in oceans as one of the strategies of biological CO_2 sequestration. However, this method cannot be adopted until there is a full understanding of their complete effects on the marine ecosystem. Plants play an important role in terrestrial sequestration as they naturally store carbon in their biomass through the process of photosynthesis. Hence, restoring and establishing new forests, wetlands, and grasslands coupled with reduced CO_2 emissions would be a practical way to enhance terrestrial carbon sequestration.

Significant efforts need to be put in for improving long-term solutions for carbon sequestration as natural methods of CO_2 uptake take a long time to fruition. However, till these solutions become effective, we need to implement temporary solutions by considering the above four modes of sequestration along with natural processes depending on the geographical, social and economic conditions of the locale to buy some time for long-term competent solutions. Further, mitigative measures including phasing out fossil fuels by switching to low-carbon energy sources, such as renewable and nuclear energy may be combined with these efforts. One such practical measure in terrestrial sequestration also recommended in 1997 Kyoto protocol climate convention is expanding and managing natural sinks like forests to remove greater amounts of CO_2 from the atmosphere (Reddy et al., 2010).

1.5 Terrestrial carbon sequestration by plants

Photosynthetic assimilation of atmospheric CO_2 by land plants is the foundation of terrestrial carbon sequestration by plants. During photosynthesis, carbon from atmospheric CO_2 is assimilated into different components necessary for plants to live and grow. The carbon present in the atmosphere as CO_2 is stored in vegetative and reproductive structures of the plant. As part of the natural process, some of the carbon is released into the

atmosphere by respiration, but some carbon is captured within the soil and increases the soil's organic matter content (**Fig. 1.7**). Carbon sequestered is the difference between carbon gained by photosynthesis and carbon lost or released by respiration and the overall gain of carbon is usually represented by net plant productivity (Nair and Nair 2003).

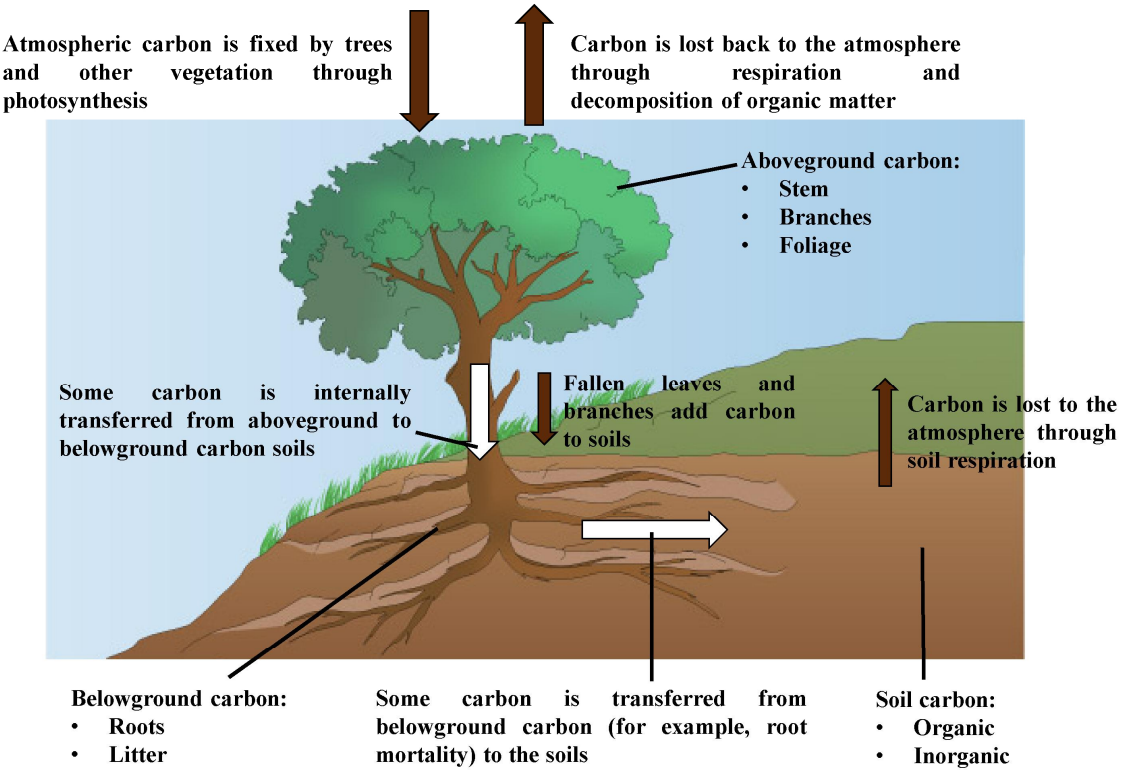


Fig. 1.7. Model depicting carbon exchange between a tree, atmosphere and soil and carbon storage sites in terrestrial CO_2 sequestration (adapted from: carbon sequestration in agriculture and forestry, US EPA, 2017).

Plants can play dual roles as carbon sinks. It can store large amounts of organic carbon assimilated through photosynthesis in above and below ground biomass which is the case for perennial trees and herbaceous plants with extensive root systems. This is a short-term sequestration strategy ranging from decades to centuries and when the plants decay, carbon is returned to the atmosphere. However, if they are well managed, plants in an ecosystem

can continue to act as a sink for several centuries. Long-term sequestration can be achieved when carbon from aboveground biomass transfers to the roots and enters the pool of soil carbon. Additionally, plants can also act as sinks by use as bioenergy crops, thereby displacing GHG emissions from fossil fuels. Long-lived plants like trees might keep the carbon sequestered for a long period of time which makes the studies on carbon sequestration potential among different tree species of greater significance. Over the lifetimes of some long-lived trees, terrestrial sequestration can be significant. For example, over the 70-year life span of a 410-acre grove of trees planted for a terrestrial sequestration project, over 70,000 tons of CO_2 will be sequestered (Jansson et al., 2010). The actual rate of carbon sequestration will vary with species, climate and site, but in general, younger and faster growing forests have higher annual sequestration rates.

Some possible methods to promote terrestrial carbon sequestration through plants include sustainable forest management, land management practices such as no-till agriculture, reforestation, and afforestation. Forest management, reforestation and afforestation involve practices which expand and enhance the growth of trees on lands that have been used for other purposes like cropland and pasture. No-till agriculture on agroecosystems would limit the release of carbon and other GHG precursors like nitrogen that are present in soil organic matter. However, decisions about terrestrial carbon sequestration require careful consideration of priorities and trade-offs among multiple resources. For example, converting farmlands to forests or wetlands may increase carbon sequestration, enhance wildlife habitat and water quality, and increase flood storage and recreational potential but the loss of farmlands will decrease crop production. Similarly, converting existing conservation lands to intensive cultivation, may diminish wildlife habitat, reduce water quality and supply, and increase CO_2 emissions. Further studies should be performed on the effects of climate and land-use change on potential carbon sequestration and ecosystem benefits, and the information about these effects should be provided for use in resource planning and development.

1.6 Elevated atmospheric CO_2 effects on plant growth and productivity

Evolutionary data suggest that plants have been experiencing the variations in atmospheric CO_2 but the rapidity of increase significantly poses a challenge on their adaptive capacity. It will be highly significant to gain knowledge on the effects of elevated atmospheric CO_2 on growth of different plant species and their capacity to adapt. Further, it is an essential initial step in understanding the full impact that the multiple interacting factors of global change (e.g. drought, temperature, ozone) will have on terrestrial ecosystems which will aid in designing strategies for terrestrial sequestration in an ecosystem. There has been lot of efforts made in predicting and measuring the scale of response that plants demonstrate in elevated CO_2 environment as evidenced by large number of reports on both short-lived crops and long-lived tree species. The first experiment on elevated CO_2 effects on plant was performed on pea which demonstrated better growth than control plants in ambient air (de Saussure, 1890). Since then the research on responses of plants to elevated CO_2 has come a long way with advancements on the scientific technologies employed to perform the studies. These include from greenhouses, closed growth chambers and sunlit controlled-environment chambers to the current technologies of eddy covariance, open top chambers (OTCs) and free-air CO_2 enrichment (FACE) (Chabbi and Loescher, 2017).

C3 photosynthesis is known to operate at less than optimal CO_2 levels. Theoretically, elevated [CO_2] should result in a stimulation of carbon uptake through photosynthesis by the C3 plants in the range of ~35 and 60%, primarily due to a decrease in photorespiration as the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation reaction is favoured in these conditions (Farquhar et al., 1980; von Caemmerer and Farquhar 1981; Long 1991). This positively connotes better growth and productivity of the plants. On the contrary, C4 plants utilize their specific leaf anatomy to bypass the photorespiratory pathway and ensuring efficient delivery of CO_2 to Rubisco. CO_2 is first converted to a four carbon compound by the enzyme PEP-carboxylase which has no affinity for oxygen in the mesophyll cells and CO_2 is released from this compound in bundle sheath cells for the normal fixation process. C4 photosynthesis can operate at low CO_2 concentrations, high

temperatures and water limited environments. Hence, theoretically if C4 plants were grown under elevated CO_2, carbon fixation would be little affected due to inherent CO_2 concentrating mechanism (Sage and Kubien, 2003). However, experimental analyses demonstrated some variations from these predictions, particularly C3 plants. Some C3 plants showed an acclimation after an initial stimulation of photosynthesis which is discussed in detail below. C4 plants, in general are insensitive to elevated CO_2 but respond positively, particularly at elevated temperature and arid conditions where they are currently common and under nutrient-limited situations as well (Ghannoum et al., 2000; Sage and Kubien, 2003). These reports on C3 and C4 plant differential responses to elevated CO_2, could be attributed to differences in experimental technologies (either OTCs or FACE), plant species used for the experiments (crops or trees), age of the plant (seedling or mature) as well as duration of the treatment (short or long).

Majority of C3 plants sense and respond to rising [CO_2] through increased photosynthesis and reduced stomatal conductance (**Fig. 1.8**). All other effects of elevated [CO_2] like growth and productivity of plants and ecosystems are derived from these two fundamental responses (Long et al., 2004).

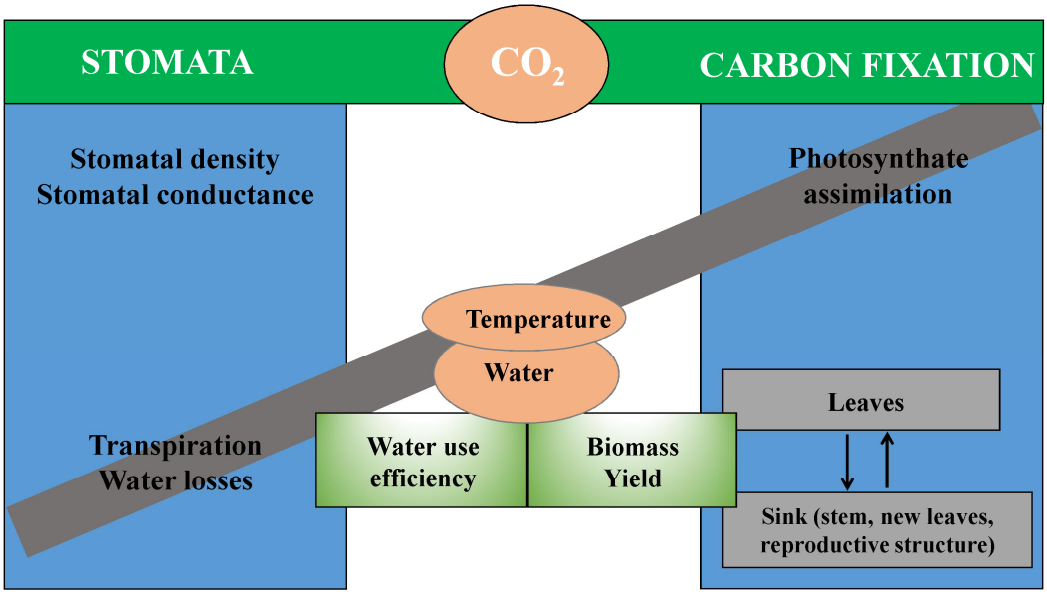


Fig. 1.8. A schematic representation of the primary responses of C3 plants involving stomatal conductance and carbon fixation under elevated CO_2 (modified from Xu et al., 2016).

Another such effect is the decrease in canopy evapotranspiration which results in decreased water use at both leaf and canopy scales at elevated [CO_2]. However, crops and tree species are diverse in their growth and behavior which is also reflected in the variability of their long-term responses to elevated CO_2. A comprehensive meta-analysis of the recent studies reporting the responses of C3 plants to elevated [CO_2] for the past seven years reveals distinct responses of crops and tree species (**Table 1.1**). These studies performed with either CO_2 as the single variable or in combination with other associated factors like temperature, nitrogen and water reflect on the growth, productivity, species diversity and stress tolerance in relation to photosynthesis. An initial stimulation of photosynthesis was recorded under elevated [CO_2] which was followed by acclimation in photosynthetic capacity with long-term growth by many crop plants (Drake et al., 1997; Long et al., 2004). The reason for this down-regulation in photosynthetic potential has been linked to limited sink strength and nitrogen status of crop plants grown at elevated [CO_2] (Fischer et al., 1997; Ray and Jarvis 1998; Heineke et al., 1999; Stitt and Krapp 1999; Isopp et al., 2000; Ainsworth et al., 2002). The increased production of carbohydrates together with reductions in total nitrogen, Rubisco protein, Rubisco activity, and/or Rubisco small subunit (rbcS) mRNA levels on a unit leaf-area basis has been correlated to this acclimatory response (Rogers et al., 1998; Moore et al., 1999; Rogers and Ellsworth 2002; Ainsworth and Long 2005). The increased levels of soluble sugars has been shown to induce feedback inhibition which in turn down-regulates photosynthetic gene transcription mediated via hexose-cycling in elevated CO_2 environment (Moore et al., 1998; Long et al., 2004). However, the extent and nature of acclimatory response depends on the species and growth conditions (Ainsworth et al., 2003; Herrick and Thomas 2001). Trees, on the other hand, lock up large amounts of carbon in their wood owing to their larger sink capacity. The earlier works have reported sustained enhanced photosynthesis for many years irrespective of the nitrogen status (**Table 1.1**).

One can construe from the studies on C3 plants responses to elevated CO_2 that carbon uptake is enhanced by elevated [CO_2] despite acclimation of photosynthetic capacity. However, the degree of stimulation of photosynthesis varies among species and experimental conditions. A deeper insight into the physiological mechanisms behind the

Table 1.1. A concise review of differential responses of growth, photosynthesis and productivity in plants to elevated CO_2 including some variables like nitrogen, temperature and moisture as reported in earlier works during the last 7 years.

Plant	Treatment	Type and duration of treatment	CO_2 concentration used in study	Response observed	Reference
Populus tremuloides	CO_2 and moisture	Natural increase in CO_2 across 45 years	[CO_2] across a period of 45 years from 1958-2003	Enhanced net primary productivity; CO_2 induced growth is enhanced during periods of high moisture availability	Cole et al., 2010
13 perrennial grassland species	CO_2 and Nitrogen	11 years	Ambient [CO_2] +180 ppm	Elevated CO_2 induced photosynthetic enhancement of 10% on average compared with ambient CO_2 across the 13 species and did not depend on soil N treatment.	Lee et al., 2011
Pinus taeda	CO_2	10 years	Ambient [CO_2] +200 ppm	Increased mineralization of nitrogen in the organic and 0-15 cm mineral horizon and deeper rooting are sustaining the elevated CO_2 enhanced plant productivity.	Hofmockel et al., 2011
Mahihot esculenta	CO_2	5 months	585 ppm	High photosynthetic rates and photosynthetic stimulation by elevated [CO_2], larger canopies, and large sink capacity all contributed to cassava's growth and yield stimulation.	Rosenthal et al., 2012
South African savannahs	CO_2	>50 years	[CO_2] across a period of 50 years from 1954-2004	Increase in wood biomass and density indicating higher sink capacity in savannah grassland in response to increasing CO_2 over >50 years.	Buitenwerf et al., 2012
Eucalyptus saligna	CO_2	2 years	Ambient [CO_2] +240 ppm	During high vapour pressure deficit (VPD), elevated [CO_2] had large effect on photosynthesis and a small effect on transpiration rate while vice-versa at small VPD.	Barton et al., 2012

Table 1.1 contd.

Plant	Treatment	Type and duration of treatment	CO_2 concentration used in study	Response observed	Reference
Pinus taeda and *Liquidambar styraciflua*	CO_2	11 years	Ambient [CO_2] +200 ppm	Sustained net photosynthetic enhancement of both species under elevated [CO_2]; elevated CO_2 has the potential to enhance the mixed-species composition of planted pine-stands.	Ellsworth et al., 2012
9 perennial alpine glacier forefield species	CO_2	3 years	580 ppm	None of the species were stimulated by elevated CO_2, irrespective of mineral nutrient addition; not carbon limited at current CO_2 concentrations	Inauen et al., 2012
Tallgrass prairie species	CO_2	4 years	500 ppm	CO_2 enrichment increased community average net primary productivity on three different soil types irrespective of species change.	Polley et al., 2012
Rice	CO_2 and temperature	6 months	760 ppm	Heat tolerance behaviour in hybrids grown under elevated [CO_2] can ameliorate the negative impacts of high temperature on seed setting and yields of rice.	Madan et al., 2012
Wheat	CO_2	3 months	700 ppm	Elevated [CO_2] only increased plant growth and photosynthesis in the genotype with the largest harvest index.	Aranjuelo et al., 2013
Rice	CO_2 and nitrogen	2 years	560-580 ppm	Future [CO_2] levels probably stimulate the grain growth of inferior spikelets which are limited in size at the expense of grain nitrogen reduction in superior spikelet.	Zhang et al., 2013

Table 1.1 contd.

Plant	Treatment	Type and duration of treatment	CO_2 concentration used in study	Response observed	Reference
Eucalyptus globulus	CO_2 and temperature	15 months	Ambient [CO_2] +240 ppm	Upper limit for photosynthetic capacity under elevated [CO_2] of *E. globulus* outside its native range to acclimate to growth temperatures >25°C.	Crous et al., 2013
Alnus glutinosa, Betula pendula and *Fagus sylvatica*	CO_2 and species diversity	4 years	580 ppm	Fine root biomass and morphology responded differentially to the elevated CO_2 when grown in monoculture; positively affected by elevated CO_2 but not by species diversity in polyculture.	Smith et al., 2013
Six tree species of subtropical forest ecosystem	CO_2 and nitrogen	4 years	700 ppm	Elevated CO_2 and N addition could facilitate tree species to mitigate phosphorous limitation by more influencing P dynamics than N in subtropical forests.	Liu et al., 2013
Schoenoplectus americanus Spartina patens	CO_2 and nitrogen	1 year	Ambient [CO_2] +300 ppm	Nitrogen addition enhanced plant growth and the growth effect was larger in combination with elevated CO_2.	Langley et al., 2013
Maize	CO_2	7 years	550-585 ppm	Elevated [CO_2] decreased evapotranspiration (ET); transpiration contributed less to total ET for maize indicating smaller role of stomata in dictating ET response to elevated [CO_2].	Hussain et al., 2013
Phragmites australis	CO_2, temperature and soil salinity	70 days	700 ppm	Negative effects of salinity were less severe at elevated CO_2 and temperature than at the ambient climactic conditions.	Eller et al., 2014

Table 1.1 contd.

Plant	Treatment	Type and duration of treatment	CO_2 concentration used in study	Response observed	Reference
Mojave desert winter annual community	CO_2	10 years	550 ppm	Elevated [CO_2] does not increase productivity of annuals in most years	Smith et al., 2014
Subtropical tree species	CO_2 and nitrogen	5 years	700 ppm	Differential responses of net primary productivity among different tree species to elevated [CO_2] and nitrogen addition will affect the species composition of subtropical forests.	Yan et al., 2014
Arabidopsis thaliana	CO_2, heat wave and drought	45 days	730 ppm	Elevated CO_2 significantly mitigated the negative impact of a combined heat and drought on plant growth and productivity.	Zinta et al., 2014
Triticum durum	CO_2	90 days	700 ppm	Grain biomass produced at elevated CO_2 was larger and less N rich, suggesting that nitrogen use efficiency rather than photosynthesis is an important target for improvement, even in good CO_2-responsive wheat cultivars.	Aranjuelo et al., 2015
Pinus taeda	CO_2	14 years	Ambient [CO_2] +200 ppm	CO_2 enrichment resulted in the development of a fine-root pool that was less dichotomous and more exploratory under nitrogen limited conditions.	Beidler et al., 2015
Wheat	CO_2	3 years	550 ppm	Elevated CO_2 induced superior performance in terms of biomass and yield and efficient water use in semi-arid environment.	O'Leary et al., 2015

Table 1.1 contd.

Plant	Treatment	Type and duration of treatment	CO_2 concentration used in study	Response observed	Reference
Wheat and Rice	CO_2 and temperature	Two growing seasons across 2 years	500 ppm	Increase in CO_2 was unable to compensate for the negative impacts of increase in temperature on biomass and yield of wheat and rice.	Cai et al., 2016
Tidal marsh (both C_3 and C_4 grasses)	CO_2 and nitrogen	9 years	Ambient [CO_2] +340 ppm	Elevated CO_2 is unlikely to increase long-term nitrogen (N) accumulation and circumvent progressive N limitation without additional N inputs in N-limited ecosystems.	Pastore et al., 2016
Coffee	CO_2	2 years	550 ppm	Upregulation of net photosynthetic rate by elevated [CO_2] took place with no signs of photosynthetic downregulation, even during the period of low sink demand.	DaMatta et al., 2016
Soybean	CO_2 and temperature	2 years	600 ppm	Overexpression of cyanobacterial, bifunctional fructose-1,6/sedoheptulose-1,7-bisphosphatase of the photosynthetic carbon reduction cycle led to increased yields under both elevated [CO_2] and temperature.	Köhler et al., 2017
Sunflower	CO_2	42 days	1000 ppm	Reduction of carbon use efficiency at high CO_2; reduced inhibition of leaf respiration by light and a diminished leaf mass ratio.	Gong et al., 2017

acclimation of photosynthetic capacity reveals decreased maximum carboxylation rate of Rubisco (V_{cmax}) and maximum electron transport rate leading to ribulose-1,5-bisphosphate (RuBP) regeneration (J_{max}) (Nowak et al., 2004; Ainsworth and Rogers, 2007; Leakey et al., 2009a). These two parameters, V_{cmax} and J_{max} are derived from a response curve of net CO_2 assimilation rate (A) vs intercellular concentration of CO_2 (C_i) in substomatal spaces [also known as A/C_i curve] which predicts the biochemical limitations imposed on photosynthesis (Farquhar et al., 1980). In species and functional groups (i.e. groups of plants that share functional characteristics with or without phylogenetic relatedness such as C4 or legumes) with Rubisco-limited photosynthetic capacity at elevated [CO_2], there is a larger potential for the stimulation of A because elevated [CO_2] both increases Rubisco carboxylation rates and decreases rates of photorespiration. Thus, trees and grasses, which were limited by Rubisco capacity at elevated [CO_2], showed greater stimulation of A compared to legumes, shrubs, and non-leguminous C3 crops that were limited by RuBP regeneration capacity at elevated [CO_2] in which increase in A was due to inhibition of photorespiration (Ainsworth and Rogers, 2007). However, the stimulation of A is affected by environmental, genetic factors and management practices (Rogers and Ainsworth, 2006; Lüscher et al., 2006; Ainsworth and Rogers, 2007).

Another critical aspect in plant's responses to elevated [CO_2] is the coupled cycling of carbon (C) and nitrogen (N) as highlighted in many studies involving both crop plants and tree species (**Table 1.1**). Crop plants have demonstrated foliar N content limiting the CO_2 fertilization effect which has been attributed to a concept proposed as progressive N limitation (Luo et al., 2004). Progressive N limitation refers to limitation of available soil N in CO_2-enriched ecosystems during allocation of C and N in long-lived biomass or soil organic matter pools. This limits the plant growth and productivity response under elevated [CO_2] as corresponding increased demand for N will magnify any existing N limitation within the ecosystem. However, some crop plants demonstrated sustained increased photosynthesis even at low N turnover at foliar levels indicating a physiological adjustment conducive with a higher photosynthetic nitrogen-use efficiency (PNUE), i.e. an increase in the rate of carbon assimilation per unit nitrogen in the foliage (Davey et al., 1999; Leakey et al., 2009a). Trees also have shown variability in responses to N content in

relation to growth and photosynthesis under elevated CO_2 which depends on the geographical location, soil N availability, tree age and growth season (**Table 1.1**).

1.7 Aspects of long-term C storage in trees and forest ecosystem

Natural forests have been implicated to store large amounts of C in their biomass in the wake of rising global CO_2 levels. However, differences in climate regimes, nutrient cycling and biological complexity of tree species at the different forests ecosystem worldwide will likely impact the carbon storage capability in these ecosystems under increasing [CO_2]. Most of the studies on tree responses to elevated CO_2 have reported photosynthetic enhancement with a few studies reporting photosynthetic acclimation which was observed at the end of the growing season, when sink strength is reduced and many environmental factors become more limiting (Medlyn et al., 1999; Körner, 2003). This downregulation reduces the positive response of photosynthesis to elevated [CO_2] but rarely completely eliminates it. Hence, photosynthetic enrichment in trees is likely to continue for increasing [CO_2] which can be partially explained by source-sink relationships. It will be of significant importance to understand, on the physiological level, the resource allocation within the trees in correlation with nutrient availability which sustains enhanced photosynthesis in tree species. Further, the regulation of photosynthetic rate and capacity, V_{cmax} and J_{max}, by source–sink relationships suggests that photosynthetic downregulation may be more prevalent in trees whose sink strength are limited annually or seasonally by environmental factors (Jach and Ceulemans, 2000; Tissue et al., 2001; Rogers and Ellsworth, 2002). In fact, the mechanistic relationship of key physiological processes of photosynthesis and respiration with nutrient availability affects carbon allocation and growth under elevated CO_2 (**Fig. 1.9**). The fine balance in source-sink relationship while also taking into account litter, soil biota and soil organic matter will likely result in increased net primary productivity in tree species with efficient C turnover and storage under elevated [CO_2]. These findings need to be extended to the ecosystem-scale to enhance the understanding of potential C storage feedbacks responses to elevated CO_2 and nutrient manipulation.

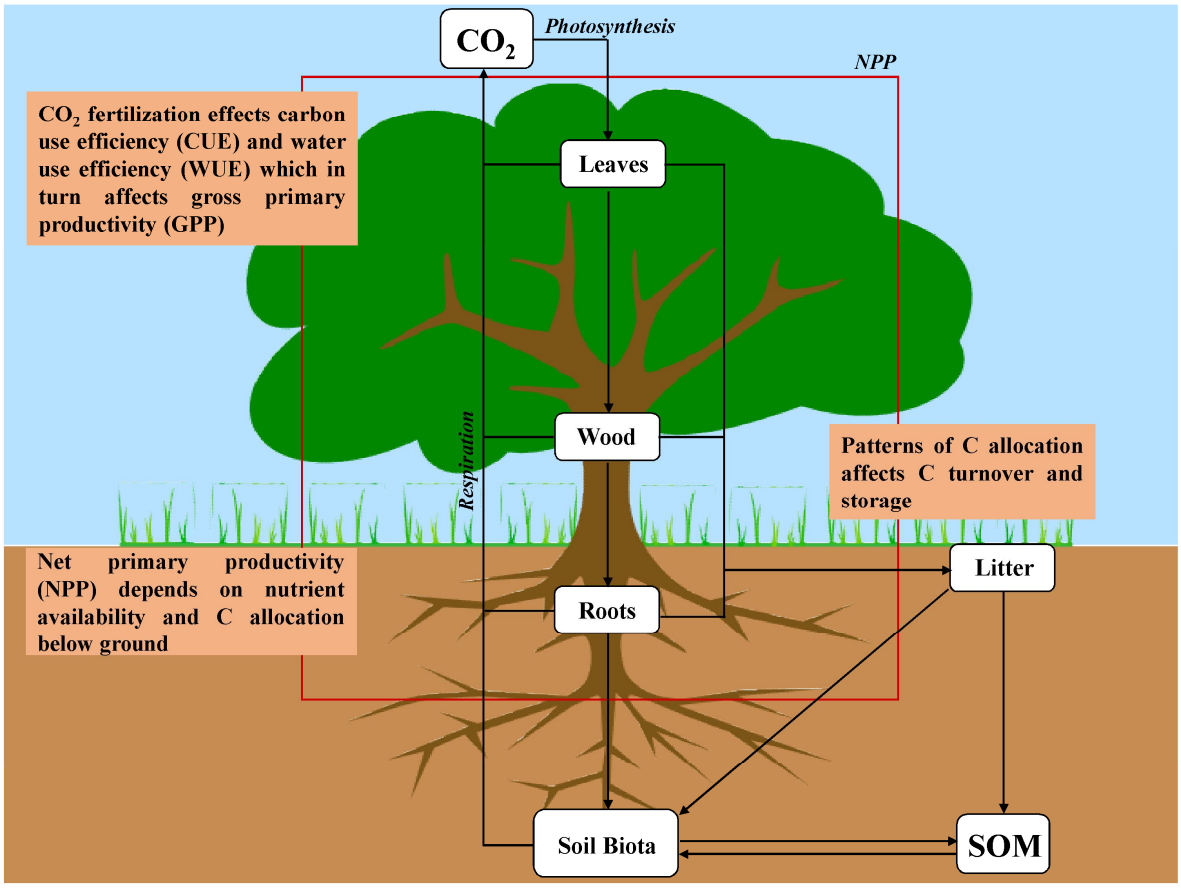


Fig. 1.9. Mechanistic model depicting major carbon pools and fluxes in tree species in a forest ecosystem under elevated CO_2 (adapted from Hofhansl et al., 2016).

A different conundrum is posed by closed-canopy forests where leaf production is constrained, which may limit the continued development of carbon sinks in elevated [CO_2] (Körner et al., 2005; Warren et al., 2015). A management strategy that has been in practice is the identification and plantation of fast growing short rotation woody crops planted as purpose-grown wood on forest sites that enable high productivity and proximity to the processing plant. Short-rotation, purpose-grown trees have a variety of inherent logistical benefits and economic advantages relative to other lignocellulosic energy crops. While many hardwood species have full management rotations that may approach 50 years or more in length, short rotation woody crops can be harvested every two to four years. Many

of the advantages are driven by the fact that trees can typically be harvested year-round and continue growing year after year providing a "living inventory" of available biomass (Hinchee et al., 2009). Short rotation trees can be utilized for the production of liquid fuels, such as ethanol, diesel, and jet fuels. Identification and management of fast growing coppice systems producing repeated biomass could be one of the ways that would remove the CO_2 from the atmosphere at least over a period of decades, potentially slowing the rate of rise in atmospheric [CO_2] and buying time for longer-term solutions. Further, the rapid growth of these trees also allows the analysis of the response to elevated [CO_2] over the complete production cycle, from planting through canopy closure and harvest. However, knowledge about the short rotation plantations tree species under elevated [CO_2] is very limited with only one plant, poplar exclusively studied (Liberloo et al., 2010). Fast-growing Populus trees (poplars) grown for coppice exposed to elevated [CO_2] in the PopFACE experiment sustained a 55% stimulation in *A* at elevated [CO_2] (Bernacchi et al., 2003; Scarascia-Mugnozza et al., 2006). Poplars exported >90% of their photosynthate during the day and stored the rest of the overflow photosynthate as starch (Davey et al., 2006), which enabled the trees to avoid acclimation of photosynthetic potential, and maintain maximal stimulation of *A* at elevated [CO_2]. Hence, it is imperative to identify and design strategies to manage such fast growing short rotation coppice plants to ensure efficient mitigation of CO_2 in different geographical realms. Moreover, an integrated understanding of long-term, leaf-level, whole plant physiological responses and functional control of key traits facilitating carbon sequestration will aid in achieving better productivity in these plants under future high [CO_2] climactic conditions.

1.8 *Jatropha curcas*: a non-edible oleaginous bioenergy plant – our experimental model plant

Increase in atmospheric carbon dioxide and rapid depletion of fossil fuels are components of a double-edged sword. To meet energy demands, deforestation for agriculture has led to reduction in the green cover. Depletion of limited fossil fuel reserves has emphasized the importance to tap into alternative and renewable sources of energy. One such source is the

oil derived from bio-fuel plants. Many tree-borne oilseed species including *Jatropha curcas*, *Pongamia pinnata*, *Madhuca indica*, *Calophyllum inophyllum* and *Simarouba glauca* are being investigated for the production of biodiesel, which can replace traditional diesel obtained from crude oil. The large-scale cultivation of such plants also increases the green cover, thereby earning carbon credits for the propagator. Focus on renewable sources of energy will allow mitigation of excess atmospheric CO_2 and lessen the strain on fossil fuel reserves. Thus, managing carbon sinks to offset the potential increasing atmospheric CO_2 concentrations has become crucial. Current interest in biofuel cropping is the potential environmental benefit through the reduction in greenhouse gas emissions in the atmosphere. Bioenergy cropping has the potential to benefit biodiversity by mitigating against climate change both by sequestering CO_2 from the atmosphere and producing eco-friendly biofuels. Oil – bearing tree species may act as potential sinks for mitigation of increasing atmospheric CO_2. Since the ratification of the Kyoto protocol, a significant effort has been made world-wide to boost biofuels with the expectation of a positive contribution to renewable fuel and greenhouse gas reduction. The utilization of energy crops as a source of renewable fuels is of great relevance to current ecological and economic issues at both national and global scales.

Jatropha curcas is a multipurpose, drought tolerant, semi-evergreen, perennial small tree species belonging to family *Euphorbiaceae* which has gained worldwide attention for its potential biofuel application. It was indigenous to Mexico and Central America (Sujatha et al., 2008); however, it has now been domesticated around the world. It is a tropical plant that can be grown in low to high rainfall areas either in the farms as a commercial crop or on the boundaries as a hedge to protect fields from grazing animals and to prevent erosion. *Jatropha* is easy to establish, grows relatively quickly and is hardy, being drought tolerant. It can reach a height of 3-5 meters, but under favourable conditions it can attain a height of 8 or 10 m. The plant shows articulated growth, with a morphological discontinuity at each increment. Flowering occurs during the wet season with the inflorescence being axillary paniculate polychasial cymes. The plant is monoecious and flowers are unisexual. The seeds mature about 3‑4 months after flowering. The seeds are black and the seed weight per 1000 is about 727 g, there are 1375 seeds/kg in the average (Kumar and Sharma,

2008). *J. curcas* fruits house two or three seeds that contain up to 40% oil and up to 35% protein, and the rest is mainly water and ash (Gübitz et al., 1999; de Oliveira et al., 2009). The seed oil can be processed to produce a high-quality biodiesel fuel, usable in a standard diesel engine (Achten et al., 2008). Apart from its use as biodiesel, the oil can also be used for soap production. Other potential benefits of *Jatropha* include efficacy of various parts of the plant (seeds, leaves and bark) as medicinal value and seed cake's potential use as a manure and fertilizer. It is also known for preventing soil erosion and improves the soil fertility in addition to sequestering carbon from the atmosphere and enhances the water holding capacity of the soil (Wani et al., 2012). Thus, it would be highly significant to understand physiological, biochemical and molecular aspects in relation to carbon gain and biomass productivity in this multifaceted plant under elevated [CO_2].

1.9 Tree Oils India Limited – source of *Jatropha curcas* germplasm

Tree Oils India limited (TOIL) is a non-government organisation in the energy sector with an objective to build an environment friendly and sustainable energy system based on plant sources and contribute to waste land utilisation. An experimental farm comprising *Jatropha*, *Pongamia*, *Madhuca* and *Simarouba* plantations are efficiently maintained at Zaheerabad, Medak district, Telangana, India (latitude 17°36′ N; longitude 77°31′ E; 622 m MSL) to fulfil the above objective. Diverse germplasm of the above plants collected from different parts of India and other countries are grown and sustained. The selection of genotype of *J. curcas* for the experimental analyses pertaining to elevated [CO_2] was carried out by a preliminary screening on the basis of morphological and productivity performance for two years (2010 and 2011). These plants were maintained with optimum fertilizer treatment (recommended NPK dose), regular watering during summer months and no watering during rainy season. The different parameters considered were plant height, collar diameter, number of secondary and tertiary branches, leaf characters, and flower, fruit and seed characteristics. Based on the superior yield performance in field conditions, Chhattisgarh variety was selected for the experimental analyses in the present

Table 1.2. Screening of seven different genotypes of field grown *Jatropha* based on morphological and yield parameters (2010 and 2011) in the farms of Tree Oils India Limited. Data is represented as mean ± SD.

	TOIL - J001 Chhattisgarh	TOIL - J002 Chittoor	TOIL - J003 Neemuch	TOIL - J005 Chintapally	TOIL - J007 Nasik	TOIL - J008 Churu	TOIL - J009 Shillong
Plant Height (in m)	3.4±0.5	2.9±0.3	2.9±0.3	2.4±0.2	2.8±0.4	2.8±0.2	3.3±0.3
Collar Height (in cm)	11±1.26	8±0.78	11±2.14	6±0.85	7±0.74	10±2.02	11±1.79
Collar Diameter (in cm)	27.045±3.26	24.5±3.67	19.72±2.83	20.68±2.54	19.09±3.12	25.45±3.78	19.09±2.36
No. of Secondary Branches	21.2±2.38	19.4±2.15	21.3±3.25	23.2±2.46	21.5±2.37	21.8±1.62	20.1±2.32
No. of Tertiary Branches	56.2±4.74	46.4±5.37	52.5±4.47	57.4±5.36	53.6±5.48	59.2±6.48	55.2±4.54
Leaf Length (in cm)	12.7±1.28	10.2±0.48	12.3±1.12	12.3±0.80	10.9±0.68	12.3±1.04	12.4±1.14
Leaf Width (in cm)	12.6±1.6	9.8±1.2	12.4±0.92	11.2±0.84	10.3±1.02	12.2±1.3	12.4±1.18

Table 1.2 contd.

	TOIL - J001 Chhattisgarh	TOIL - J002 Chittoor	TOIL - J003 Neemuch	TOIL - J005 Chintapally	TOIL - J007 Nasik	TOIL - J008 Churu	TOIL - J009 Shillong
Fruits per Cluster	7.1±1.25	4.2±0.27	3.2±0.18	5.1±0.48	2±0.06	3±0.05	4.1±0.58
Fruit Length (in cm)	2.9±0.1	2.5±0.2	2.7±0.2	2.7±0.2	2.6±0.1	2.6±0.2	2.6±0.3
Fruit Width (in cm)	2.7±0.05	2.4±0.06	2.5±0.1	2.4±0.03	2.6±0.06	2.7±0.02	2.3±0.04
Seed Viability per Fruit	3	2	2	2	2	1	2
Total No. of Flowers per Apex (Male:Female flower)	82.2±9.4 (20:4)	56.4±4.8 (20:2)	37.3±3.8 (19:1)	36.8±4.6 (20:2)	44.8±5.3 (19:1)	52.8±3.4 (20:1)	75.3±7.2 (20:3)
Total seed Yield per Tree (in g)	3500.5±300.2	800.4±80.3	756.2±73.6	532.5±84.4	743.8±100.3	200.4±38.2	1500.4±263.2

dissertation work (**Fig. 1.10, Table 1.2).**

Fig. 1.10. *Jatropha curcas* plantations maintained at the experimental farms of TOIL, Zaheerabad, Medak district, Telangana State, India with a spacing of 2×2 m^2.

1.10 Framed objectives

In this PhD dissertation, whole plant morphology and photosynthetic responses in field-grown *Jatropha*, grown under elevated CO_2, both short-term and long-term, were critically investigated. The following research objectives were addressed in the thesis:

1. Investigating growth, biomass and seed yield in relation to photosynthesis and source-sink relationship in *J. curcas* grown under short-term elevated [CO_2] for successive production cycles.

2. Insights into the expression analysis of regulatory genes associated with photosynthesis and carbohydrate metabolism in leaves of *J. curcas* grown under elevated [CO_2] for one year using transcriptome sequencing and assembly.

3. Characterizing growth and photosynthetic capacity in relation to *in vivo* biochemical capacity and nitrogen content in *J. curcas*, grown under long-term elevated [CO_2] for a four year period.

4. Expression analysis of certain key regulatory genes associated with carbon sequestration and their *in vitro* enzyme activity in *J. curcas* grown under long-term elevated [CO_2].

Chapter II

Investigating growth, biomass and seed yield in relation to photosynthesis and source-sink relationship in *Jatropha curcas* grown under elevated [CO_2] for successive production cycles

Chapter 2

2.1 Introduction

An unprecedented rise in the global atmospheric concentration of carbon dioxide ([CO_2]), from ~280 µmol mol^{-1} during the pre-industrial era to the current levels of ~400 µmol mol^{-1} (NOAA-ESRL, www.CO_2now.org) is one of the major issue of global significance relevant to climate change. The global atmospheric CO_2 concentration is projected to reach ~550 µmol mol^{-1} by 2050 (Prentice et al. 2001), which may be expected to have significant impact on managed and natural ecosystems. The Kyoto protocol in 1997 aimed for the containment of emission of greenhouse gases (GHGs) and also implementation of stabilization of atmospheric CO_2 by increasing the sinks for its absorption (UNFCCC 2008). Evolutionary data suggests that plants have been experiencing the changes in [CO_2] (Post et al. 1990), but the current pace will raise many questions on the adaptive photosynthetic capacity of plants in the near future as the global scenario of [CO_2] is exceeding the highest IPCC emissions (IPCC, 2007). Understanding plant's behaviour and their capacity to adapt to rapidly changing [CO_2] will be an initiative in understanding the full impact of multitude of interacting factors of global climate change to the terrestrial ecosystem.

Many studies have shown plant responses to elevated CO_2 in the past decade which were performed either in enclosures (green houses, open top chambers) or natural microclimate (FACE: Free Air CO_2 Enrichment). Two fundamental responses are quite common including increased photosynthetic rate (P_n) and decreased stomatal conductance (g_s) followed by stimulation of growth (Tissue et al. 1997; Long et al. 2004; Körner 2006; Leaky et al. 2009a). All other responses to elevated CO_2 by plants were derived from these two responses (Ainsworth and Long 2005). The increased stimulation of photosynthesis has been hypothesized to preferred carboxylation reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the presence of elevated [CO_2] with subsequent reduction in photorespiration and increase in carbon fixation by 35-60% in the absence of any photosynthetic downregulation (Farquhar et al. 1980). Some plants have shown significant increment in photosynthesis and biomass gain under elevated CO_2 (Gunderson et al. 2002; Norby et al. 2005). However, CO_2 enrichment experiments do not always support the theoretical predictions of stimulation of photosynthesis as shown in some studies that in long term, the initial stimulation of carbon fixation declined following an acclimatory downregulation of photosynthetic capacity (Nowak et al. 2004; Ainsworth and Long 2005; Norby et al. 2010). The acclimation of photosynthetic capacity can be due to number of factors including limited sink capacity, end-product limitation, decreased Rubisco content and activation state caused by decreased N content as well as genetic factors (Luo et al. 2004; Ainsworth and Long 2005; Ainsworth and Rogers 2007; Leaky et al. 2009a). Therefore, identifying and predicting mechanisms to understand growth and productivity of plants in future high-CO_2 conditions remain difficult with such variable responses reported in the literature.

Plant reproduction and development are also affected by elevated CO_2. Previous studies have reported variable responses in reproductive events and development of some plant species to CO_2 enrichment (Ward and Strain 1997; Ward et al., 2000). An exhaustive study conducted by Jablonski et al. (2002) revealed that CO_2 enrichment in plants across many species resulted in 19% more flowers, 18% more fruits, 16% more seeds, 4% greater individual seed mass, 25% greater total seed mass (equivalent to yield), and 31% greater total mass. Recent studies also approve this observation that elevated CO_2 increases

reproductive potential and hence, fertilization and viable seed formation (Bunce 2005; Ladeau and Clark 2006a, b; Darbah et al. 2008). Flowering time is an important phenological trait which affects reproductive success and survival of plants in the process of natural selection (Schemske 1977). Elevated CO_2-induced variations in flowering time will have consequences at various levels of ecological organization (Springer and Ward 2007). However, there is no clear consensus on the responses of elevated CO_2 on flowering time. The earlier studies documented on certain annual and perennial plant species (both intraspecific as well as interspecific) described three possible events: early flowering, late flowering and no change in flowering time (Nakamoto et al. 2004; Cleland et al. 2006; Heinemann et al. 2006; Rogers et al. 2006). These elevated CO_2-induced differential responses in flowering time among plant species possibly reflect strategies to harness optimum potential for sustainable growth and development in the changing climatic conditions. The flowering time can be promoted in plants as a physiological adaptation to avoid stresses such as nutrient deficiency, drought, heat or cold which ensures maximum utilization of natural resources to sustain floral development and seed set while delayed flowering time allows extended vegetative growth in plants to acquire and allocate more resources for reproductive success (Roux et al. 2006). It is thus necessary to understand the mechanisms underlying the growth and reproductive development responses of certain economically important plant species grown under elevated CO_2 for predicting the diversity of evolutionary trajectories and their impact on terrestrial ecosystem in future CO_2 enriched environmental regimes.

The ever growing energy demand of humans triggered burning of fossil fuels and deforestation resulting huge emission of GHGs and reduction in the green cover of the planet. Depletion of limited fossil fuel reserves has triggered the importance to tap into alternative and renewable sources of energy. Accordingly, plants that produce non–edible oil rich seeds namely *Jatropha curcas*, *Pongamia pinnata*, *Madhuca indica*, *Calophyllum inophyllum*, *Simarouba glauca* and *Camelina sativa* are being targeted to produce bio – diesel, which can be blended with diesel obtained from crude oil. The large-scale cultivation of such plants also increases the green cover, thereby earning carbon credits for the propagator which will also allow mitigation of excess atmospheric CO_2 and lessen the

strain on fossil fuel reserves (IPCC 2011). *Jatropha curcas* L. (Family: Euphorbiaceae), a semi-evergreen perennial shrub or small tree native to Central America, is being cultivated in the tropics and subtropics as a biofuel crop as its seed oil is highly suitable for production of biodiesel (Jules and Paull 2008; Jain and Sharma 2010). Thus, it is imperative to study the response of this multifaceted plant to elevated CO_2 and monitor the growth and yield in response to environmental changes.

In the present study, we examined the responses of *Jatropha*, grown during successive production cycles under elevated CO_2 conditions. The main objectives were to determine (1) whether CO_2 enrichment was able to stimulate growth and photosynthesis in *J. curcas* grown in field conditions; (2) variation in flowering and yield and (3) to understand source-sink relationships in *J. curcas* under elevated CO_2.

2.2 Materials and Methods

2.2.1 Plant material, growth conditions, study site and experimental design

The present work was conducted at University of Hyderabad (UH) (17.3°10′N and 78°23′E at an altitude of 542.6 m above MSL), located 20 km north of Hyderabad metropolitan in the Telangana region of Andhra Pradesh, India. The regional climate is hot-steppe (Köppen climate classification) characterized by distinct hot dry summer lasting from March until June followed by south-west monsoonal rain during July-September and winter during November-January with an occasion temperature dip to ~10°C. The daily variations in local climate were recorded at the experimental site by an automatic data logger **(Fig. 2.1)**.

Four octagonal shaped Open Top Chambers (OTCs) of 4×4 meter dimensions were constructed in the experimental fields of UH (**Fig. 2.2A**). Two OTCs were assigned for CO_2 enrichment studies in which the mean CO_2 concentration was maintained at ~550 μmol mol^{-1} and two for ambient CO_2 in which mean CO_2 concentration was ~380 μmol mol^{-1} and ranged from 360-390 μmol mol^{-1} depending on weather conditions. The octagonal shape of OTCs reduces any temperature build up in chambers when compared to external environment. The OTCs were covered with double walled 5mm thick polycarbonate sheets which allows ~85% transmission of light. OTCs were provided with

sensors to record temperature and humidity, NDIR based CO_2 analyzer for measurement of CO_2 concentration in the OTC chamber, thermal mass flow meter for measurement of CO_2 injection flow rate and SCADA (Supervisory Control and data Acquisition) based OT-matic system to regulate and monitor CO_2 concentration (**Fig. 2.2B, 2.2C**).

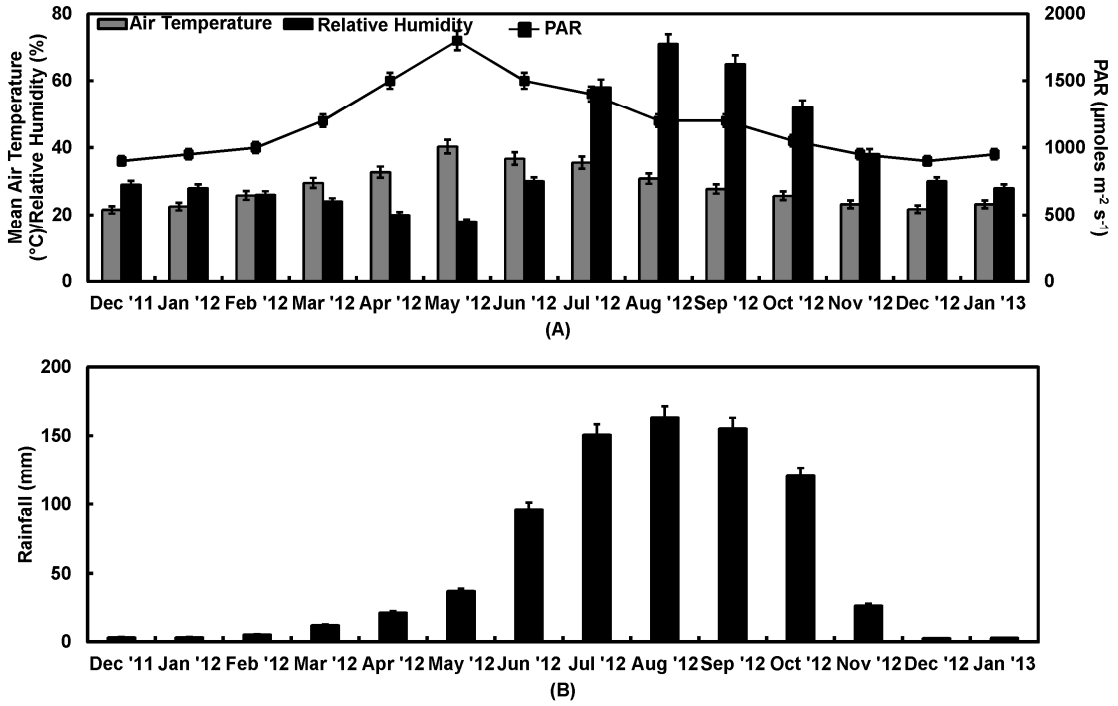
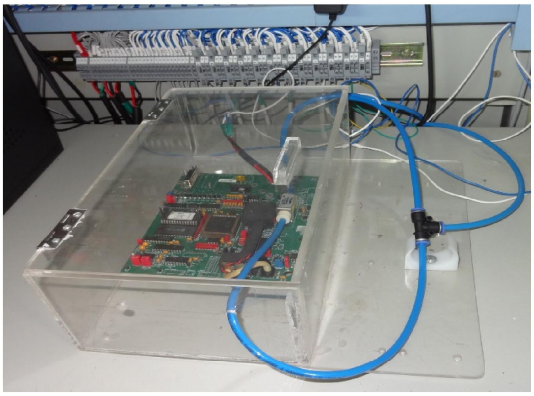


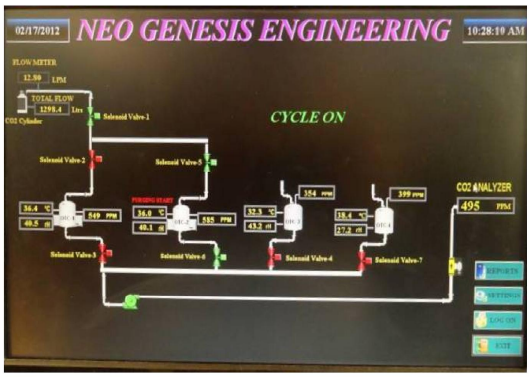
Fig. 2.1. Monthly average of (A) air temperature (°C), relative humidity (%) and mid-morning photosynthetically active radiation (PAR mol m^{-2} s^{-1}) and (B) rainfall (mm) at the study site for two experimental growth periods (I – December 2011 to June 2012; II - July 2012 to January 2013).

The OTCs were connected independently to the OT-MATIC system by underground Cu piping through which CO_2 was pumped from cylinders (**Fig. 2.2D**). Pure CO_2 was injected inside OTC through choked jets at sonic speed to allow rapid mixing of CO_2 and air. An experimental plot of 4×4 m having similar edaphic features as the chambers was established in the fields of UH near to OTCs to grow *Jatropha* without any enclosure in natural conditions to compare the growth and phenology with the plants grown in ambient OTCs so as to assess any chamber effect on the growth, phenology and ontogenic development with respect to flowering time, development and maturation of fruits in plants.

(A) Open Top Chambers



(B) CO_2 Analyzer



(C) CO_2 Monitoring Facility

(D) CO_2 Source

Fig. 2.2. Figure depicting the CO_2 treatment set-up constructed and installed to analyse the elevated CO_2 responses of *J. curcas*. The set-up comprises (A) OTCs, (B) NDIR based CO_2 analyser, (C) CO_2 monitoring system and (D) CO_2 cylinders for the supply of CO_2.

Jatropha curcas shows optimal vegetative growth in all seasons except winter during which leaf shedding occurs. Flowering and seed yield can occur throughout the year depending on the environmental conditions (Gour 2006). Our study intended to investigate elevated CO_2-induced vegetative and reproductive responses in *J. curcas* during a complete production cycle and it involved two experimental study periods: growth period – I (December 2011 to June 2012), growth period – II (July 2012 to January 2013) which included two experimental harvests. Superior quality, disease free seeds of *Jatropha curcas* were obtained from Tree Oils India Ltd. (TOIL), Zaheerabad, Medak district, Andhra Pradesh, India (latitude 17º36'N; longitude 77º31'E; 622m MSL). The seeds were surface sterilised with 0.5% sodium hypochlorite and grown in polythene bags. After ten days, three *Jatropha* seedlings were transplanted in three pits with a spacing of 2 m × 2 m

in each OTC and the experimental plot. The CO_2 treatment was started after 7 days of growth of seedlings inside the OTCs. Surface irrigation was applied regularly to retain soil moisture content of 80 - 85% at the rooting zone (30 – 50 cm soil depth) in both the OTCs and outside field plot and the extent was increased to match transpiration during the hottest months (April – June) so that the plants experience optimal growth conditions. The soil pH was ~7.5 and the texture red sandy loam. Soil available nitrogen was ~100 kg ha^{-1} and organic carbon content was ~1.5%. Soil was enriched with farmyard manure added at the rate of 12 kg $plant^{-1}$ in two equal splits (at the time of plantation of seedlings and after three months of growth) during both experimental growth periods. The soil in the OTCs was surfaced with polyethylene liners to avoid absorption of CO_2 by soil. Important soil physicochemical characteristics measured during the experimental growth period in both OTCs and outside plot are listed in **Table 2.1**. After the completion of first growth period, all the plants of OTCs and field were coppiced to 1m and again the growth of plants monitored for second growth period. The harvesting of fruits was done in the last months of both the growth periods i.e. June 2012 and January 2013.

Table 2.1. Soil characteristics of the study site during experimental time span (Dec 2011-Jan 2013).

Soil characteristics (at 0 – 30 cm soil depth)	
Texture	Sandy loam
pH	7.2
Organic carbon (%)	1.5
Available N (kg ha^{-1})	100
Available P (kg ha^{-1})	10
Available K (kg ha^{-1})	115.4

2.2.2 Photosynthesis measurements

Net CO_2 fixation rate (P_n; µmol m^{-2} s^{-1}), leaf transpiration (E; mmol m^{-2} s^{-1}) and stomatal conductance (g_s; mol m^{-2} s^{-1}) were measured simultaneously accessing fully-expanded and healthy sunlit leaves of the upper canopies (4 leaves per plant at the 3rd - 4th position from shoot apex in each branch) using a portable infrared CO_2/H_2O gas analyzer (IRGA) (LCpro-32070, ADC Bioscientific Ltd. U.K.). The gas analyzer was equipped with a

detachable broad leaf chamber (LCpro-32070, U.K.) with PAR sensor (silicon based sensor, LCpro-32070) and leaf thermistor probe (ADC, M.PLC-011) attached to it. The measurements were made periodically during both growth periods between 10:00 and 11:00 h solar time at an interval of 30 days. Measurements were made after steady state condition was obtained, typically requiring around 2 min under standard conditions. The instant water use efficiency (WUE_i) was calculated using the data on photosynthesis and transpiration taken at regular intervals during the plant growth ($WUE_i = P_n/E$; mmol CO_2 mol^{-1} H_2O). The incident photosynthetically active radiation (PAR) on leaf while performing photosynthesis measurements (10:00 – 11:00 h solar time) varied depending on the season (**Fig. 2.1**).

2.2.3 Measurement of stomatal density and stomatal index

Leaf samples (n=6) of 3 × 3 mm sizes were fixed for 4 h in FAA (10% formalin: 0.5% acetic acid: 50% ethyl alcohol: 35% H2O), dehydrated in ethyl alcohol – acetone series, dried in critical point drier (EMS 850) and mounted on to copper stubs using double stick cellophane tape. The mounted samples were coated with gold in a sputter coater (FC-1100, Jeol) and observed under scanning electron microscope (EDAX, Philips XL 30 ESEM). The behavioural changes in stomata were observed using a photomicroscope system (MPS 60, Leica, Wetzlar, Germany). The samples were fixed at an equivalent growth stage of the plants (120 days after plantation), at equivalent leaf ages and at the same time of day (09:00-10:00h). Stomatal and cell counts were averaged for each sample and subsequently for each replication. Stomatal index was calculated according to Salisbury (1927). Stomatal index (%) = [Stomatal density/(Stomatal density + Epidermal cell density)] × 100

2.2.4 Measurement of leaf area and specific leaf area (SLA)

Leaf area was measured using CI-202 portable laser leaf area meter (CID Biosciences, Inc.) equipped with a laser scanner with built-in control unit. The CI-202 yields instant results for leaf area, length, width, perimeter, ratio, and shape factor measurements. Leaves were further dried in an oven at 60°C for 24hrs. The dried leaves were used for estimation of specific leaf area (ratio of leaf area to dry weight).

2.2.5 Estimation of chlorophylls and carotenoids

Chlorophyll and carotenoids were estimated according to Hiscox and Israelstam (1979). Leaf discs of 1cm diameter (surface area: ~315 mm^2) were punched from the leaves used for determining photosynthetic rates during 10:00 – 11:00 h. The discs were extracted with DMSO in hot water bath at 60°C for 1 hr. Later the tubes were cooled in the dark and optical density (O.D.) values read at 663 nm, 645 nm, 480 nm and 510 nm using a UV – Visible spectrophotometer (UV – 1800; Shimadzu Corporation). Chlorophyll and carotenoid contents were calculated according to Arnon (1949). The values were expressed as mg m^{-2}.

2.2.6 Measurement of foliar carbohydrates

Leaf discs of 1cm diameter (surface area: ~315 mm^2) were punched from the leaves used for determining photosynthetic rates during 10:00 – 11:00 h. The total carbohydrates and starch were extracted and estimated according to Hedge and Hofreiter (1962) while soluble sugars extraction and estimation followed Ebell (1969). Total carbohydrates and starch from dried leaf discs were extracted with 2.5N hydrochloric acid and 52% (v/v) perchloric acid respectively. The soluble sugars were extracted with 80% ethanol and subjected to analysis with phenol-sulphuric acid. The values were reported as μg mg^{-1} dw.

2.2.7 Plant morphology: vegetative growth, flowering time and fruit related development

The phenological development of the plants was monitored and the events were recorded for both experimental growth periods. Plant height was regularly recorded at regular interval of 30 days with the help of a ruler. Plant height growth rate (PHGR) was calculated every month: PHGR = $(H_2 – H_1) / (t_2 – t_1)$ where H_1 = height of the plant in cm at time t_1 and H_2 = height of the plant in cm at time t_2. The number of branches at the end of each growth period was recorded for each plant. During the course of growth of plants, individual branches were selected at random in all the plants from the chambers and from field (fifteen per plant) and were tagged to record the chronology of events pertaining to onset of flowering, pod formation and development and pod maturation. The inflorescence

intensity and the number and array of male and female flowers in the inflorescence were also recorded.

2.2.8 Destructive harvest, biomass yields and carbon content in the above ground biomass

At the end of first cycle of growth, the plants in both the chambers and field were coppiced. The fresh weight of both stem and leaves were recorded immediately. Thereafter, they were dried in an incubator at 70ºC for 4–5 days till constant weight was reached which was recorded as dry biomass. The plants were up-rooted at the end of second growth cycle and the soil removed from roots. Both fresh and dry biomass measurement of roots were performed as mentioned above. Carbon content in the stem was estimated by measuring the wood volume for each plant according to Newbould (1967) and specific gravity according to Negi et al. (2003). The amount of carbon dioxide sequestered in the above ground biomass was calculated as the weight of the carbon × 3.663 (IPCC 2003; FAO 2004).

2.2.9 Seed yield characteristics and oil content

Mature fruits (dark brown in colour) were harvested twice during both the production cycles (June 2012 and February 2013). The pods were sun dried and thereafter data on various seed yield parameters including number of fruits per plant (both mature and immature), number of seeds per fruit, fruit weight and seed weight were recorded. Oil content was estimated according to AOAC 16th edition (1995). Seeds were ground to make fine powder. The powder was subjected to hexane extraction by soxhlation (distillation temperature range was 55-70ºC) for 3 h in the soxhlet extractor using a heating mantle. Hexane was removed from the extracted oil using a rotary evaporator (Heidolph, Germany) at 55ºC under reduced pressure for 30 min.

2.2.10 Statistical analysis

One way analysis of variance (ANOVA) was carried out on growth, photosynthetic physiology, biochemical parameters and biomass data obtained from 6 representative trees (n=6) of field, ambient and elevated CO_2 conditions with CO_2 treatment as the independent

factor. Results were represented as mean ± SD (standard deviation) of n (n = 6-15) measurements. The significance of the differences between mean values of tested variables was determined using the Dunnett's multiple comparison analysis during both experimental growth periods. Whenever there was statistically significant difference, means were separated using least significant difference (LSD) test at the 5% level of significance. All statistical analysis was performed using Sigma Plot 11.0.

2.3 Results

2.3.1 Photosynthetic leaf gas exchange measurements

Significant variations were recorded for gas exchange characteristics of *Jatropha* grown in CO_2-enriched conditions when compared to ambient CO_2 and field conditions during both experimental growth periods (**Fig. 2.3**). The P_n of *Jatropha* under field and ambient CO_2 atmosphere at 30 DAP showed no significant difference (~18.23 and ~19.11 µmol m^{-2} s^{-1}) during growth period I and ~20.38 and ~22.37 µmol m^{-2} s^{-1} during growth period II respectively (**Fig. 2.3A and 2.3B**). However, the elevated CO_2-grown plants showed a slightly higher P_n which was recorded to be ~22.38 and ~26.72 µmol m^{-2} s^{-1} for growth period I and growth period II respectively (**Fig. 2.3A and 2.3B**). A significant increase ($p<0.001$) in P_n of plants grown in elevated CO_2 atmosphere was observed as the growth of the plant progressed during both growth periods when compared to plants grown under field and ambient CO_2 atmosphere. At 120 and 150 DAP the P_n of plants grown in elevated CO_2 atmosphere was ~54% higher for both growth periods [120 DAP – ~31.91 µmol m^{-2} s^{-1} (growth period I); ~31.39 µmol m^{-2} s^{-1} (growth period II) and 150 DAP – ~28.81 µmol m^{-2} s^{-1} (growth period I); ~28.44 µmol m^{-2} s^{-1} (growth period II)] in comparison to plants grown in both field and ambient CO_2 atmosphere (**Fig. 2.3A and 2.3B**). Significant reduction ($p<0.05$) was observed in both g_s and E of plants grown in elevated CO_2 atmosphere (**Fig. 2.3C, 2.3D, 2.3E and 2.3F**). At 30 DAP, g_s was recorded as ~0.45 mol m^{-2} s^{-1} for both growth period I and growth period II in plants grown in CO_2-enriched conditions but at 150 DAP g_s was ~0.28 mol m^{-2} s^{-1} for both growth periods showing a reduction of ~75% in comparison to plants grown in field and ambient CO_2 conditions (**Fig**

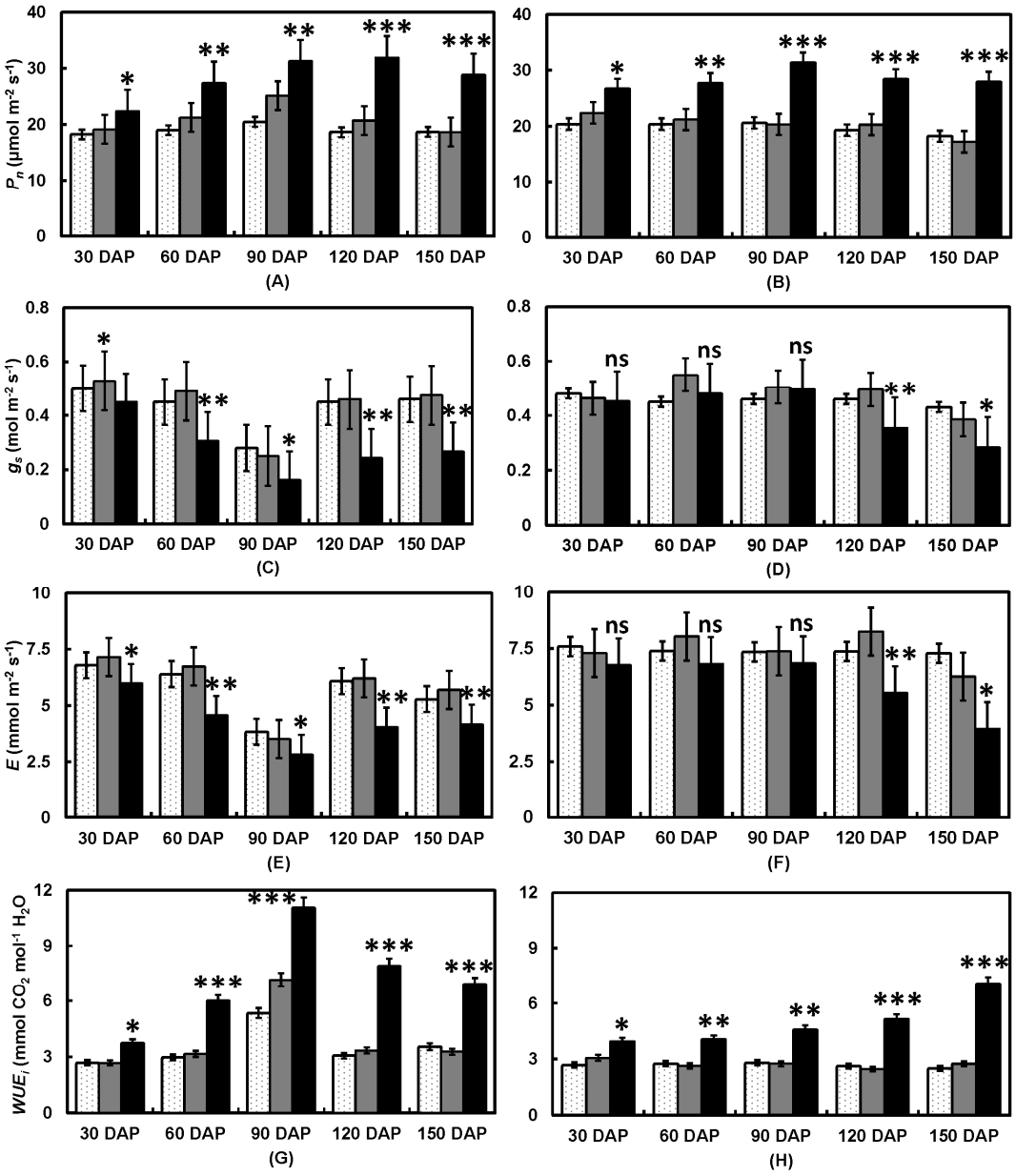


Fig. 2.3. Progressive changes in (A-B) net photosynthetic rate (P_n), (C-D) stomatal conductance to H_2O (g_s), (E-F) transpiration rate (E) and (G-H) instantaneous water use efficiency (WUE_i) in *Jatropha curcas* grown under elevated CO_2 (*black bar*), ambient CO_2 (*grey bar*) and field conditions (*dotted bar*) as recorded on different time interval (30, 60, 90, 120 and 150 DAP; DAP – Days After Plantation) in two experimental growth periods. The P_n, g_s, E and WUE_i of *Jatropha curcas* as exhibited during growth period I (A, C, E, G) and growth period II (B, D, F, H) are depicted separately. Values are mean ± SD (n=6-15). Analysis of variance (ANOVA) was performed to test significant differences among the CO_2 treatments and between the growth periods (ns – not significant, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$).

2.3C and 2.3D). A similar concomitant decrease of ~40% in *E* was observed in elevated CO_2-grown *Jatropha* plants at 150 DAP (**Fig. 2.3E and 2.3F**). Relative to field and ambient CO_2-grown plants, a significant increase of ~80% ($p<0.001$) in WUE_i was recorded in elevated CO_2-grown plants for both growth periods (**Fig. 2.3G and 2.3H**). The P_n, g_s, *E* and WUE_i values showed statistically significant difference ($p<0.01$) among the treatments (elevated vs field and elevated vs ambient).

2.3.2 Stomatal density and stomatal index

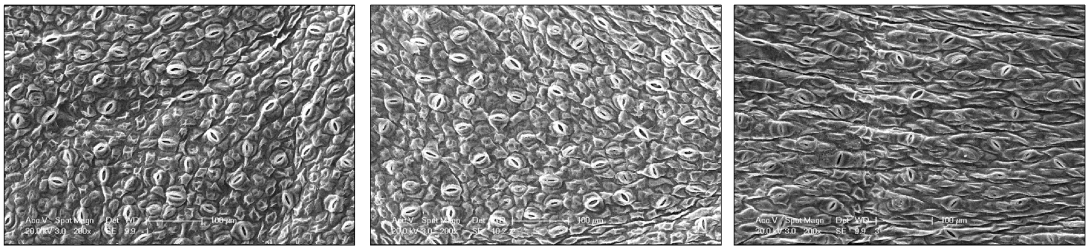
There was no significant variation observed among the stomatal density and stomatal index in *Jatropha* leaves of the plants grown in field, ambient and elevated CO_2 during both growth periods (**Table 2.2**). There was a marginal reduction of ~10-15% in stomatal density of elevated CO_2-grown plants in comparison to field and ambient CO_2 atmosphere which was not statistically significant. However, there was a significant variation ($p<0.001$) in the number of open and closed stomata in the leaves of plants grown in elevated CO_2 compared with field and ambient CO_2 for both growth periods. The number of open stomata in the leaves of elevated CO_2-grown plants was found to be significantly reduced (~60-75%) when compared to field and ambient CO_2 environment grown plants (**Table 2.2**, **Fig. 2.4**).

2.3.3 Specific leaf area

The leaf area of *Jatropha* plants grown in elevated CO_2 atmosphere was marginally high compared to those in plants grown in field and ambient CO_2 atmosphere (**Fig. 2.5A**). The leaf area of elevated CO_2 grown plants (~108 cm^2 for both growth periods) exhibited an increase of ~15% and ~12% in comparison to plants grown in field and ambient CO_2 conditions. The specific leaf area (SLA) of *Jatropha* plants grown in CO_2-enriched atmosphere consistently decreased ($p<0.05$) with the progression of plant age than in the plants grown in field and ambient CO_2 atmosphere (**Fig. 2.5B**). The SLA of plants grown in field, ambient and elevated CO_2 atmosphere at 30 DAP was measured to be ~180 $cm^2\ g^{-1}$. After 150 DAP the SLA of plants grown in elevated CO_2 atmosphere showed ~22%

Table 2.2. Variation in stomatal behavior in *Jatropha* leaves as observed under ambient and elevated CO_2 environment recorded during two growth periods. The parameters investigated were stomatal density and stomatal index. Values are the means of two growth periods (±SD). Different letters indicate that the values are significantly different at $p<0.001$.

	Stomatal density (stomata mm^{-2})	Number of fully opened stomata (mm^{-2})	Number of partially opened stomata (mm^{-2})	Number of closed stomata (mm^{-2})	Stomatal index (%)
Field	237.54±10.36[a]	150.32±8.93[a]	54.16±6.83[a]	33.34±2.32[a]	27.19±2.38[a]
Ambient	245.83±12.53[a]	139.16±9.53[a]	67.50±7.56[a]	39.16±2.63[a]	28.37±3.82[a]
Elevated	212.50±12.72[a]	87.52±9.46[b]	54.16±6.86[a]	70.83±3.83[b]	27.83±3.17[a]



(A) Field (B) Ambient (C) Elevated

Fig. 2.4. Comparative differences in the degree of stomatal opening between (A) field, (B) ambient and (C) elevated CO_2 grown *Jatropha* plants. Notably, more number of open stomata is observed in field and ambient CO_2 grown plants compared to elevated CO_2 plants. (Scale bar = 100 µm)

decrease compared with plants grown in field and ambient CO_2 conditions and was recorded to be ~140.21 cm^2 g^{-1}.

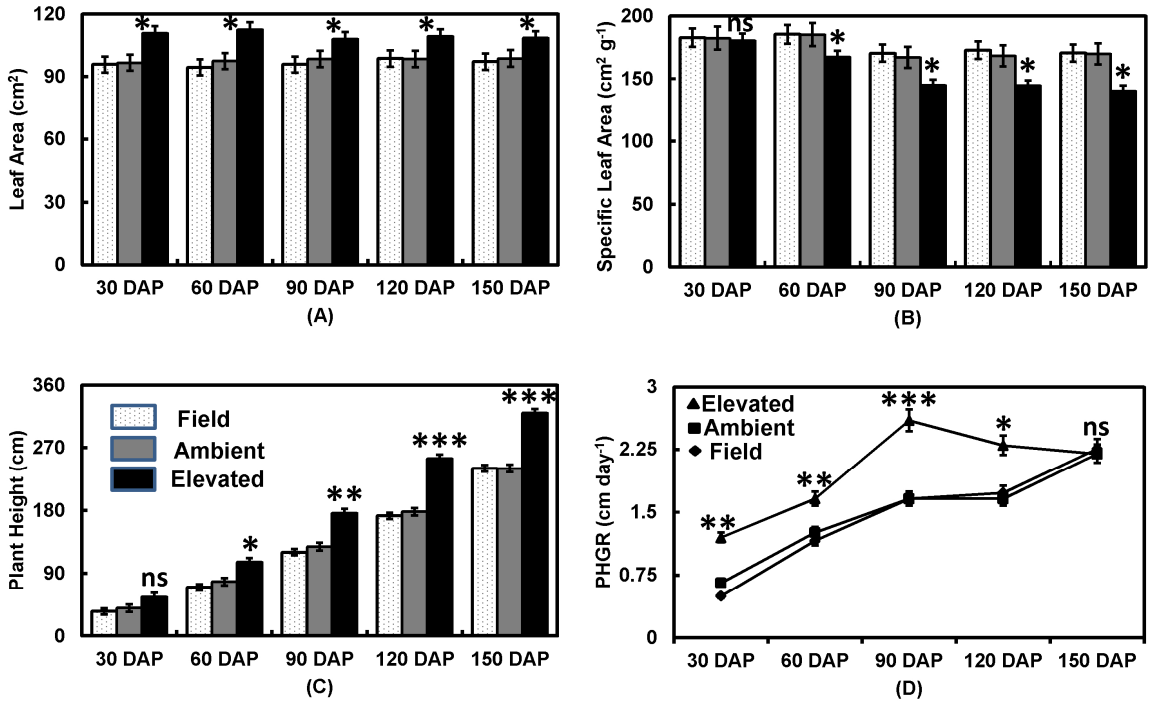


Fig. 2.5. Leaf area (A), Specific leaf area (B) and Plant height (C) measured periodically at different time intervals of growth of *Jatropha curcas* under field (*dotted bar*), ambient (*grey bar*) and elevated CO_2 (*black bar*) conditions. The values are Mean ± SD of two growth periods. Plant height growth rate (D) is also depicted. Analysis of variance (ANOVA) was performed to test significant differences among the CO_2 treatments and between the growth periods (ns – not significant, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$).

2.3.4 Estimation of chlorophylls and carotenoid content

The chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents of *Jatropha* plants grown in elevated CO_2 atmosphere did not show any significant variation with the progression of plant age when compared to its counterparts grown in field and ambient CO_2 atmosphere (**Table 2.3**). At 30 DAP and 60 DAP, the chlorophyll a, chlorophyll b and total chlorophyll content of field, ambient and CO_2-enriched atmosphere grown plant was recorded to be ~0.26 mg m^{-2}, ~0.06 mg m^{-2} and ~0.32 mg m^{-2} respectively.

Table 2.3. Variation observed in the biochemical parameters as influenced by CO_2 (field and ambient, ~390 µmol mol^{-1}; elevated, 550 µmol mol^{-1}) in *Jatropha curcas* recorded during two growth periods at periodic intervals (DAP, days after plantation). Values are the means of two growth periods (±SD).

	Chlorophyll a (mg m^{-2})	**Chlorophyll b (mg m^{-2})**	**Total Chlorophyll (mg m^{-2})**	**Total carotenoids (mg m^{-2})**	**Total carbohydrates (µg mg^{-1} dw)**	**Soluble sugars (µg mg^{-1} dw)**	**Starch (µg mg^{-1} dw)**
30 DAP							
Field	0.266±0.01	0.064±0.02	0.321±0.02	0.163±0.06	137.05±12.05	56.03±1.04	56.03±3.04
Ambient	0.261±0.20	0.063±0.02	0.325±0.20	0.172±0.51	143.84±15.03	54.05±0.53	54.05±4.03
Elevated	0.263±0.10 n.s.	0.063±0.01 n.s.	0.326±0.12 n.s.	0.154±0.17 n.s.	186.70±16.06**	75.07±0.96**	85.07±5.06**
60 DAP							
Field	0.248±0.04	0.063±0.01	0.313±0.05	0.171±0.21	159.44±13.04	50.02±1.24	60.02±3.04
Ambient	0.258±0.10	0.066±0.03	0.325±0.08	0.173±0.53	158.44±12.04	53.94±0.47	63.97±3.07
Elevated	0.263±0.05 n.s.	0.063±0.03 n.s.	0.327±0.06 n.s.	0.244±0.08*	188.68±13.03**	75.68±0.83**	96.63±3.03**
90 DAP							
Field	0.202±0.05	0.083±0.01	0.293±0.05	0.214±0.28	155.84±14.03	52.44±0.63	63.62±2.23
Ambient	0.201±0.10	0.085±0.09	0.297±0.08	0.214±0.55	160.45±12.04	53.45±0.45	64.62±2.74
Elevated	0.195±0.09 n.s.	0.075±0.02 n.s.	0.270±0.12 n.s.	0.214±0.16 n.s.	197.70±11.06**	76.70±1.96**	96.80±3.79**
120 DAP							
Field	0.214±0.15	0.083±0.01	0.297±0.14	0.200±0.63	168.45±14.06	53.45±1.03	63.53±3.73
Ambient	0.225±0.06	0.107±0.01	0.332±0.10	0.202±0.78	165.48±14.04	55.48±2.04	65.46±4.52
Elevated	0.189±0.17 n.s.	0.093±0.05 n.s.	0.281±0.16 n.s.	0.198±0.50 n.s.	238.69±14.06**	78.69±1.86**	98.75±6.85**
150 DAP							
Field	0.214±0.06	0.063±0.04	0.276±0.07	0.220±0.08	160.45±12.01	52.45±2.06	64.53±2.05
Ambient	0.216±0.20	0.069±0.01	0.284±0.15	0.220±0.48	168.43±13.02	53.43±1.08	63.73±3.98
Elevated	0.186±0.17 n.s.	0.060±0.04 n.s.	0.245±0.21 n.s.	0.218±0.34 n.s.	240.72±14.04**	78.72±2.04**	97.83±3.79**

n.s., not significant; *p<0.05; **p<0.01

There was a marginal decrease in chlorophyll a and total chlorophyll content at 150 DAP for elevated CO_2 enriched grown plants which showed ~15% reduction in comparison to plants grown in field and ambient CO_2 atmosphere. The chlorophyll b content of elevated CO_2 grown plants also showed marginal decrease of ~12% and ~14% in comparison to plants grown field and ambient CO_2 atmosphere at 120 and 150 DAP respectively (**Table 2.3**). The carotenoid content of plants grown in field, ambient and elevated CO_2 atmosphere showed the same pattern as exhibited by chlorophylls with the progression of plant age. A reduction of ~23% was observed in the carotenoid contents of plants grown in elevated CO_2 atmosphere when compared to field and ambient CO_2 grown plants at 150 DAP (**Table 2.3**).

2.3.5 Estimation of foliar carbohydrates

CO_2 enrichment had a pronounced influence on the foliar carbohydrate content of *Jatropha* plants when compared to plants grown in field and ambient CO_2 environment (**Table 2.3**). Total carbohydrates, soluble sugars and starch content in leaves of plants grown in CO_2 enriched environment were significantly ($p<0.01$) high with the progression of plant age in comparison to plants grown in field and ambient CO_2 environment. At 30 DAP the total carbohydrates, soluble sugars and starch content of plants grown in elevated CO_2 atmosphere were ~30-35%, ~36% and ~55% high respectively in comparison to its counterparts grown in field and ambient CO_2 atmosphere (**Table 2.3**). This trend was also observed at 150 DAP with a significant ($p<0.01$) increase recorded for elevated CO_2 grown plants which was ~43-50%, ~39% and ~54% respectively for total carbohydrate, soluble sugar and starch contents in comparison to field and ambient CO_2-grown plants (**Table 2.3**). The total carbohydrate, soluble sugars and starch content showed statistically significant differences ($p<0.01$) among the treatments (elevated vs field and elevated vs ambient).

2.3.6 Non –destructive growth characteristics - Plant growth and morphology

Plant height was significantly ($p<0.001$) increased in elevated CO_2-grown plants with the progression of plant age in *Jatropha* in comparison to plants grown in field and ambient

CO_2 atmosphere (**Fig. 2.5C**). The plants grown in CO_2-enriched environment attained a height of ~300cm during both growth periods in comparison to plants grown in field and ambient CO_2 environment (~250cm) at 150 DAP (~33% higher). Similarly, there was a significant increase ($p<0.01$) in PHGR of *Jatropha* grown in CO_2-enriched conditions in comparison to plants grown in field and ambient CO_2 atmosphere at 30 DAP (~115%), 60 DAP (~38%), 90 DAP (~56%) and 120 DAP (~33%) (**Fig. 2.5D**). Concomitantly, number of tertiary branches also increased significantly ($p<0.01$) in plants grown in CO_2-enriched environment in comparison to plants grown in field and ambient CO_2 atmosphere during both growth periods (**Table 2.4**). The increment was more pronounced during growth period II (~45%) than in growth period I (~34%). However, there was no significant difference in the number of secondary branches of plants grown in elevated CO_2 atmosphere in comparison to plants grown in field and ambient CO_2 atmosphere during both experimental growth periods (**Table 2.4**).

The most striking response of *Jatropha* plants was observed in the reproductive morphology and development in elevated CO_2 conditions. The appearance of inflorescence primordia in elevated CO_2-grown plants was seen to be earlier than the field and ambient CO_2-grown plants during both the growth periods (I and II) in all the plants grown in elevated OTCs (**Fig. 2.6**). The flowering time (appearance of the first flower) was seen to be accelerated by ~8 – 10 days in the plants grown in elevated CO_2 atmosphere when compared to its counterparts in field and ambient CO_2 atmosphere (**Fig. 2.6**). There were conspicuous changes observed in the inflorescence morphology in plants grown under elevated CO_2. The number of flowers per apex during growth period I was significantly ($p<0.01$, ~40%) high in plants grown in CO_2-enriched atmosphere when compared to plants grown in field and ambient CO_2 conditions. However, in growth period II there was no significant variation in the number of flowers (**Table 2.4**). The ratio of male to female flower was also influenced under elevated CO_2 conditions. The plants grown in CO_2-enriched atmosphere showed significantly lower ($p<0.01$) male to female flower ratio per apex when compared to plants grown in field and ambient CO_2 atmosphere (**Table 2.4**). The fruit setting was also accelerated by ~20 days in *Jatropha* in CO_2-enriched atmosphere during both growth periods (**Fig. 2.6**).

Appearance of Inflorescence primordia

F - 94±3DAP
A - 95±3DAP
E - 85±3DAP

Appearance of first open flower

F - 105±3DAP
A - 108±3DAP
E - 93±3DAP

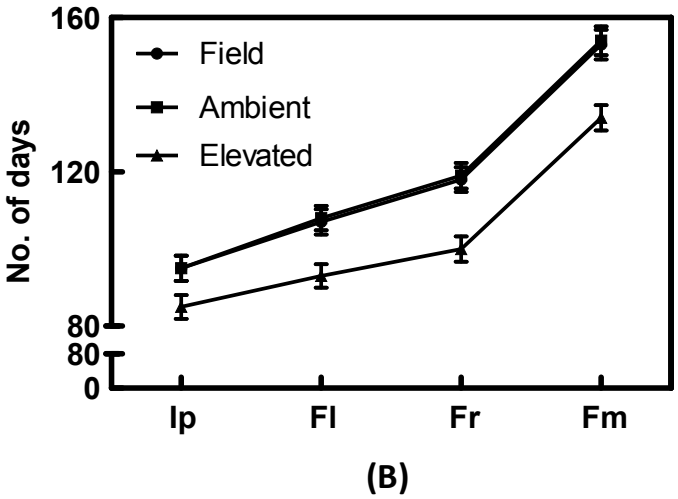
Pollination and appearance of pods

F - 120±3DAP
A - 119±3DAP
E - 100±3DAP

Maturation of pods

F - 152±3DAP
A - 154±3DAP
E - 134±3DAP

(A)



(B)

Fig. 2.6. (A) The different stages of reproductive phenology comprising appearance of inflorescence primordia, open first flower, pollination and appearance of pods, and maturation of pods in *Jatropha curcas* from the day of plantation in field, ambient and elevated CO_2 conditions. (B) The chronology of reproductive events during different stages of reproductive development. (F – Field; A – Ambient; E – Elevated; Ip – appearance of inflorescence primordia; Fl – appearance of first open flower; Fr – appearance of first fruit/pod after pollination; Fm – completion of fruit maturation; DAP – days after plantation).

Table 2.4. Flower morphology and growth characteristics (secondary and tertiary branches per plant, number of flowers per apex and male to female flower ratio) of *Jatropha curcas* influenced by CO_2 treatment. Values are mean ± SD for both growth periods (I and II). Different letters indicate that the values are significantly different at $p < 0.01$. (DAP – Days after plantation)

	Secondary branches plant^{-1}		Tertiary branches plant^{-1}		Number of flowers apex^{-1}		Male:Female flower ratio	
	I	II	I	II	I	II	I	II
Field	12±2.5^a	16±1.2^a	17±3.6^a	22±2.5^a	11±2.5^a	24±4.5^a	3.5±0.05^a	4.5±0.04^a
Ambient CO_2	13±1.6^a	16±1.5^a	18±2.5^a	24±3.8^a	12±4.4^a	25±4.3^a	3.3±0.04^a	4.2±0.05^a
Elevated CO_2	17±2.6^b	17±1.3^a	25±2.6^b	35±4.2^b	17±2.3^b	28±2.2^a	2.2±0.05^b	2.4±0.03^b

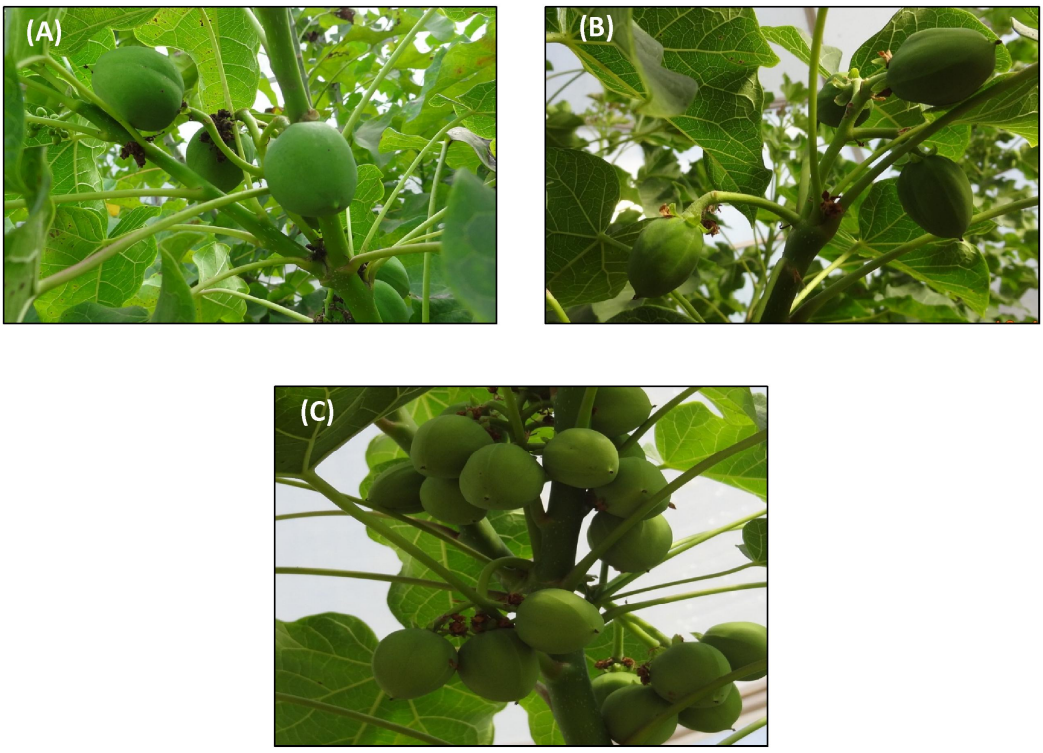


Fig. 2.7. Comparative fruit morphology of *Jatropha* plants grown in elevated and ambient CO_2 conditions. The numbers of fruits formed per inflorescence were higher in (C) elevated CO_2 grown plants when compared to (A) field and (B) ambient CO_2 grown plants which resulted in higher yield in elevated CO_2-grown plants.

The pod development and maturation was subsequently faster in all the *Jatropha* plants grown in elevated CO_2 atmosphere in comparison to field and ambient CO_2-grown plants (**Fig. 2.6**). Also, the number of pods per fruit cluster was higher in plants grown under elevated CO_2 as depicted in **Fig. 2.7A** (field), **Fig. 2.7B** (ambient) and **Fig. 2.7C** (elevated).

2.3.7 Biomass measurement and estimation of carbon sequestration

Biomass accumulation differed significantly in elevated CO_2-grown *Jatropha* plants when compared to field and ambient CO_2-grown plants during both growth periods ($p<0.01$, **Fig. 2.8**). Elevated CO_2 significantly increased ($p<0.01$) fresh weight of leaf (**Fig. 2.8A**), stem (**Fig. 2.8B**) and root (**Fig. 2.8C**). At the time of harvest, the fresh weight of leaves of elevated CO_2-grown plants showed an increase of ~75% in comparison to field and ambient CO_2-grown plants during both growth periods (**Fig. 2.8A**). The stem fresh weight was ~10 kg in elevated CO_2-grown plants which was ~45% and ~65% more in comparison to ambient CO_2 and field-grown plants respectively during the course of the experiment (**Fig. 2.8B**). The roots were harvested after completion of both growth periods and the fresh weight was ~8 kg for elevated CO_2-grown plants which was ~38% higher than field and ambient CO_2-grown plants (**Fig. 2.8C**). A similar trend in dry biomass was recorded in elevated CO_2-grown plants ($p<0.001$) (**Fig. 2.8D**, **2.8E** and **2.8F**). The leaf dry weight of high CO_2-grown plants was found to be ~100% more in comparison to field and ambient CO_2-grown plants during both growth periods (**Fig. 2.8D**). The stem dry weight of elevated CO_2-grown plants was recorded to be ~3 kg for both growth periods which was ~188% and ~114% more in growth period I and ~61% and ~41% more in growth period II in comparison to plants grown in field and ambient [CO_2] respectively (**Fig. 2.8E**). Similarly, the root dry weight of elevated CO_2-grown plants was ~65% and ~45% more in comparison to plants grown in field and ambient CO_2 (**Fig. 2.8F**). The above ground fresh and dry biomass of *Jatropha* in elevated CO_2 environment after harvest showed the same trends as fresh and dry weights of individual organs and was significantly higher ($p<0.01$) (**Fig. 2.8G and 2.8H**).

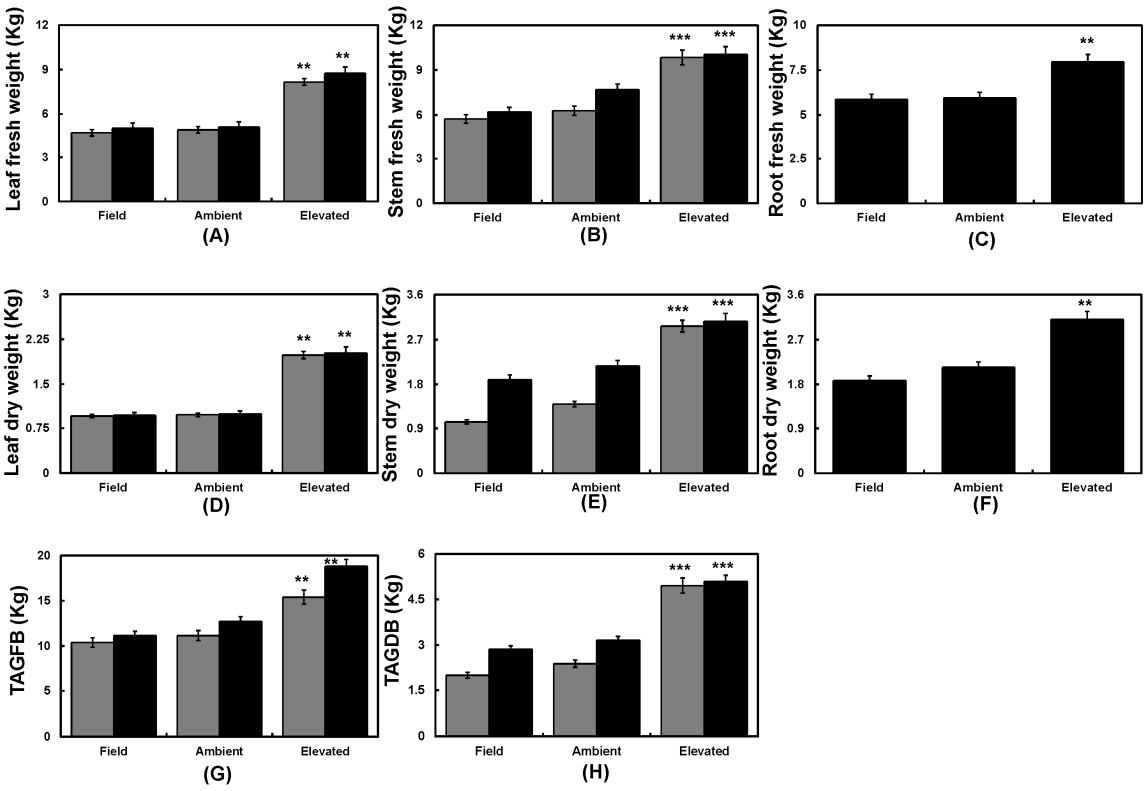


Fig. 2.8. Analysis of biomass through destructive harvest of *Jatropha* plants. (A) Leaf fresh weight, (B) stem fresh weight, (C) root fresh weight, (D) leaf dry weight, (E) stem dry weight, (F) root dry weight, (G) total above ground fresh biomass (TAGFB), (H) total above ground dry biomass (TAGDB) determined by the destructive harvest at the end of both growth periods (Growth period I – *grey bar*; Growth period II – *black bar*). Values are mean ± SD (n = 6). Analysis of variance (ANOVA) was performed to test significant differences among the CO_2 treatments and between the growth periods (** - $p<0.01$).

Total above ground fresh biomass (TAGFB) was recorded to be ~15 kg and ~19 kg for elevated CO_2-grown plants during growth period I and growth period II respectively. It was significantly ($p<0.01$) high and was ~32% and ~38% for growth period I and ~65% and ~48% for growth period II when compared with plants grown in field and ambient CO_2 conditions respectively (**Fig. 2.8G**). Similarly, total above ground dry mass was significantly ($p<0.001$) high for plants grown in elevated CO_2 environment accounting for ~150% and ~110% increase during growth period I and ~78% and ~70% for growth period II in comparison to field and ambient CO_2-grown plants respectively (**Fig. 2.8H**).

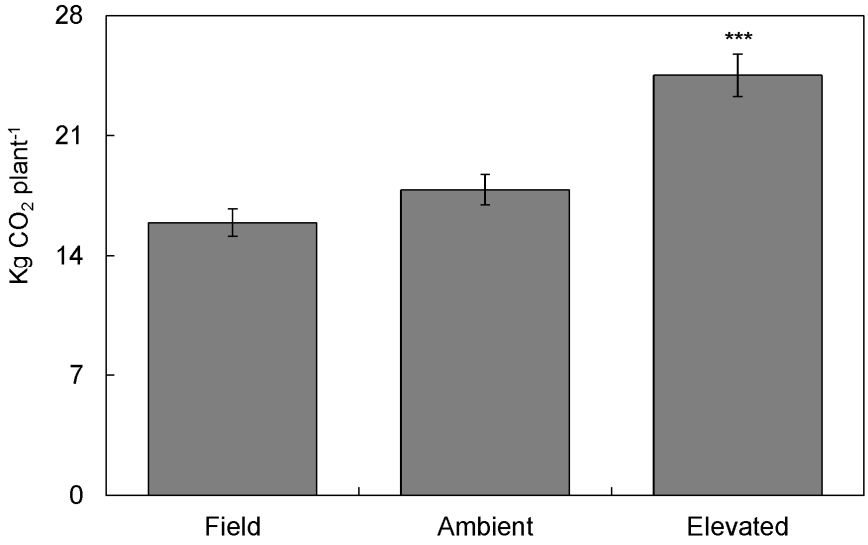


Fig. 2.9. Carbon dioxide sequestered in *Jatropha curcas* grown under field, ambient and elevated CO_2 environments. Values are mean ± SD (n = 6). Data was analyzed using one way analysis of variance (ANOVA) to test significant differences among the CO_2 treatments and between the growth periods (*** - $p<0.001$).

The carbon sequestration potential of *Jatropha* plants was estimated to be significantly higher ($p<0.001$) in elevated CO_2 conditions when compared to its counterparts grown in field and ambient CO_2 regimes (**Fig. 2.9**). *Jatropha* plants grown in elevated CO_2 atmosphere sequestered ~24 kg of CO_2 $tree^{-1}$ in one year which was ~54% and ~37% more in comparison to plants grown in field and ambient CO_2 growth regimes respectively.

2.3.8 Fruit development and yield parameters

Elevated CO_2 induced significant variations in number and development of reproductive organs of *Jatropha* in comparison to field and ambient CO_2-grown plants (**Table 2.5**). The number of fruits harvested from a single plant differed significantly ($p<0.01$) in comparison to field and ambient CO_2-grown plants. An interesting trend was observed in the number of fruits borne by the plants over the two growth periods grown in field, ambient and elevated CO_2 environment. In growth period I, a highly significant ($p<0.01$) difference in number of fruits borne by the plants was recorded. The elevated CO_2-grown

plants had ~128 fruits per plant which was ~573% and ~653% more in comparison to field and ambient CO_2-grown plants (**Table 2.5**). We observed that many flowering branches of field and ambient CO_2-grown plants did not bear female flowers. This resulted in very less number of fruits recorded for field and ambient CO_2-grown plants which was ~19 and ~17 respectively. However, during growth period II the number of fruits recorded for all the plants were recorded to be very high ($p<0.01$) in elevated CO_2-grown plants. The number of fruits recorded was ~320 in elevated, ~210 in ambient and ~182 in field-grown plants (**Table 2.5**). The number of mature fruits in elevated CO_2-grown plants was found to be significantly higher ($p<0.01$) in growth period II and it was recorded ~158. This was ~216% and ~172% higher in comparison to field and ambient CO_2-grown plants (**Table 2.5**). This showed the development and maturation of fruits was faster in elevated CO_2-treated plants in comparison to field and ambient CO_2-grown plants. The number of immature fruits exhibited no significant variation and it was recorded as ~131 for field, ~151 for ambient CO_2 and ~161 for elevated CO_2-grown plants (**Table 2.5**). The number of seeds per fruit also did not show any significant variation in either field, ambient and elevated CO_2-grown plants. However, there was a significant increment ($p<0.01$) of number of seeds harvested per plant in elevated CO_2-grown plants in comparison to field and ambient CO_2-grown plants. The number of seeds per plant harvested from elevated CO_2-grown plants was ~690% (growth period I) and ~75% (growth period II) higher in comparison to field and ambient CO_2-grown plants (**Table 2.5**). There was also a significant difference ($p<0.01$) in 100 fruit weight between the elevated CO_2 and its counterparts grown in field and ambient CO_2 conditions. A significant increase of ~50% was observed in the 100 fruit weight of elevated CO_2-grown plants for both growth periods in comparison to field and ambient CO_2-grown plants (**Table 2.5**). However, the 100 seed weight of elevated CO_2-grown plants did not show any significant variation during both growth periods when compared to field and ambient CO_2-grown plants (**Table 2.5**).

There was no significant variation in oil content (%) in seeds of field, ambient or elevated CO_2-grown plants (**Table 2.5**). However, the seeds of plants of all the three environments accumulated more oil in growth period II in comparison to growth period I (**Table 2.5**).

Table 2.5. Yield traits of *Jatropha curcas* grown under elevated CO_2. Field and OTC grown *Jatropha* plants were harvested completely at the end of both growth periods. Harvested mature fruits were carefully preserved in plastic packets, carried to laboratory and selected yield parameters [Fruits per plant (both mature and immature), seeds per fruit, 100 fruit weight, seed per plant, 100 seed weight and oil content] were measured. Values are the means ±SD for both growth periods (I and II). Different letters indicate that the values are significantly different at $p < 0.01$.

	Number of fruits plant^{-1}		**Number of mature fruits plant^{-1} post harvest**		**Number of immature fruits plant^{-1} post harvest**		**Number of seeds fruit^{-1}**		**100 fruit weight (g)**		**Number of seeds plant^{-1}**		**100 seed weight (g)**		**Oil content (%)**	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Field	19.48± 12.36^{a}	182.66± 27.36^{a}	19.48± 12.36^{a}	50.67 ± 10.53^{a}	0.00±0.00^{a}	131.99± 24.65^{a}	2.04± 0.02^{a}	2.68± 0.02^{a}	125.48± 18.56^{a}	209.49± 15.67^{a}	45.58 ± 5.63^{a}	489.52± 23.54^{a}	61.50± 9.36^{a}	78.16± 8.46^{a}	38.46± 2.58^{a}	47.04± 3.52^{a}
Ambient CO_2	17.33± 11.01^{a}	210.33± 34.53^{ab}	17.33± 11.01^{a}	58.65 ± 12.53^{a}	0.00±0.00^{a}	151.68± 35.37^{ab}	2.15± 0.02^{a}	2.69± 0.02^{a}	128.43± 20.46^{a}	224.37± 18.75^{a}	45.92 ± 6.36^{a}	565.78± 25.42^{a}	59.73± 10.45^{a}	83.40± 9.82^{a}	38.01± 3.42^{a}	48.27± 2.35^{a}
Elevated CO_2	128.33± 17.63^{b}	320.66± 32.23^{b}	84.43± 21.73^{b}	158.7 ± 24.43^{b}	43.9± 12.9^{b}	161.90± 28.33^{b}	2.78± 0.02^{a}	2.82± 0.02^{a}	194.64± 17.62^{b}	320.76± 21.22^{b}	356.7 ± 31.74^{b}	961.98± 36.28^{b}	70.01± 8.02^{a}	86.74± 11.82^{a}	42.27± 2.85^{a}	50.27± 3.55^{a}

2.4 Discussion

The rising CO_2 levels in the atmosphere and its repercussions on global climate change warrants an urgent need in increasing the size of carbon reservoirs and endorsing the use of alternative and renewable sources of energy to account for decreased greenhouse gas (GHG) emissions (IPCC, 2011). Deforestation is attributed for over 20% of the world's GHG emission and therefore, it is of utmost importance to revert these areas in order to restore better carbon sequestration. Recent studies have indicated that *Jatropha* can be grown with minimal inputs to reclaim wastelands and sequester large amounts of carbon from the atmosphere (Becker and Makkar, 2008; Wani et al., 2012). In the present study, we report significant variations observed in morphophysiological, growth and yield responses of *Jatropha* under elevated CO_2 for a complete production cycle which provides a positive connotation towards better performance in terms of growth and development of this potential biofuel tree species.

Plant growth and yield is influenced by various internal and external stimuli inducing morphological and biochemical adjustments within the plants to ensure sustained development. We provide evidence that enhanced plant growth and improved seed yield (including fruit yield) in the present investigation recorded for *Jatropha* in CO_2-enriched conditions were due to enhanced photosynthetic CO_2 fixation, efficient source sink balance, optimal partitioning of dry matter into individual plant organs, plasticity in phenological events and improved plant canopy growth. The effects of elevated CO_2 on leaf assimilation physiology have received much attention and the existing information shows a primary response in increased photosynthetic rates and reduced stomatal conductance (Long et al. 2004; Ainsworth and Long 2005; Ainsworth and Rogers 2007). In our study, the P_n of *Jatropha* was significantly high during both growth periods showing no seasonal variation under elevated CO_2 atmosphere. Elevated CO_2-grown *Jatropha* maintained high P_n even under the reduced g_s and E during two growth periods. The increase in P_n of *Jatropha* grown under elevated CO_2 with concurrent decrease in E resulted in dramatic increases in leaf-level WUE_i (Hamerlynck et al. 2002; Possell and Hewitt 2009). The rate of CO_2 exchange between the plants and its external environment is

tightly regulated by stomatal dynamics and stomatal conductance imposes limitation on CO_2 assimilation, particularly when plants are exposed to enriched CO_2 atmosphere (Wullschleger et al. 2002; Ainsworth and Rogers 2007). Stomatal conductance (g_s) generally decreased under elevated CO_2 conditions and depends on the stomatal opening and stomatal density**.** It is however reported that changes in stomatal aperture rather than density determine the response of g_s to elevated CO_2 conditions (Tricker et al. 2005; Ainsworth and Rogers 2007) which is also true in our study with a significant variation in number of open and closed stomata in elevated CO_2 grown *Jatropha*. The responses of g_s to elevated CO_2 were variable and subject to environmental feedback (Leaky et al. 2006). Elevated CO_2 concentrations also reduced transpiration rates which are likely to be through partial stomatal closure, a direct response of guard cells to increased intercellular CO_2 concentration (Paoletti and Grulke 2005). This adaptation allows the plants to preserve greater soil moisture contents in CO_2-enriched environments, which positively feeds back to increase plant growth (Leaky et al. 2009a). The increased P_n in leaves in spite of decreased g_s in elevated CO_2-grown plants can be attributed to enhanced biochemical activity in leaves (Herrick et al. 2004). Other studies have suggested that the expression of certain regulatory carbon assimilation enzymes, including Rubisco, and modulation of their activities probably plays an important role in influencing the P_n in under high CO_2 atmosphere (Caemmerer and Quick 2000; Messinger et al. 2006). The rates of instantaneous photosynthesis increased in many C_3 species exposed to elevated atmospheric CO_2 due to increased carboxylation efficiency of Rubisco as the enhanced CO_2 concentration at the active site of Rubisco repressed its oxygenation function (Urban 2003; Ainsworth and Rogers 2007). Increase in photosynthetic pigment contents has been considered to be a precondition for higher photosynthesis. However, our results exhibited no significant changes in the chlorophyll and carotenoid concentrations in elevated CO_2 conditions regardless of high photosynthetic rates. Previous reports on elevated CO_2 studies demonstrate a highly varied pattern of response with chlorophyll pigment concentration (Pritchard et al. 2000; Stylinski et al. 2000; Rasineni et al. 2011). The varied pigment concentration in *Jatropha* at the end stages of growth in elevated CO_2 might be to

maintain the balance of light absorption capacity of the photosystems to sustain high photosynthetic rates and increased growth patterns.

In our experiment, elevated CO_2 induced significant changes in morphological components associated with growth and productivity of *Jatropha*. The *Jatropha* plants grown in elevated CO_2 atmosphere were ~34% taller in comparison to field and ambient CO_2-grown plants after 150 days of growth which is in congruent to the relative growth rates of these plants. This short-term growth stimulation by elevated CO_2 has been described in certain crop plants and tree species (Zhang et al. 2006; Rasineni et al. 2011). Nonetheless, these enhanced growth rates under high CO_2 were not maintained in many plant species as they reprogram their photosynthetic processes to utilise the available abundant resources (Long et al. 2004). Many reports have suggested a possible reason for photosynthetic downregulation in plants under elevated CO_2 is the accumulation of soluble leaf carbohydrates and starch in the absence of any available potential sink. The ability of the plants to grow under elevated CO_2 not only depends on enhanced photosynthetic ability but also on the optimal allocation of available resources to sink organs (Moore et al. 1999; Reddy et al. 2010). In this study, significant increase in foliar carbohydrate content including starch and soluble sugars under elevated CO_2 conditions was observed with no downregulation of photosynthetic capacity suggesting the availability of potential sinks in *Jatropha* for better resource utilization. Our study also showed that the growth of *Jatropha* under high CO_2 atmosphere was accompanied by significant increase of tertiary branches which positively connotes towards improved resource allocation when compared to ambient CO_2-grown plants. Also, the flower and fruit yields in these tertiary branches were significantly high which might act as potential sinks, allowing *Jatropha* to perform superior photosynthesis in elevated CO_2 conditions. The presence of additional sinks suggests improved utilization of carbohydrate supply from source tissues as a result of enhanced photosynthetic rates to feed the growing sink demand under CO_2 enriched conditions (Reich et al. 2006; Hogy and Fangmeir 2009; Rasineni et al. 2011). Our study demonstrated enhanced biomass yields in *Jatropha* for both growth periods under elevated CO_2 suggesting a possible role of improved photosynthetic capacity. This positive interaction between photosynthesis and morphological components of growth in *Jatropha*

under CO_2 enriched conditions can be attributed to efficient coordination between source activity and sink strength. Subsequently, the fast growth and increased metabolic activity and sink strength probably would have allowed *Jatropha* to escape photosynthetic downregulation under elevated CO_2 conditions (Idso et al. 2002; Wittig et al. 2005; Körner 2006). The enhanced biomass accumulation and increase in morphological components of growth like tertiary branches and reproductive tissues in *Jatropha* under elevated CO_2 suggest efficient regulation in carbon capture and storage. This efficient carbon sequestration in *Jatropha* can be attributed to ecophysiological traits like increased gas exchange rates, efficient leaf area development (both individual leaf area as well as total tree leaf area), development of sylleptic branches to capture more solar radiation per unit mass which can contribute to tree growth (Dillen et al. 2010; Chen and Setter 2012). Also, biomass allocation was preferentially towards stem in *Jatropha* under elevated CO_2 atmosphere as shown in our data suggest better carbon storage.

An important phenological finding in our study was that the flowering time of *Jatropha* under elevated CO_2 was accelerated by ~8 days during both growth periods. The timing of flowering is an important stage of development during the life cycle of any plant as the seed number would be determined at this stage. Early flowering has been reported in many crop plants under elevated CO_2 which include both short day and long day plants (He et al. 2005; Cleland et al. 2006; Heinemann et al. 2006; Springer and Ward 2007). Early flowering is an adaptation strategy allowing the plant to ensure resource accumulation in order to avoid a period of dry or cold for improved yield and quality (Roux et al. 2006). However, the mechanisms controlling these responses are not clearly understood. A plausible reason for accelerated flowering in plants under elevated CO_2 conditions was that the increase in the relative growth rates of plants due to enhanced photosynthetic process (He et al. 2005). Alternatively, flowering time can be modulated in plants as carbohydrates which act as regulatory signal molecules during the course of growth allow physiological and developmental plasticity in plants depending on environmental conditions (Rolland et al. 2006). In our study, the increased growth rates due to stimulated photosynthesis and levels of foliar carbohydrates in *Jatropha* grown in elevated CO_2 plants which may act as signalling molecules would have played a possible dual role in early initiation of

flowering. Also, the intensity of flowering response in *Jatropha* under elevated CO_2 was significantly high. Similarly, accelerated fruit setting and fast development and maturation of fruits can be attributed to increased foliar carbohydrate molecules which might have played a signaling role in their fast development in elevated CO_2 conditions.

Seed yield in *Jatropha* plants is dependent on floral morphology and development which relies on number of flowering branches, number of female flowers and most importantly the ratio of male to female flowers within an inflorescence (Wu et al. 2010). In the present study, *Jatropha* demonstrated significant increase in most of the yield associated characters which contributed towards overall seed (~690% in growth period I and ~70% in growth period II) and fruit yield (~600% in growth period I and ~52% in growth period II) improvement in elevated CO_2 conditions. We observed that even though there were a sizable number of flowers in the branches of *Jatropha* plants grown in field and ambient CO_2-grown plants during growth period I, the fruit setting in many branches was not observed. This observation is supported in our data in fruit yield for elevated CO_2-grown plants which shows an unprecedented high numbers in growth period I. While during growth period II, the yield was better in field and ambient CO_2 grown plants but still the elevated CO_2 grown plants performed better in terms of fruit and seed yield. The environmental conditions in which the experiment was performed may be attributed for this peculiar behavior. *Jatropha* is a day-neutral plant (Heller, 1996); so there is no possibility of role of photoperiod in *Jatropha* flowering. Temperature and rainfall are the known factors which govern the flower development and fruit formation in *Jatropha* (Brittaine and Lutaladio 2010). As observed by us, an optimum temperature of 20 - 28°C and a good rainfall is sufficient for *Jatropha* to flower year round. At high temperatures (>35°C), the vegetative growth of *Jatropha* is unaffected while the reproductive development may be impeded with repressed yields (Gour 2006). In the growth period I, when flowering was initiated the daily maximum temperatures of the experimental site was ranging from 40 - 44°C which might have hindered the female flower development and subsequently there was no fruit setting in many of the flowering branches of field and ambient CO_2-grown plants. However, there was no such trend observed in elevated CO_2 grown plants as evidenced through lower ratio of male to female flowers in each flowering

branch and subsequently fruit setting was higher resulting in greater number of fruits and seeds. We implicate that elevated CO_2 might have played a role in offsetting the effect of temperature on floral development in elevated CO_2 plants (Hamilton et al. 2008; Wang et al. 2008). Since plant growth and reproduction is dependent on photosynthesis, the interactive effects of elevated CO_2 and warmer growth temperatures might have contributed to the normal floral development in elevated CO_2-grown plants which also showed better photosynthetic performance. In growth period II, the mean daily maximum temperatures of experimental site ranged from 22 - 37°C and the plants grown in field, ambient and elevated CO_2 exhibited normal flowering and fruit development. However, plants grown in elevated CO_2 atmosphere again outperformed its counterparts in terms of fruit and seed yield. One of the possible reasons attributed to this observation is that the number of male to female flowers in an inflorescence was significantly high in elevated CO_2 plants as shown in our data (**Table 2.3**). The increased number of female flowers might have led to increased pollination and subsequently higher fruit and seed yield.

Our study clearly demonstrated that elevated CO_2 can effectively enhance biomass productivity, higher seed and fruit yield in *Jatropha*. These data can significantly contribute in describing the elevated CO_2 induced responses of perennial semi-evergreen small trees like *Jatropha*, which should be grown as a potential biodiesel yielding tree species in a future high CO_2-environment.

Chapter III

Insights into the gene expression analysis of regulatory genes associated with photosynthesis and carbohydrate metabolism in leaves of *Jatropha curcas* grown under elevated [CO_2] for one year using transcriptome sequencing and assembly

Chapter 3

3.1 Introduction

Plants adjust their photosynthetic machinery in response to elevated levels of [CO_2] to sustain growth and productivity. The adaptive capacity of plants to increased [CO_2] in atmosphere depends on a range of physiological processes which vary among different plant species. Earlier studies have reported that the initial stimulation in photosynthetic potential under prolonged exposure to elevated [CO_2] was followed by an acclimatory down-regulation in photosynthesis, leading to less than predicted productivity in many plant species (Ainsworth and Long 2005; Körner, 2006; Kimball, 2016). However, some fast growing coppice plantations with high yield potential have been able to sustain increased growth and productivity under elevated [CO_2] over a long period of time suggesting that maintenance of short rotation plantations is an effective strategy for mitigation of increasing atmospheric [CO_2] proportionally (Bernacchi et al., 2003; Sekhar et al., 2014). The rapid growth of these trees also allows the analysis of their responses to elevated CO_2 over a complete production cycle (Davey et al., 2006). The ability of such plants to sense and respond to the elevated CO_2 environment requires transcriptional casca-

-des operating at cellular level to adjust their morphology, physiology and phenotype accordingly (Leakey et al., 2009b). Hence, studying the molecular mechanisms associated with growth of these trees to predicted elevated [CO_2] can provide an insight into certain key candidate genes and the pathways controlled by them in order to understand the relationship between gene expression and adaptation to varying external environments.

The depletion of fossil fuel reserves and anthropogenic increase in emission of greenhouse gases have developed worldwide interest in renewable sources of energy including biofuels obtained from both carbohydrate- or oil-based feedstock and biomass. Plants producing non-edible oils like *Jatropha curcas* and *Pongamia pinnata* which can be grown with minimum agricultural inputs are being promoted for production of biodiesel (Chaitanya et al., 2015). Apart from its usage in biofuels, bioenergy cropping also has an additional advantage of increasing the green cover of planet and sequestering excess carbon from atmosphere through photosynthesis thereby partially affecting the commitments of the Kyoto protocol (IPCC, 2011). *J. curcas*, a member of family *Euphorbiaceae*, has been advocated as a plant with high potential for biofuel plantations because of its high seed oil content, easy propagation, rapid growth, short gestation period and adaptation to a wide range of agro-climatic conditions (Kumar and Sharma, 2008). It would be noteworthy to understand the molecular aspects associated with growth and productivity in *Jatropha* in different growth conditions which is crucial in domesticating and developing *Jatropha* cultivars for a wide variety of applications (Maghuly and Laimer, 2013).

Previous investigations on molecular responses in plants under elevated CO_2 have been limited to small plants with only one or two studies undertaken on tree species. The studies reported on the responses of tree species to elevated CO_2 reveal insights about secondary metabolism during delayed senescence and enhanced radial growth (Taylor et al., 2005; Ainsworth et al., 2006; Tallis et al., 2010; Wei et al., 2013; Niu et al., 2016). Recent advancements in next generation sequencing technologies like Solexa/Illumina platform based *de novo* RNA-sequencing and high throughput deep sequencing have allowed discovery of new genes, analysis of specific transcripts, gene expression and generation of transcript sequences of non-model organisms (Wang et al., 2009; Garg and Jain, 2013).

This approach has accelerated better understanding of complex transcriptional patterns and measurements of gene expression in different tissues or at different stages of plant development in response to varying external environments (Qu et al., 2016). Realizing its importance as an economic plant, it is only recently a few genomic resources have been generated for *Jatropha* (Sato et al., 2011; Hirakawa et al., 2012; Wu et al., 2015). However, studies on transcriptome analysis of *Jatropha* were limited to regulatory aspects of oil biosynthesis in developing seeds with little emphasis on growth and photosynthesis (Costa et al., 2010; Jiang et al., 2012; Grover et al., 2014). Furthermore, few transcriptome studies, reported for growth of *Jatropha* under different environmental conditions, were limited to seedlings or pot-grown plants with no reports on plants grown for longer durations in field conditions (Wang et al., 2013; Juntawong et al., 2014; Zhang et al., 2014; Sapeta et al., 2015).

We have earlier reported growth, yield responses and carbon sequestration in *J. curcas* grown for two complete production cycles under elevated CO_2 (~550 ppm) which demonstrated that *Jatropha* was able to sustain enhanced levels of photosynthesis throughout an year of growth in elevated CO_2 atmosphere resulting in increased fruit and seed yields (Kumar et al., 2014). In this study, we sequenced the transcriptome of *Jatropha* which was grown for a year under elevated CO_2 using Illumina technology and integrated the findings with the morphophysiological data which was recorded continuously for the year comprising two growth seasons to verify the consistency in response to CO_2 enriched conditions. The goal of this study was to gain insights into the expression analysis of certain crucial genes associated with photosynthesis and growth in elevated CO_2 grown *Jatropha*. This study is the first of its kind on the transcriptomic analysis of *J. curcas* grown and maintained in field under elevated CO_2 environment.

3.2 Materials and Methods

3.2.1 Plant material and CO_2 treatment

Jatropha curcas seeds (Variety: Chhattisgarh) were surface sterilized with 0.5% sodium hypochlorite and grown in polythene bags. After 10 days, three *Jatropha* seedlings were transplanted in three pits with a spacing of 2 × 2m in octagonal-shaped open top chambers

(OTCs). The establishment of plant growth, experimental setup, microclimatic growth conditions and CO_2 treatment inside the OTCs were same as described in our previous study (Kumar et al., 2014). Two OTCs were used for elevated CO_2 treatment (mean CO_2 concentration – 550 ppm) and two for ambient CO_2 (mean CO_2 concentration – 395 ppm) in this study. Three *Jatropha* plants were grown and maintained in each OTC for one year. The plants were maintained as coppice plantations after completion of harvest of seeds which was done once every six months. The experiment started in the month of January and the first growth season ended in June and the second growth season was from July to December. The two growth seasons were chosen with respect to the growth behaviour of *Jatropha* as described earlier (Gour, 2006).

3.2.2 Morphophysiological and biochemical measurements

Leaf gas exchange and chlorophyll a fluorescence measurements were performed regularly on the upper canopy young leaves (preferably 3rd and 4th leaf from top in the canopy; n - 15) of *Jatropha* grown under ambient and elevated CO_2 during the one year growth period (**Fig. 3.1**). Light saturated photosynthetic rates (A_{sat}) were measured by performing photosynthetic light response curves (*A/Q*) by using a portable infra-red gas analyzer (IRGA, LCpro-32070, ADC Biosynthetic Ltd. UK). The microclimate conditions maintained during the measurement of A_{sat} were as follows: air temperature of 25 – 26°C, CO_2 concentration of 550 and 395 μmol mol^{-1} for elevated and ambient CO_2 grown plants respectively and relative humidity of 55–60%. *A/Q* studies were performed with an automated program of eight steps [0, 300, 600, 900, 1200, 1500 and 1800 μmol m^{-2}s^{-1} photosynthetic photon flux density, PPFD supplied by a LED light source (LCpro Lamp 32070 – Broad, ADC Bioscientific Ltd. UK) attached to leaf chamber] and duration of each step was three minutes. *A/Q* curves were used to estimate A_{sat} from a three-component exponential function equation $A = [a(1 - e^{-bx}) + c]$, [where A = photosynthetic rate and x = PPFD and a, b and c were parameters estimated by the nonlinear regression] (Watling et al., 2000).

Photosynthetic leaf gas exchange by Infrared Gas Analyzer

Photochemical efficiency by Mini PAM

Fig. 3.1. Portable infrared CO_2/H_2O gas analyzer, IRGA (LCpro-32070, ADC Biosynthetic Ltd., UK) and Mini-PAM (Heinz Walz GmbH, Germany) for measurement of leaf gas exchange and photosynthetic efficiency respectively.

The same leaves were used to measure the chlorophyll a fluorescence with the miniaturized pulse-amplitude-modulated photosynthesis yield analyzer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) using a special leaf clip holder (model 2030-B, Walz) described by Bilger et al. (1995). This can be used to estimate the photosystem II operating efficiency (ΦPSII), which translates photochemistry efficiency (Genty et al., 1989). The maximal photochemical efficiency of photosystem II [$F_v/F_m = (F_m - F_o)/F_m$] was estimated from these measurements. F_o and F_m represent the minimum and maximum fluorescence yield, respectively, measured in dark-adapted leaves by illuminating the leaves with a beam of saturating light (intensity – 4000 µmol m^{-2} s^{-1} of 650 nm peak wavelength, an excitation intensity sufficient to ensure closure of all PS-II reaction centers; between 09:00 h and 10:00 h). After this initial measurement, the actinic light is switched on (intensity – 800 µmol m^{-2} s^{-1}) and leaves illuminated with saturating pulse to record fluorescence values in light-adapted state. The effective quantum yield of PS II [$\Delta F/F_m' = (F_m' - F)/F_m'$], non-photochemical quenching [NPQ = $(F_m - F_m')/ F_m'$] and photochemical quenching [qP = $(F_m' - F)/(F_m' - F_o)$] were also calculated where F is fluorescence yield of the light-adapted sample and F_m' is the maximum light-adapted fluorescence yield. Apparent rate of photosynthetic electron transport of PS II (ETR) was obtained as ETR = $\Delta F/F_m' \times$ PPFD ×

0·5 × 0·84, where the factor 0·5 assumes equal excitation of both PS II and PS I and 0.84 is the ETR correction factor which accounts for the fraction of incident light really absorbed by photosystems (Klughammer and Schreiber, 2008).

Morphological parameter like plant height, and biochemical parameters like chlorophyll, soluble carbohydrates and starch content were measured on the same leaves used for physiological measurements were performed after 90, 180, 270 and 360 days of growth on both ambient and elevated CO_2 grown plants as described previously in chapter 2 (Kumar et al., 2014). Similarly, above ground biomass and reproductive yields were assessed at 180 and 360 days after coppicing the plants. These morphological, physiological and biochemical measurements were performed to analyse the consistency in response of *Jatropha* under elevated CO_2 at different seasons. Further, upper canopy young green leaves (preferably 3rd from top) used for physiological measurements were collected at the above mentioned four time points (90, 180, 270 and 360 days) from both ambient and elevated CO_2 grown *Jatropha* plants during its one year of growth and stored at -80°C until use. Three independent biological replicates of stored young green leaves of 360 days from each ambient and elevated grown plants were used for sequencing (each replicate from individual tree). The transcriptome generated at this time point was used for deciphering differential regulation of key regulatory genes associated with photosynthesis and carbohydrate metabolism at the four different time points and correlate their expression patterns with morphophysiological analysis. The four time points selected for morphophysiological, biochemical and molecular analyses reflect two distinct stages of *Jatropha* across two seasons. The 90 and 270 days are the stages when the vegetative growth rate is at maximum for both seasons. At 180 and 360 days for both seasons, both vegetative and reproductive growth simultaneously occur.

3.2.3 RNA extraction, Illumina sequencing and quality control

Agilent plant RNA isolation kit (Agilent Technologies, USA) was used to isolate total RNA from leaf tissue of ambient (sample A) and elevated (sample E) CO_2-grown *Jatropha* plants respectively. The concentration, intactness and purity of RNA were checked with Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Samples having RNA integrity

number (RIN) value greater than 8 were used for library preparation. Three biological replicates were sequenced from both ambient and elevated CO_2-grown *Jatropha* plants. Paired end cDNA library preparation for ambient and elevated samples was performed by the genomics facility (Genotypic Technology Pvt. Ltd., Bangalore, India) following Illumina TruSeq RNA library protocol outlined in "TruSeq RNA Sample Preparation Guide" (Illumina Technologies, San Diego, CA). The prepared library was quantified using Nanodrop and validated for quality by running an aliquot on High Sensitivity Bioanalyzer Chip (Agilent Technologies, USA). Sequencing of constructed cDNA library was performed on Illumina HiSeq 2000 sequencer on high output mode and RNA-Seq data were generated in Fastq format. Sequencing resulted in generation of 101 bp raw reads having attached adapter sequences in tissues from both sample A and E. These raw reads were subjected to filtering through the standard Illumina pipeline. The filtered Fastq files were further subjected for quality control using SeqQC 2.1 and BBDuk (https://sourceforge.net/projects/bbmap/) to remove adapters, B-block, low quality bases towards 3′ ends and contaminant reads (Annadurai et al., 2013).

3.2.4 Sequence assembly and analysis

Assembly was performed using the high quality reads after removing duplicate reads from libraries for both the samples using Velvet 1.2.10 and Oases 0.2.08 at different k-mer lengths (Zerbino and Birney, 2008; Schulz et al., 2012). The high quality filtered reads were *de novo* assembled for transcript generation. Various k-mer assemblies were performed and the best hash length assembly was selected (Sample A: 49, Sample E: 53) considering various parameters like total number of transcripts generated, maximum transcript length, total transcript length and less number of N's. Further, as the *Jatropha* genome is available, the reference based assembly was also performed by mapping the trimmed reads onto the *J. curcas* reference genome and exclusively to gene models using Rsubread package on the downloaded files from NCBI (NCBI GCA_000696525.1) [http://www.ncbi.nlm.nih.gov/genome/annotation_euk/Jatropha_curcas/100/] (Liao et al., 2013; Wu et al., 2015). After mapping, the mapped file in *.bam format were used to count mapped reads and to provide the genomic coordinates, GTF file was downloaded from

NCBI site. The mapped reads were counted using featureCounts program within the Rsubread package (Liao et al., 2014). The unaligned reads to the genome were also assembled. The clustering of assembled transcripts from both libraries to generate unigenes was performed using CD-HIT (Fu et al., 2012). The length of the assembled unigenes for further study were selected as ≥200. Gene IDs were assigned to unigenes according to *Jatropha* genome version JatCur_1.0 (http://www.ncbi.nlm.nih.gov/genome/jatropha curcas).

3.2.5 In silico differential gene expression analysis

The differential gene expression (DGE) analysis in the sequenced cDNA library generated from leaves of both sample A and E was carried out using DESeq tool, considering sample A as control and sample E as treated from the unigenes generated (Love et al., 2014). The alignment of reads of both sample A and E was performed using Bowtie tool (Langmead et al., 2009). The read count profile for reads from both A and E was generated and DGE analysis carried out using shrinkage estimation for dispersions and fold changes to improve stability and interpretability of estimates. The relative expression levels of each annotated unigene was estimated separately for both samples and also as a joint estimate from both samples and presented as its mean expression level (at the base scale), the fold change from sample A to sample E and the logarithm (to basis 2) of the fold change. The differentially expressed genes (DEGs) were identified with log-fold (log_2) expression change ≥1 or ≤ -1 using a statistically significant P-value ($P < 0.05$) and FDR (FDR < 0.01).

3.2.6 Functional annotation of transcript sequences

After assembly and clustering, transcript annotation was done by performing BLASTX analysis against the non-redundant protein database (N_r) and Swiss-Prot (Altschul et al., 1990; Boeckmann et al., 2003). The hits with an E-value ≤ 1E-05 and blast score ≥ 80 were considered to be significant. GO (Gene Ontology) terms were assigned to transcripts on the basis of best significant match with proteins of members of Euphorbiaceae to impart a broad overview of their functions and categorized into biological process, molecular

function and cellular component using in house Perl scripts. Also, KOG (Eukaryotic Orthologous Groups) was used to identify the transcript homologues from other organisms and thus assigning a probable function to transcripts. KAAS [KEGG (Kyoto Encyclopedia of Genes and Genomes) Automatic Annotation Server] was used for metabolic pathway analysis using *Arabidopsis thaliana* and *Oryza sativa* L. ssp. *japonica* as reference organisms to identify the enriched metabolic pathways in various gene sets (Moriya et al., 2007). The unigenes were classified into various transcription factors (TFs) using Transcription factor Family Data Base (TFDB) (Jin et al., 2014).

3.2.7 Quantitative PCR analysis

Validation of differential gene expression data was carried out using the qRT-PCR analysis at the four different time points. Gene specific primers were designed for certain key regulatory enzymes involved in photosynthesis, carbohydrate and nitrogen metabolism (**Table 3.1**). Total RNA was isolated from the leaves of both ambient and elevated CO_2-grown *Jatropha* plants using Sigma Spectrum^{TM} Plant Total RNA kit (Sigma, USA). 1µg of RNA was used for cDNA synthesis by Revert aid first strand cDNA synthesis kit (Thermo Scientific, USA). qRT - PCR was performed on Eppendorf thermal cycler using KAPA SYBR FAST qPCR Master Mix (2X) Universal (KAPA Bio systems, USA). Each reaction contained 1 µL of the first-strand cDNA as template from both sample A and sample E in a total volume of 10 µL reaction mixture. The amplification program was performed at 95°C for 30 s followed by 95°C for 5 s and 55°C for 30 s (40 cycles). The relative expression was calculated using the formula, $F = 2^{-(\Delta Ct \text{ treated}-\Delta Ct \text{ control})}$ with 18SrRNA as housekeeping gene for normalization of data (Livak and Schmittgen, 2001). The fold change values were log transformed with base 2 so that ~1.00 fold was used to identify differentially expressed genes. Three independent biological replicates with three technical replicates of each biological replicates for both samples were used for analysis. Total RNA was extracted from the same group of samples that were used in transcriptome analysis.

Table 3.1. List of primers for selected genes used for qRT-PCR analysis for the confirmation of gene expression. [The gene IDs were assigned according to *Jatropha* genome version JatCur_1.0 (http://www.ncbi.nlm.nih.gov/genome/jatrophacurcas); F – Forward primer (5′-3′); R – Reverse primer (5′-3′)].

Description	Gene ID	Primer Sequence
Carbonic anhydrase (CA)	105640386	**F** – CCGGCTTCGTCCACTTTAAG **R** – CTGGCAATGTTTCGGACCAT
Sedoheptulose 1,7-bisphosphatase (SB)	105646676	**F** – TTGGGGAATCATTGCGGTTG **R** – CTCCCATGCACATCAGAAGC
Cytosolic fructose 1,6-bisphosphatase (FB)	105629115	**F** – GATGCACCAAAGCCACTTGA **R** – GGGGATCTGTTACCACCACA
Sucrose phosphate synthase 1 (SPS1)	105633156	**F** – GTCTGATGTCATGCGGTTCC **R** – TGTCATCCCTGTTCCCCATT
Granule bound starch synthase (GBSS)	105639084	**F** – ACTCTATGGTCCTCAGGCAG **R** – GGTAGCAGGGAAGAAGAGCA
Glutamate dehydrogenase (GD)	105632353	**F** – AGCGAAAGGAGGGATTGGAT **R** – GCCTCTCTACCCAGTGATCC
Glutamine synthetase (GS)	105631892	**F** – TTGTTGCCGAAGAGTGTTGG **R** – ATGCCTTGTAGTGGGAGTCC
Nitrate reductase (NR)	105638879	**F** – GTGAAACGTGGCAAGTCTGT **R** – GCAGTTGTTCATCATGCCCA
Hexokinase 3 (HXK3)	105631535	**F** – GGTTTGCATCCTTCACCTGG **R** – TCATTGACCAAAGCTGCCAC
Rubisco large subunit (R_L)	7564870	**F** – TAAAACTTTCCAAGGGCCGC **R** – AAATCAAGTCCACCGCGAAG
Rubisco small subunit (R_S)	105642030	**F** – CCGGCTTCGTCCACTTTAAG **R** – TCCACATTGTCCAGTAGCGT
Rubisco activase (RA)	105647331	**F** – ATCTTGGGGATTTGGGGAGG **R** – TGCGTAGTTCCACCCATTCT
F-type H^+-type transporting ATPase subunit beta (FTA)	7564869	**F** – GTACCTGCGGACGATTTGAC **R** – AACGATCTGAGGTTGGAGCA
Phosphofructokinase (PFK)	105632412	**F** – TGCTTCAGGATGTTGGGCTA **R** – GCAAGGAGTGTGCAGAAGAC
Phosphoglucomutase (PGM)	105634080	**F** – GCTCGGTCTATGCCAACAAG **R** – ACAGCCCATATGCCATCCTT
Ribose-5-PO_4 isomerase (RI)	105647678	**F** – AGTCTGGTATGGTTCTCGGC **R** – ATCGACGTTGGGGTGAGAAT
Glyceraldehyde-3-PO_4 dehydrogenase (GAP)	105630249	**F** – AGCTCCTTCAAGAATCCCGG **R** – TTGATGCTGCTACCTTGGGA
Ferredoxin-NADP reductase (PetH)	105635331	**F** – AGACCTGGCACATGGTCTTC **R** – TGAGCAGACTCCCTTCACAA
Hexokinase 1 (HXK1)	105631535	**F** – AACCCTAGCTGGAGGCAAAT **R** – TCAGTGGAAGATGCGATGAC
Triose phosphate/phosphate translocator (TPT)	105649590	**F** – GCTCTCTTTGCTTGCATTCC **R** – ACATTGCCAACTGCATGTGT

Table 3.1 contd.

Description	Gene ID	Primer Sequence
Cytosolic triose phosphate isomerase (TPICY)	105647635	**F** – GCCGTCATCTGATGTTGTTG **R** – CCGAGAATGACCCACAGAAT
Chloroplastic triose phosphate isomerase (TPICH)	105643783	**F** – TTGATGTTGTTGTCGCACCT **R** – ACCCACTTGCAGCCAATATC
Sucrose phosphate synthase 3 (SPS3)	105633156	**F** – ACGGAGCTCTTGCTCACATT **R** – TTCTTCCAAGCGAGTGTCCT
Photosystem (II), PsbP	105641916	**F** – ATACACGGATGCCAAGGAAG **R** – GCTTGTCAGCCACAAACTGA
PsbC, chloroplast	7564849	**F** – GATCAACGTCTTGGGGCTAA **R** – GGCGCATGGGTCATATATTC
PsbB, chloroplast	7564760	**F** – TCGAAGGGCAGAGTCAAAGT **R** – AAGAGCAGAGCAAACGAAGC
PsbA, chloroplast	7564824	**F** – AGGCTGAGCACAACATCCTT **R** – AATAACCATGAGCGGCTACG
PsaA, chloroplast	7564856	**F** – CAAGTGGTTTGGCCGATAGT **R** – CATCTTGGAACCAAGCCAAT
Phosphoribulokinase, chloroplastic (PRK)	105631767	**F** – AAGTTTTGCCAACCCAACTG **R** – CAGGGTAAGAGCAGGTGAGC
Glucose-6-phosphate dehydrogenase, chloroplastic (G6PD)	105641505	**F** – CACGTTTCAGTCCTTGCTCA **R** – CATCCTGCAAAAAGACAGCA
Glucose-6-phosphate/phosphate translocator 1, chloroplast (GPT)	105645263	**F** – GAACCTGGGAAGGCAACATA **R** – ATGTCACCGGATTCCTGAAG
Cytosolic aldolase (ALD)	105639139	**F** – TTCTGATGGAAAACCCTTCG **R** – CGGGCACCTGCCTTATAGTA
Transketolase (TKL)	105643137	**F** – GCAATGTCAGGTTTGGTGTG **R** – AAGCTTGCCAAGTGCTCAAT
NAD(P)H-quinone oxidoreductase subunit 2, chloroplastic (NDHB)	7564781	**F** – TGGGGCAAGCTCTTCTATTC **R** – GCTTGAACCCGATTCCTACA
NAD(P)H-quinone oxidoreductase subunit K, chloroplastic (NDHK)	7564864	**F** – GTCCTAGACAAGCGGACCTG **R** – CTTATCGACTCCCCGAACAG
18SrRNA	105629717	**F** – CCTGCGGCTTAATTTGACT **R** – TTAGCAGGCTGAGGTCTC

3.2.8 Enzyme activity measurements

The activity measurements of certain key regulatory enzymes of photosynthesis and carbohydrate metabolism like Rubisco, SPS, HXK, FB and RA were performed on leaves of both ambient and elevated CO_2 grown plants as described previously at all four time points (Holaday et al., 1992; Cheng and Fuchigami 2000; Lee et al., 2014). Leaf discs of 1cm diameter (surface area: ~315 mm^2) were punched out from the same group of leaves used for physiological and transcriptomic analysis. The enzymes were extracted with

different extraction buffer and the activities were measured with a UV-Visible spectrophotometer (UV-1800; Shimadzu Corporation, Kyoto, Japan).

3.2.8.1 Rubisco: For Rubisco extraction, two leaf discs were homogenized in a pre-cooled mortar with 3 ml ice-cold extraction buffer [100 mM Bicine (pH 7.8 at 25°C), 5 mM EDTA, 0.75% (w/v) polyethylene glycol (20,000), 14 mM β-mercaptoethanol, 1% (v/v) Tween 80, and 1.5% (w/v) insoluble polyvinylpyrrolidone (PVP)]. The extract was then centrifuged at 13,000 g for 40 s in an Eppendorf centrifuge (5430 R) and the supernatant used immediately for Rubisco activity assay. Rubisco activity was measured at 25°C by enzymatically coupling RuBP carboxylation to NADH oxidation which was monitored spectrophotometrically at 340 nm. The assay solution for both initial and final activity measurements contained 100 mM Bicine (pH 8.0 at 25°C), 25 mM $KHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 80 nkat glyceraldehyde-3-PO_4 dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase, 80 nkat creatine phosphokinase, and 0.25 mM NADH. For initial activity, 50 µl of sample extract was added in a 1 ml quartz cuvette containing 900 µl of the assay solution, immediately followed by adding 50 µl 0.5 mM RuBP and mixed well. The change of absorbance at 340 nm was monitored for 40 s. For final activity, 50 µl of 0.5 mM RuBP was added 15 min later, after a sample extract was combined with the assay solution to activate all the Rubisco fully and the decrease in absorbance recorded at 340 nm.

3.2.8.2 Rubisco activase: RA was isolated from leaf discs using a modified method of Wang et al. (1992). Leaf discs were ground to a fine powder with a pre-cooled mortar and a pestle in liquid nitrogen and then extracted in the extraction buffer containing 50 mM 1,3-bis[tris(hydroxymethyl) methylamino] propane (BTP) (pH 7.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1mM EDTA, 0.5 mM ATP, 10 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM benzamidine, 0.01 mM leupeptin, 1.5% PVPP and 3 mM mercaptobenzothiazole (MBT). The leaf slurry was filtered through five layers of Miracloth. The filtered solution was centrifuged at 30,000g for 40 min. $(NH_4)_2SO_4$ powder was slowly added into the supernatant to 35% saturation and stirred for 30 min. The supernatant and pellet were collected by centrifugation at 8,000g for 10 min. The supernatant was discarded and the resuspended pellet contains RA. To isolate RA in

the resuspended pellet, 50% (w/v) PEG (10,000) was added to 5 ml of 20 mM BTP (pH 7.0) buffer (containing 0.2 mM ATP, 10 mM $MgCl_2$ and 2 mM MBT) to a final concentration to 18%, and centrifuged at 8,000g for 10 min. The pellet was dissolved in the above buffer. This solution was centrifuged at 20,000g for 10 min. The resulting pellet was resuspended in the same buffer, and centrifuged. The collected supernatants were loaded onto a 20 ml Q-Sepharose column equilibrated with 20 mM BTP (pH 7.0). The column was eluted with 20 mM BTP (pH 7.0) at a flow rate of 1 ml/min at 4°C before continuing with a linear gradient from 0 to 0.5 M NaCl in 20 mM BTP (pH 7.0). 3 ml fractions were pooled, and assayed for RA activity. RA activity was assayed as the ability to produce ADP in an ATP-dependent reaction in absorption at 340 nm. The isolated RA solution was added to a total volume of 0.4 ml of the activation reaction mixture containing 50 mM Tricine (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 40 units/ml pyruvate kinase, and 40 units/ml lactate dehydrogenase.

3.2.8.3 Hexokinase, cytosolic fructose 1,6-bisphosphatase and sucrose phosphate synthase: The frozen leaf discs were ground to a fine powder with liquid nitrogen in a mortar and pestle. The fine powder was extracted in 1 ml ice-cold extraction medium that always contained 2% (w/v) PVP and 0.1% (v/v) Triton X-100. In addition, the medium for each enzyme extraction contained: HXK, 50 mM Tris-HCl (pH 8.1), 5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol; FB, 50 mM Hepes (pH 7.0), 5 mM $MgCl_2$, 1 mM EDTA and 15 mM 2-mercaptoethanol; SPS, 50 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA and 10% (v/v) glycerol. The extract was centrifuged for 10 s in an Eppendorf microfuge (Eppendorf 5415R). All enzymes except SPS (assayed at 20°C) were assayed spectrophotometrically at 30°C. The assays were as follows: HXK (measured spectrophotometrically at 340 nm), 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.2 mM NAD^+, 10 mM glucose, 1 mM ATP, 3 units glucose 6-PO_4 dehydrogenase and 100 µL extract; FB (measured spectrophotometrically at 366 nm), 50 mM Hepes (pH 7.0), 2 mM $MgC1_2$, 0.1 mM fructose 1,6-bisphosphate, 0.5 mM $NADP^+$, 10 units each of glucose-PO_4 isomerase and glucose 6-PO_4 dehydrogenase and 100 µL extract; SPS, full activity under limiting conditions with 5 mM Pi, except that 5% (v/v) glycerol was included and 100 µL extract

was used. SPS assays were terminated after 15 min. by boiling. Controls without F6P and G6P were included. After centrifugation of the boiled assay solutions, the amount of UDP formed was determined spectrophotometrically at 620 nm in 50 mM Hepes (pH 7.0), 5 mM $MgCl_2$, 2.5 mM PEP, 0.1 mM NADH, 10 units each of lactate dehydrogenase and pyruvate kinase, using 100 µL SPS assay solution. The amount of UDP standard recovered in the presence and absence of Pi with plant extract was determined using the SPS assay solution but in the absence of F6P, G6P, and UDP-Glucose.

3.2.9 Statistical analysis

For qRT-PCR analysis, three independent biological replicates with three technical replicates of each biological replicates for both samples were used for analysis and the mean ± standard deviation (SD) values were calculated for each sample. The significance of the difference for all physiological (n = 6-15), biochemical (n = 6-15) and molecular were tested by using Analysis of Variance (ANOVA) and the comparisons were tested by Dunnett's multiple comparison analysis. All statistical analysis was performed using SIGMA PLOT 11.0.

3.3 Results

3.3.1. Morphophysiological and foliar biochemical analysis

Morphophysiological and biochemical variations were monitored during growth of *Jatropha* under ambient and elevated CO_2 conditions at regular intervals for both seasons. There was no seasonal variation in most of the recorded parameters with a significant variation observed in reproductive yield for both growth seasons. The light-saturated photosynthetic rate (A_{sat}) and apparent quantum efficiency (AQE; calculated as an initial slope of *A/Q* curve) were recorded to be significantly higher (~28 µmol m^{-2} s^{-1}; ~0.030) at all four time points under elevated CO_2 in comparison to ambient CO_2 grown plants (~18 µmol m^{-2} s^{-1}; ~0.020) which was sustained during both seasons ($P < 0.01$) (**Fig. 3.2A, Table 3.2**). Similarly, elevated CO_2 grown *Jatropha* showed significant variations in chlorophyll a fluorescence characteristics in comparison to ambient grown plants (**Table**

3.2). The maximal photochemical efficiency of photosystem II (F_v/F_m), efficiency of water splitting complex (F_v/F_o), electron transport rate (*ETR*), effective quantum yield of PS II ($\Delta F/F_m'$) and photochemical quenching (qP) for elevated CO_2 grown plants were recorded to be ~10, ~40, ~10, ~15 and ~12% respectively, higher than ambient grown *Jatropha* plants ($P < 0.05$) during its growth in both seasons (**Table 3.2**). However, non-photochemical quenching (NPQ) was significantly decreased by ~25% in elevated CO_2 grown *Jatropha* in comparison to ambient grown plants ($P < 0.05$) (**Table 3.2**).

Further, *Jatropha* plants were able to sustain enhanced growth in elevated CO_2 environment as demonstrated by the plant height, number of secondary and tertiary branches, and more number of flowers (**Fig. 3.2B, 3.3, 3.4 and 3.5**). At the end of both seasons, after 180 and 360 days of growth, the elevated CO_2 grown plants reached the height of ~3 m in comparison to ambient grown plants ($P < 0.01$). However, the chlorophyll content (both chlorophyll a and b) did not show any significant variation at 90 and 270 days with a non-significant decrease after 180 and 360 days recorded under elevated CO_2 conditions in comparison to ambient CO_2 grown *Jatropha* plants (**Fig. 3.2C**). The Chl a/b ratio also was unaltered (~2-3) suggesting optimum photosynthesis. Among the foliar carbohydrates, both starch and soluble sugars were recorded to be significantly higher ($P < 0.05$) at all the four time points in elevated CO_2 grown *Jatropha* plants in comparison to ambient CO_2 grown plants (**Fig. 3.2D**). The above ground dry biomass (comprising leaf, stem and fruits) at the end of both seasons, 180 and 360 days after growth, was found to be significantly higher ($P < 0.01$; ~75% and ~67% respectively for both seasons) for elevated CO_2 grown *Jatropha* plants (**Fig. 3.2E**). The most interesting observation was recorded in fruit yield of *Jatropha* with very little yield (~20) in ambient CO_2 conditions at the end of first growth season (180 days) in comparison to elevated CO_2 grown plants which recorded almost ~3 fold more yields ($P < 0.01$). However, the yield significantly improved in second growth season (360 days) for ambient grown plants (~190) but again the elevated CO_2 grown *Jatropha* plants demonstrated better yield performance with a significant ($P < 0.01$) increase of ~1.5 folds (**Fig. 3.2F**). As the *Jatropha* plants showed better photosynthetic performance in relation to source sink interaction under elevated CO_2 for both seasons, we decided to sequence its transcriptome

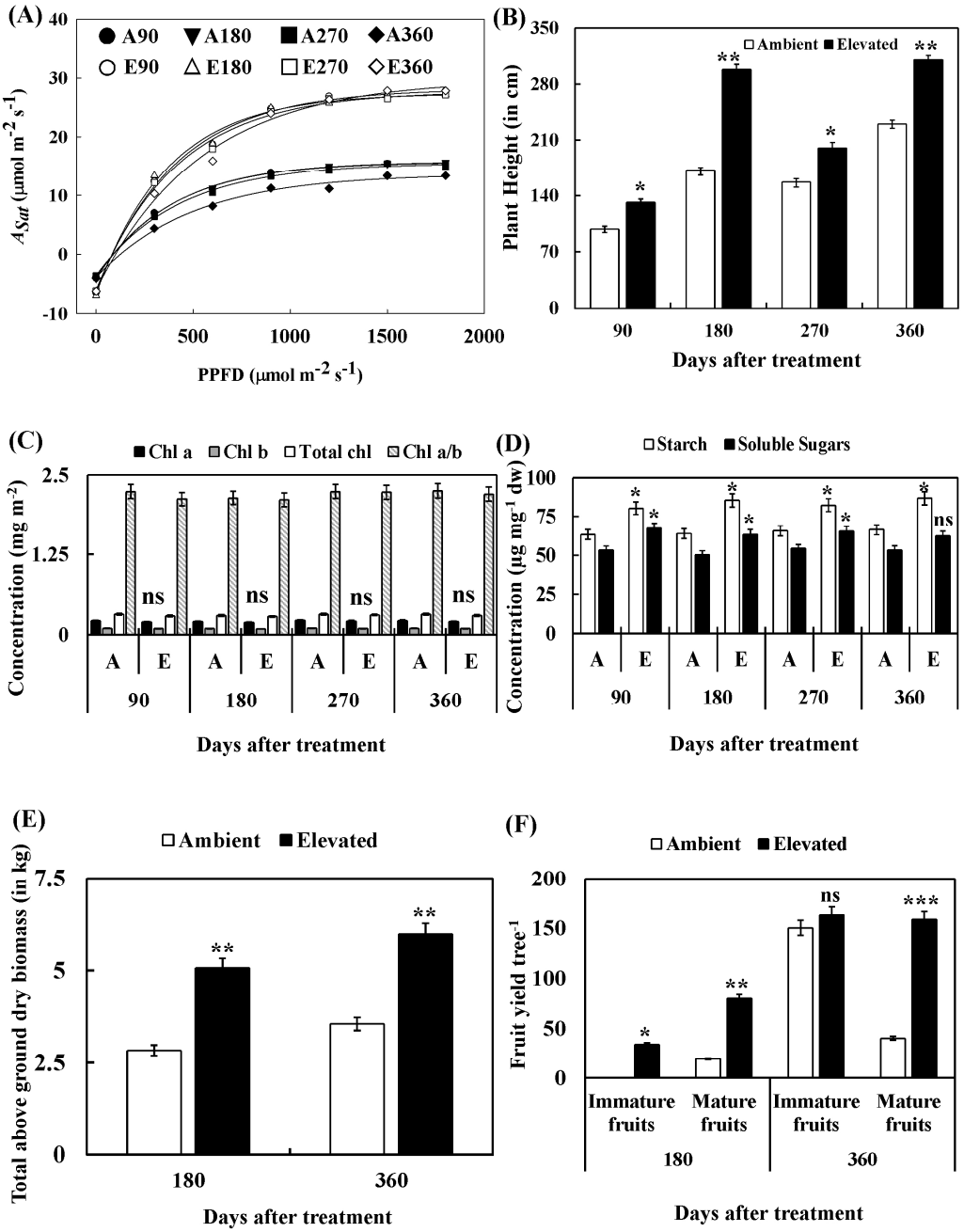


Fig. 3.2. (A) Photosynthetic A vs. Q Curve measured at saturating light intensities of 0, 300, 600, 900, 1200, 1500, 1800 and 2000 μmol photons m^{-2} s^{-1} at 90, 180, 270 and 360 days. (B) Plant height at 90, 180, 270 and 360 days of growth. (C) Chlorophyll content and Chl a/b in leaves of *Jatropha*. (D) Variations observed in the starch and soluble sugar content. (E) Above ground biomass after 180 and 360 days of growth. (F) Fruit yield per tree after 180 and 360 days of growth. The data given here represents average of six representative plants from two OTCs and values are expressed as mean ± SD. [Note – ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001].

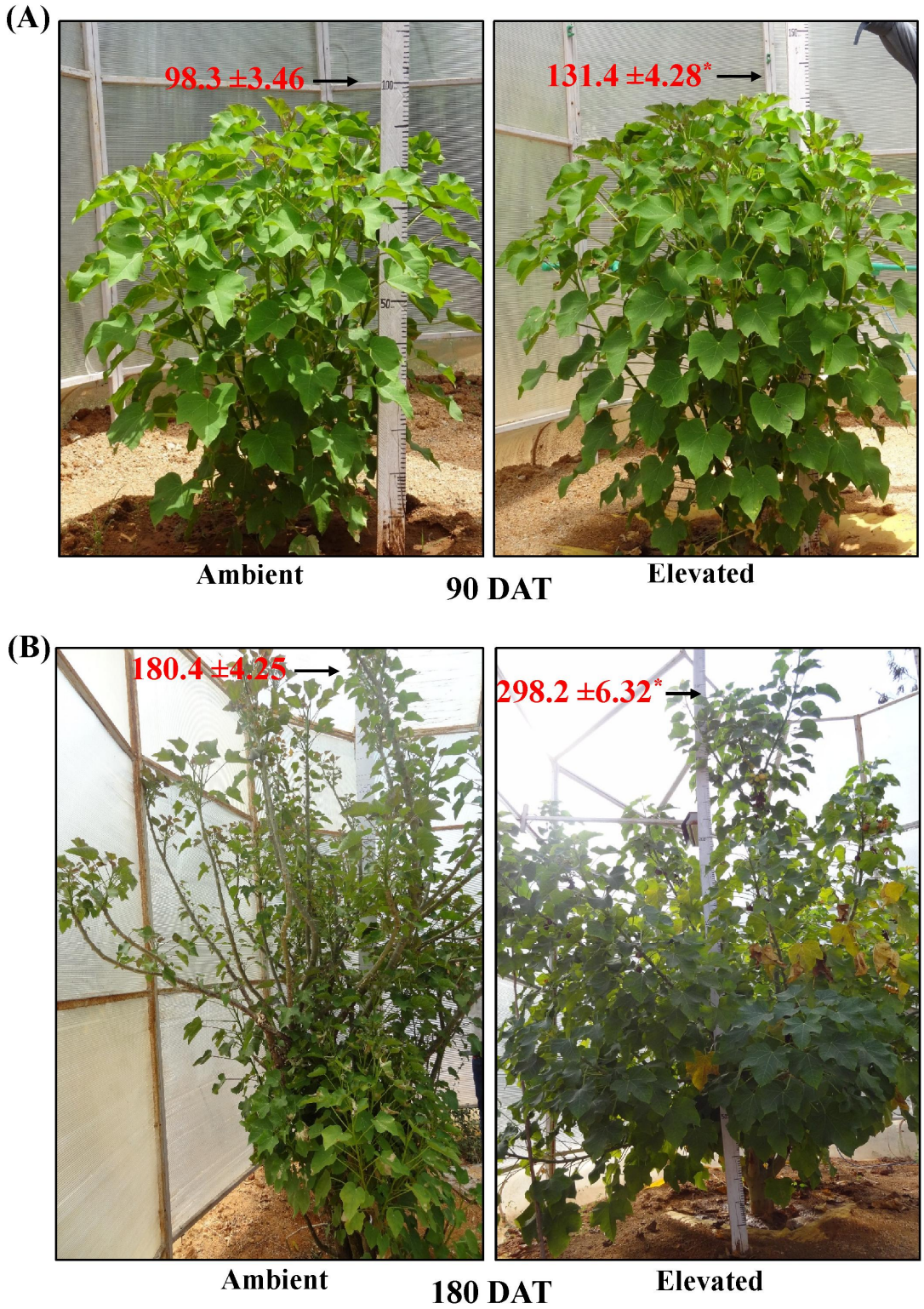


Fig. 3.3. Plant height of *Jatropha* at (A) 90 DAT, and (B) 180 DAT during first growth period. (DAT, days after treatment; *$P < 0.05$).

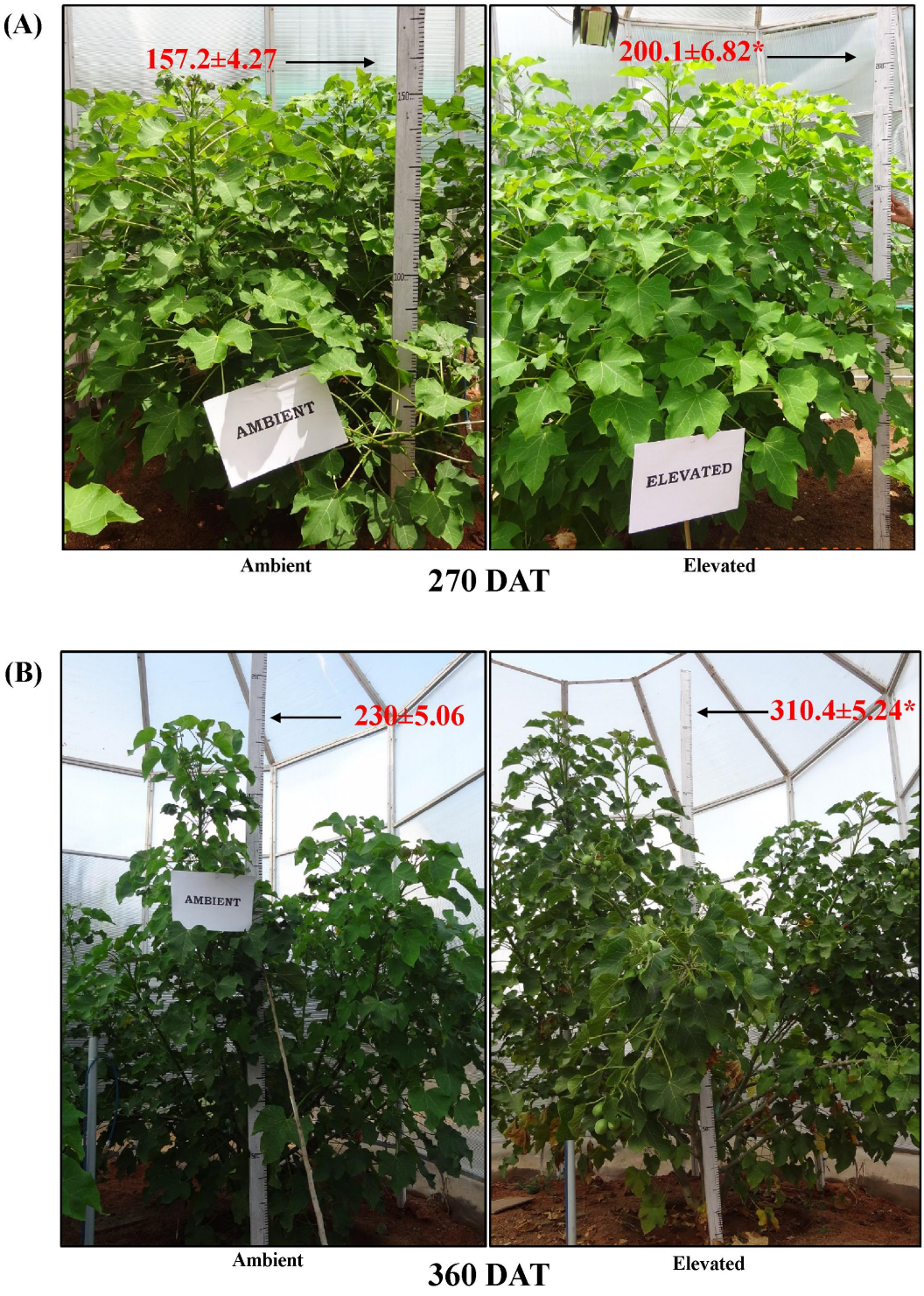


Fig. 3.4. Plant height of *Jatropha* at (A) 270 DAT, and (B) 360 DAT during second growth period. (DAT, days after treatment; *$P < 0.05$).

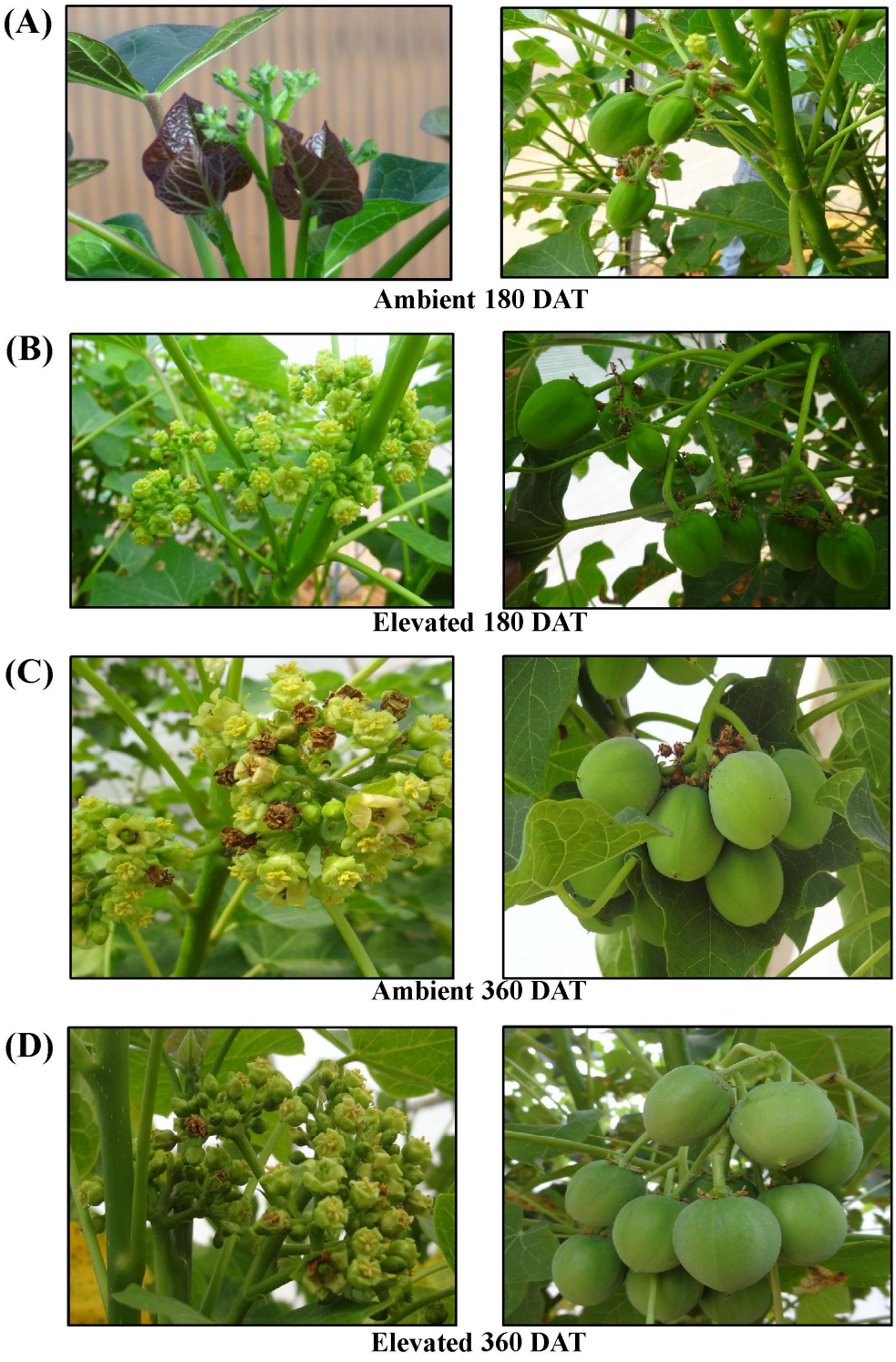


Fig. 3.5. Comparative flower and fruit morphology of *Jatropha* grown under ambient and elevated CO_2 conditions at the end of two growth seasons (180 and 360 DAT).

Table 3.2. Chlorophyll *a* fluorescence measurements on leaves of *Jatropha curcas* exposed to ambient (A) and elevated (E) [CO_2] at 90, 180, 270 and 360 days. Values were mean ± SD (n = 6), followed by significance of difference under elevated CO_2 [* $P < 0.05$; ** $P < 0.01$].

Parameter	90 d		180 d		270 d		360 d	
	A	E	A	E	A	E	A	E
AQE	0.022±0.002	0.032±0.001*	0.021±0.001	0.030±0.001*	0.21±0.001	0.030±0.003*	0.020±0.002	0.031±0.003*
F_v/F_m	0.724±0.05	0.804±0.03*	0.718±0.04	0.789±0.05*	0.728±0.05	0.804±0.02*	0.724±0.02	0.803±0.04*
F_v/F_o	2.625±0.46	3.64±0.38**	2.634±0.35	3.713±0.35**	2.768±0.45	3.930±0.45**	2.492±0.25	3.488±0.36**
$\Delta F/F_m'$	0.685±0.05	0.754±0.05*	0.680±0.03	0.748±0.03*	0.692±0.01	0.761±0.02*	0.693±0.02	0.762±0.05*
ETR	148.4±4.52	161.4±7.24*	146.4±3.05	168.3±5.05*	152.5±2.26	175.3±4.25*	150.6±4.29	173.1±4.38*
NPQ	0.589±0.05	0.464±0.05*	0.575±0.04	0.431±0.02*	0.572±0.05	0.429±0.01*	0.574±0.03	0.419±0.02*
qP	0.726±0.03	0.801±0.02*	0.718±0.02	0.825±0.03*	0.728±0.05	0.815±0.03*	0.730±0.04	0.817±0.05*

to identify probable molecular events in relation to photosynthetic physiology.

3.3.2 Sequencing and transcript assembly

Sequencing of constructed cDNA library resulted in generation of 101 bp raw reads of fastq file size of 9.98 (42.69 millions) and 9.56 GB (40.84 millions) for ambient (sample A) and elevated (sample E) respectively (**Table 3.3**). More than 85% of high quality (HQ) reads with average Phred quality score of ≥ 30 at each base position (**Fig. 3.6**) were obtained and used for downstream analyses. A total of 44,179 and 28,825 contigs with maximum contig length of 8302 and 6817 were acquired for sample A and E, respectively. A total of 61,779 and 48,775 transcripts were obtained having a maximum transcript length 10,713 and 9,793 and a N50 value of 2304 and 2032 for sample A and E respectively (**Table 3.4**). Also, the assembled transcriptome of *Jatropha* demonstrated ≥90% similarity with *Jatropha* genome (**Table 3.5**). An average of 97.86% of reads matched to the reference genome covering ~87.7% of gene models/~89% of transcripts. An average of 92% of reads completely matched gene model indicating 5% of the transcript reads are matched to the non-gene model position of the genome which may be novel transcripts identified in our study. A total of 1809 (sample A) and 808 (sample E) transcripts generated from unaligned reads to genome (**Table 3.5**). A total of 69,581 unigenes having 95% identity were obtained after primary analysis and clustering with CD-HIT with an average unigene length of 1657 and N50 value of 2225 (**Table 3.6**).

3.3.3 Functional annotation and differential gene expression analysis

Among the *de novo* assembled unigenes, a total of 16,575 unigenes were found to be differentially expressed in elevated CO_2 in comparison to ambient grown *Jatropha* plants (**Fig. 3.7**). Among them, 6685 unigenes were up-regulated and 9890 unigenes were down-regulated in elevated CO_2 grown *Jatropha* plants. Furthermore, on the basis of applied yardstick for selecting differential expressed genes (DEGs) [$P < 0.05$, FDR < 0.01 and $-1.00 \geq |\log_2 \text{foldchange}| \geq 1.00$], a total of 3023 unigenes were differentially expressed in response to CO_2 treatment in leaves which comprised 837 (~27%) up-regulated unigenes and 2186 (~73%) down-regulated unigenes. The majority of Gene Ontology (GO) terms

Table 3.3. Brief summary of sequencing and QC results.

S. No.	1	2	3	4
Fastq file name	Sample A leaf (R1)	Sample A leaf (R2)	Sample E leaf (R1)	Sample E leaf (R2)
Fastq file size	9.98 GB	9.98 GB	9.56 GB	9.56 GB
Maximum read length	101	101	101	101
Minimum read length	50	50	50	50
Mean read length	100	100	100	100
Total number of raw reads	42.69 millions	42.69 millions	40.84 millions	40.84 millions
Total number of reads	42.69 millions	42.69 millions	40.84 millions	40.84 millions
Total number of HQ reads*	38.69 millions	38.69 millions	37.13 millions	37.13 millions
Percentage of HQ reads	91%	90%	91%	91%
Total number of bases	4258412748	4258412748	4079278164	4079278164
Total number of bases (in Mb)	4258.4127	4258.4127	4079.2781	4079.2781
Total number of HQ bases**	3856372846	3859173527	3706283654	3692563428
Total number of HQ bases (in Mb)	3856.37284	3859.17352	3706.28365	3692.56342
Percentage of HQ bases	90.55%	90.62%	90.85%	90.52%
Total number of non-ATGC characters	242761 bases	205341 bases	246732 bases	214208 bases
Total number of non-ATGC characters (in Mb)	0.242	0.205	0.246	0.214
Percentage of non-ATGC characters	0.01%	0.01%	0.01%	0.01%
Number of reads with non-ATGC characters	124563	21282	137215	22356
Percentage of reads with non-ATGC characters	0.51%	0.10%	0.55%	0.10%

* >70% of bases in a read with >30 Phred score and reads which are of low quality can be trimmed and used.

** bases with >30 Phred score.

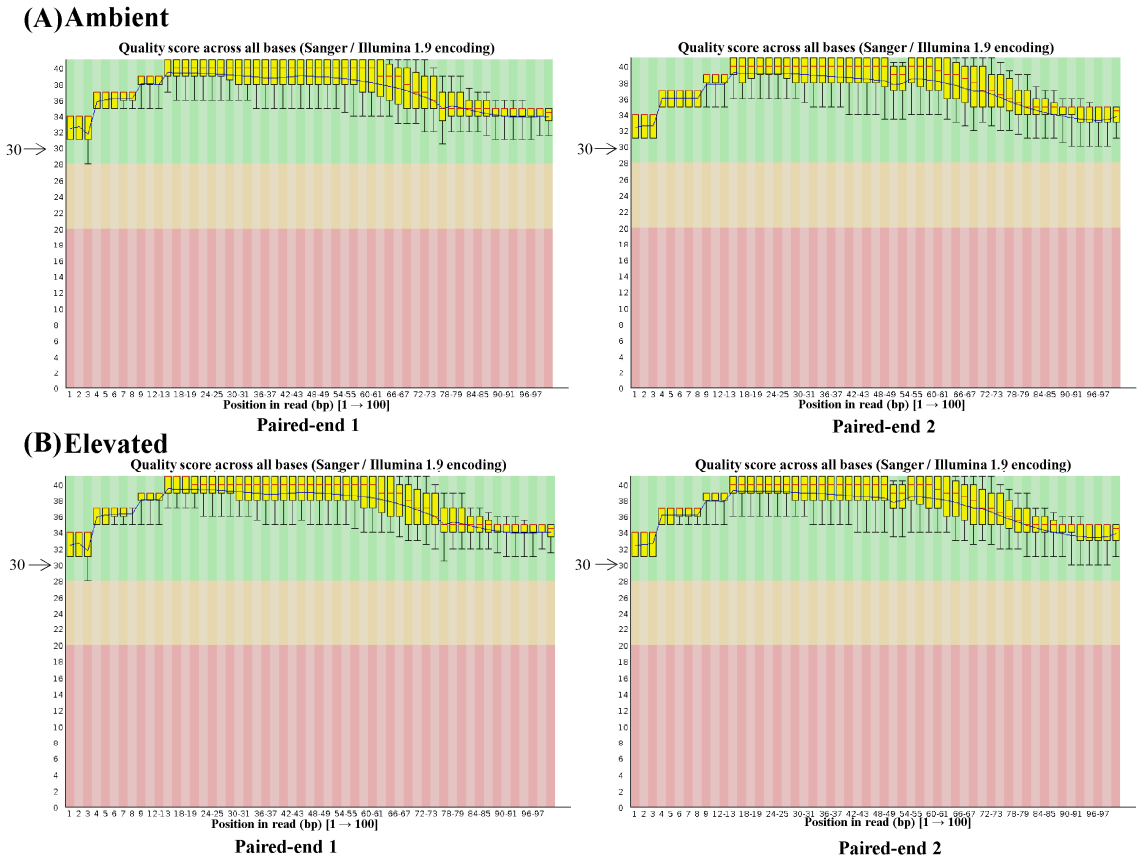


Fig. 3.6. Average Phred quality scores of each base position for filtered reads for both the paired-end reads in (A) ambient and (B) elevated conditions.

Table 3.4. Assembly statistics of *Jatropha curcas* L. transcriptome under elevated CO_2.

Transcript Statistics	Sample A		Sample E	
	Contig	Transcripts	Contig	Transcripts
K-mer	49		53	
% reads used for assembly	86.58	97.05	83.69	97.14
Number of transcripts identified	44179	61779	28825	48755
Maximum Contig Length	8302	10713	6817	9793
Minimum Contig Length	200	200	200	200
Average Contig Length	828.2 ± 723.3	1,725.4 ± 1,185.9	1,008.7 ± 810.4	1,546.0 ± 1,004.2
Median Contig Length	409	3281	2304	3417
Total Contigs Length	3,65,88,305	10,65,94,308	2,90,75,500	7,53,73,695
Total Number of Non-ATGC Characters	11918	2680	8199	1728
Percentage of Non-ATGC Characters	0.033	0.003	0.028	0.002
Contigs >= 200 bp	44179	61779	28825	48755
Contigs >= 500 bp	25145	52438	18769	41010
Contigs >= 1 Kbp	11825	42537	10907	32316
Contigs >= 10 Kbp	0	2	0	0
N50 value	1187	2304	1522	2032

Table 3.5. Reference assisted *de novo* assembly of both samples (A and E) with the *Jatropha* genome [*Jatropha curcas* cultivar: GZQX0401 RefSeq Genome; Genome version available in NCBI: JatCur_1.0, (http://www.ncbi.nlm.nih.gov/genome/Jatrophacurcas)]. The genome was downloaded from NCBI (Accession: GCA_000696525.1).

	A	E
Raw read count	42693007	40849528
Processed read count	38692273	37131099
Alignment percentage	97.67	98.06
Alignment percentage to gene models	91.61	92.9
Gene models covered	21846	21431
Transcripts expressed	33782	33159
Unaligned reads to genome	562112	352822
Number of transcripts identified	1809	808
Maximum contig length	5189	3609
Minimum contig length	200	200
Average contig length	513.7 ± 438.5	459.4 ± 410.5
Median contig length	257	367.5
Total contigs length	9,29,307	3,71,229
Total number of non-ATGC characters	140	71
Percentage of non-ATGC characters	0.015	0.019
Contigs ≥ 200 bp	1809	808
Contigs ≥ 500 bp	606	207
Contigs ≥ 1 Kbp	169	48
Contigs ≥ 10 Kbp	0	0
N50 value	617	513

Table 3.6. Unigene statistics of *Jatropha curcas* L. transcriptome under elevated CO_2.

Number of transcripts identified	69581
Maximum Contig Length	10713
Minimum Contig Length	200
Average Contig Length	1,657.4 ± 1,136.6
Median Contig Length	425
Total Contigs Length	11,53,25,317
Total Number of Non-ATGC Characters	3425
Percentage of Non-ATGC Characters	0.003
Contigs >= 200 bp	69581
Contigs >= 500 bp	58502
Contigs >= 1 Kbp	46868
Contigs >= 10 Kbp	2
N50 Value	2225

assigned to unigenes belonged to ATP binding in molecular function, integral to membrane in cellular functions and carbohydrate metabolic process in biological function category respectively (**Fig. 3.8A**). A total of 27,125 (~38 %) unigenes from ambient and elevated CO_2 grown *Jatropha* were annotated based on EuKaryotic Orthologous Group (KOG) classification (**Fig. 3.8B**). KEGG was used for classifying the unigenes into various metabolic pathways. In total, 8345 unigenes had blast hits and 6568 were assigned to 275 known pathways. The majority of annotated transcripts were associated to carbohydrate metabolism (~14.98%) followed by translation (~12.46%), protein folding, sorting and degradation (~10.65%) and amino acid metabolism (~9.08%) (**Fig. 3.9**).

The GO terms of 'carbohydrate metabolic process', 'proteolysis', 'lipid metabolic process', 'transcription' and 'regulation of transcription' were highly enriched for the DEGs within the biological process category (**Fig. 3.10**). Most of genes categorized in molecular function were involved in 'catalytic activity' and 'binding activity' (**Fig. 3.10**).

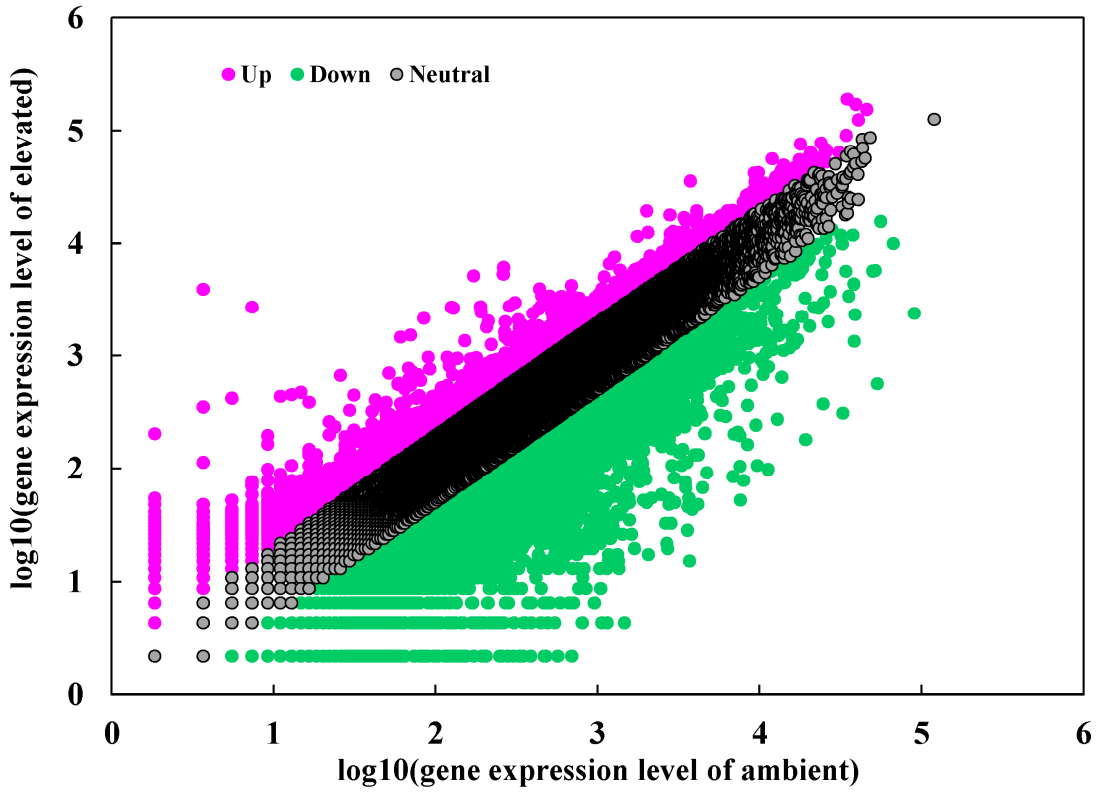


Fig. 3.7. Differentially expressed genes in the leaves of elevated CO_2 compared with those in the ambient conditions. The significantly up-regulated genes by elevated CO_2 treatment were marked in magenta pink while the significantly down-regulated genes were marked in green with the threshold of (log_2Ratio) ≥ 1.

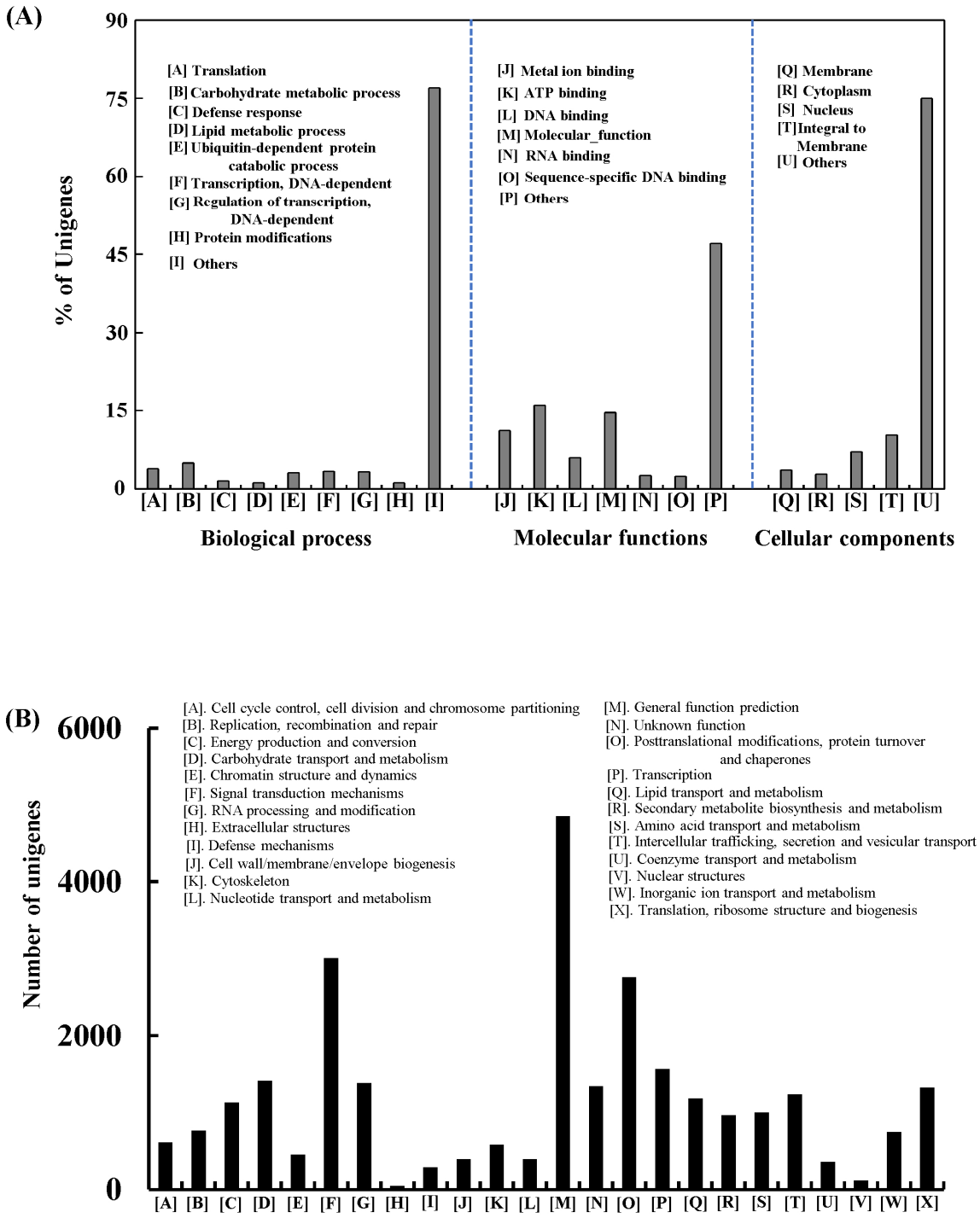


Fig. 3.8. (A) GO annotations for *Jatropha* leaf unigenes. (B) Distribution of unigenes according to KOG database.

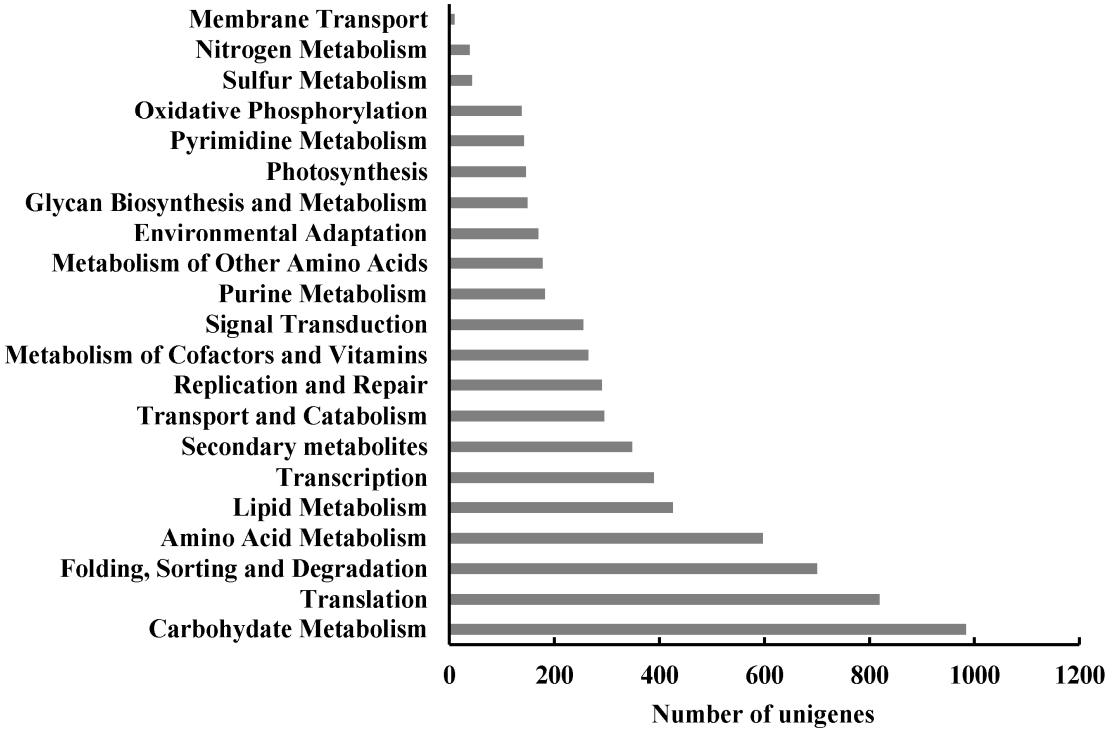


Fig. 3.9. Distribution of unigenes into biological pathways using KEGG.

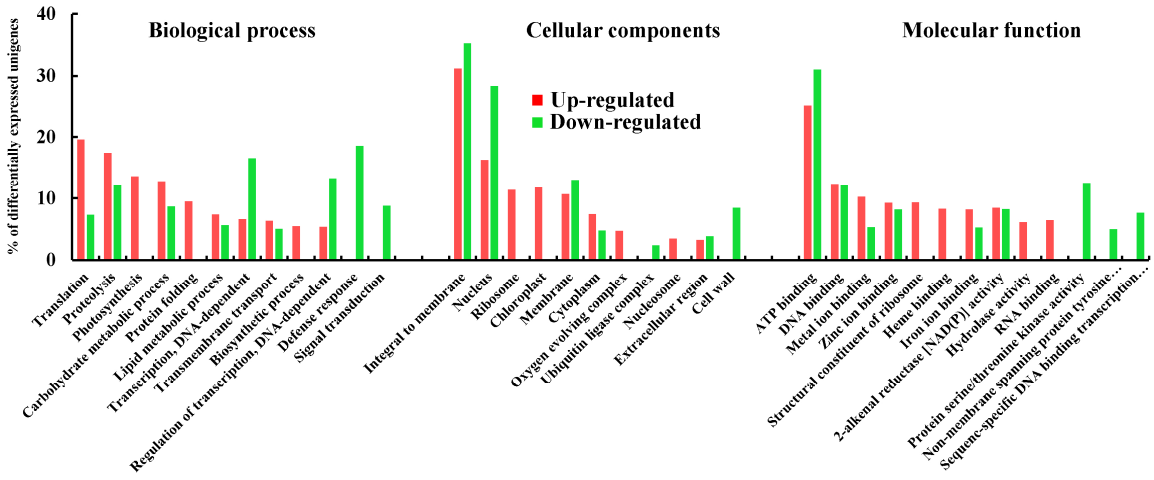


Fig. 3.10. Enriched GO terms of the differentially expressed unigenes in leaves of elevated CO_2 grown *J. curcas*. The GO terms are categorized into 'Molecular function', 'Cellular component' and 'Biological pathway' with the percentage of unigenes up-regulated depicted in red and down-regulated in green for each term.

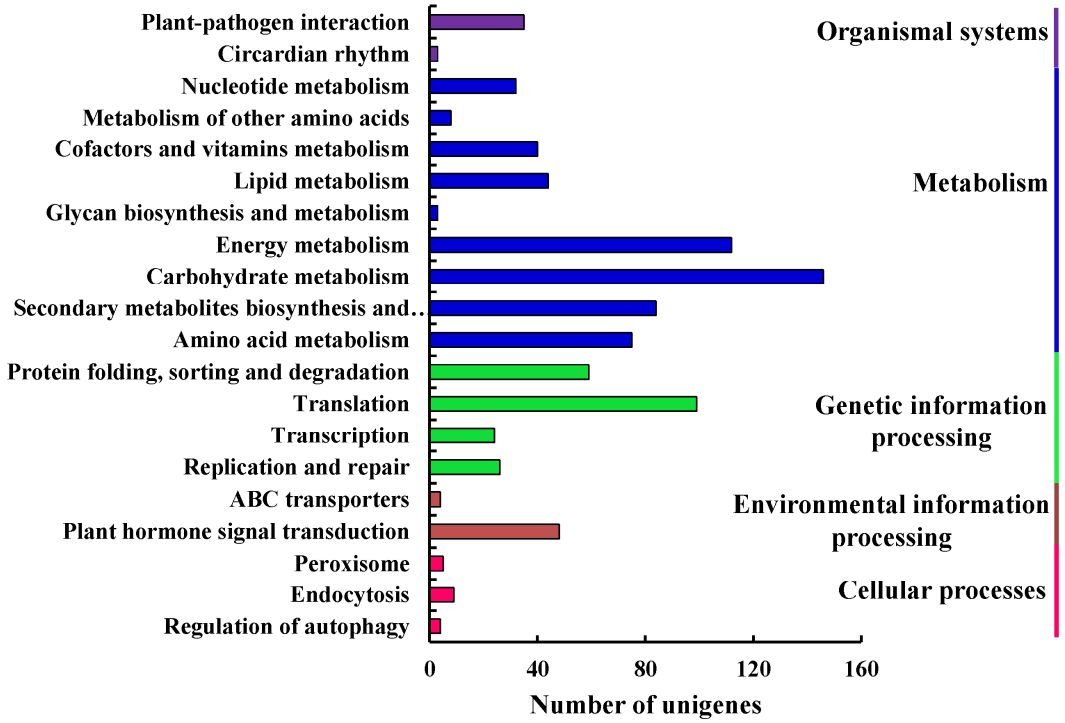


Fig. 3.11. Enriched KEGG classification terms of the differentially expressed unigenes in leaves of elevated CO_2 grown *J. curcas*. A total of 860 DEGs were assigned to 85 KEGG pathways. The number of DEGs belonging to each category are provided.

The top categories in cellular components included 'integral to membrane', 'nucleus' and 'ribosome' (**Fig. 3.10**). A majority of up-regulated DEGs belonged to 'protein synthesis and degradation', 'photosynthesis' and 'carbohydrate metabolic process', while most of down-regulated DEGs were related to 'defense response' and 'DNA-dependent regulation of transcription' in the biological process category (**Fig. 3.10**). A total of 860 DEGs from leaves of elevated CO_2 grown *Jatropha* were assigned to 85 KEGG pathways (**Fig. 3.11**). The most abundant KEGG pathways in our analysis were 'ribosome' (15%), 'photosynthesis' (11%) and 'plant hormone signal transduction' (11%) (**Fig. 3.11**).

3.3.3.1 Photosynthesis and carbohydrate metabolism

Elevated CO_2 conditions significantly induced the up-regulation of transcripts putatively encoding light harvesting chlorophyll a-b binding proteins, which are actively involved in the efficient transfer of excitation energy to the photosystems by ~1.7 folds (**Table 3.7**).

Further, there was significant up-regulation of transcripts belonging to PSII protein complex components [D1 protein (PsbA) {~2.6 folds}, PS (II) CP47 chlorophyll apoprotein (PsbB) {~1.4 folds}, PS (II) CP43 chlorophyll apoprotein (PsbC) {~1.7 folds}, oxygen evolving complex protein (PsbP) {~1.6 folds} and oxygen evolving enhancer protein 3-1 {~1.7 folds}], and PSI protein complex [PSI P700 chlorophyll a apoprotein A1 (PsaA) {~2.1 folds}] (**Table 3.7**). Similarly, there was significant upregulation of transcripts encoding NADH dehydrogenase 1-like or NDH-1 plastoquinone reductase subunits B, K and T by ~2.2 folds, ~2 folds and ~1.6 folds respectively, petH; ferredoxin-$NADP^+$ reductase (FNR) by ~1.6 folds and F-type H^+-transporting ATPase subunit beta (FTA) by ~1.6 folds, which play an important role in photosynthetic electron transport and ATP synthesis (**Table 3.7**). CO_2 enrichment promoted the expression of the genes involved in photosynthetic carbon reduction cycle. The DGE analysis showed up-regulation in transcript levels of regulatory enzymes of carbon reduction cycle and pentose phosphate pathway including rubisco, both large and small subunit by ~2.3 and ~1.5 folds respectively, sedoheptulose-1,7-bisphosphatase (SB) by ~1.5 folds, phosphoribulokinase (PRK) by ~1.8 folds, transketolase (TKL) by ~1.5 folds, glucose-6-phosphate dehydrogenase (G6PD) by ~1.1 folds, ribose-5-phosphate isomerase (RI) by ~1.7 folds, chloroplastic triose phosphate isomerase (TPI) by ~1.5 folds and chloroplastic NADP dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by ~1.5 folds in elevated CO_2 grown plants (**Table 3.7**).

A comprehensive pathway analysis of the DEGs identified in leaves under elevated CO_2 revealed enrichments in glycolysis, starch synthesis and sucrose metabolism. The expression of DEGs putatively encoding granule bound starch synthase (GBSS) which is associated with starch synthesis was found to be up-regulated by ~1.7 folds in the DGE analysis (**Table 3.7**). Also, CO_2 treatment significantly up-regulated transcripts putatively encoding for glycolysis including hexokinase3 (HXK3) by ~1.6 folds, phosphofructokinase (PFK) by ~1.4 folds, cytosolic aldolase by ~1.6 folds, and cytosolic TPI by ~1.5 folds respectively (**Table 3.7**). Interestingly, we found unigenes putatively encoding hexokinase1 (HXK1) to be down-regulated by ~1.1 folds (**Table 3.7**). We also found significant up-regulation of sequences putatively encoding enzymes of sucrose

metabolism including sucrose phosphate synthase 1 (SPS1) by ~2.4 folds, fructose 1,6-bisphosphatase (FB) by ~1.6 folds, and phosphoglucomutase (PGM) by ~2.1 folds (**Table 3.7**). However, one unigene putatively encoding for sucrose phosphate synthase 3 (SPS3) was found to be down-regulated by ~1.9 folds (**Table 3.7**). Also, significant up-regulation in transcripts encoding for chloroplastic triose phosphate/phosphate translocator by ~1.5 folds was noticed while down-regulation for chloroplastic glucose 6-phosphate/phosphate translocator by ~1.8 folds indicating increased exchange of triose phosphates between chloroplast and cytosol (**Table 3.7**). Significant up-regulation was also recorded for sequences putatively encoding for carbonic anhydrase (CA) by ~2.1 folds and rubisco activase (RA) by ~1.6 folds (**Table 3.7**).

3.3.3.2 Chlorophyll metabolism

Since chlorophyll is the driving force for the light-dependent reactions of photosynthesis, the transcript abundance of sequences associated with chlorophyll metabolism were analyzed. The chlorophyll synthase gene showed no significant expression change under CO_2 enriched conditions in *Jatropha*. Uroporphyrinogen decarboxylase, which catalyzes the first committed step of chlorophyll biosynthesis, and magnesium-protoporphyrin O-methyltransferase encoding transcripts were the only two members of chlorophyll biosynthesis which were significantly up-regulated by ~1.6 and ~1.5 folds respectively (**Table 3.7**). Also, chlorophyllase-1, which participates in chlorophyll breakdown was found to be up-regulated by ~1.6 folds in elevated CO_2 treated plants (**Table 3.7**).

3.3.3.3 Cell wall metabolism and remodelling

The cell wall metabolic machinery associated with the growth and development of plants involve numerous members of glycosyltransferases, glycoside hydrolases, carbohydrate esterases and polysaccharide lyases. We did not find any enrichment in the CesA genes, which is presumed to encode for the catalytic subunits of cellulose synthase. However, we found down-regulation of ~2.9 folds in one unigene putatively encoding for cellulose synthase-like protein D1 which is involved in the formation of hemicellulose backbones (**Table 3.7**). There was significant down-regulation identified in sequences encoding putatively for enzymes associated with xyloglucan biosynthesis, xyloglucan glycosyltransferase 4 (~2.4 folds) and xyloglucan glycosyltransferase 12 (~2.1 folds).

Table 3.7. Differentially regulated unigenes [log_2fold change ≤-1.00 or ≥1.00 (elevated versus ambient)] associated with photosynthesis and carbohydrate metabolism in *Jatropha* after one year of growth under elevated CO_2 identified in the RNA-seq analysis. The gene IDs were assigned according to *Jatropha* genome version JatCur_1.0 (http://www.ncbi.nlm.nih.gov/genome/jatrophacurcas) with a blast score ≥ 80. [Pink: up-regulation; Green: down-regulation].

Pathway	Description	No. of unigenes	Gene ID	Transcript length	% Similarity (BlastX)	Log2fold change
Photosynthesis: light-dependent reactions	Chlorophyll a-b binding proteins	2	105637992	1089; 1128	100%; 99%	1.67; 1.8
	D1 protein (PsbA)	1	7564824	1296	99%	2.63
	PS (II) CP47 chlorophyll apoprotein (PsbB)	1	7564760	2011	100%	1.44
	PS (II) CP43 chlorophyll apoprotein (PsbC)	1	7564849	4040	100%	1.75
	Oxygen evolving complex protein (PsbP)	2	105641916; 105639240	1305; 976	100%; 87%	1.59; 1.98
	Oxygen evolving enhancer protein 3-1	5	105632870	709; 791; 603; 684; 566	99%; 98%; 100%; 77%; 100%	1.84; 1.58; 1.71; 1.66; 1.84
	PSI P700 chlorophyll a apoprotein A1 (PsaA)	1	7564856	5561	100%	2.09
	NDH-1 plastoquinone reductase subunit (subunit B)	1	7564781	4635	97%	2.23
	NDH-1 plastoquinone reductase subunit (subunit K)	1	7564864	1941	99%	2.03
	NAD(P)H-quinone oxidoreductase subunit T, chloroplastic	3	105636994	624; 989; 1153	100%; 99%; 100%	1.71; 1.47; 1.65

Table 3.7 contd.

Pathway	Description	No. of unigenes	Gene ID	Transcript length	% Similarity (BlastX)	Log2fold change
Photosynthesis: light-dependent reactions	Ferredoxin-NADP$^+$ reductase (petH)	7	105635331;105629056	877; 1853; 1157; 962; 1170; 1652; 1557	99%; 100%; 100%; 100%; 97%; 81%; 100%	1.48; 1.65; 1.42; 1.67; 1.57; 1.6; 1.61
	F-type H$^+$-transporting ATPase subunit beta	1	7564869	2193	100%	1.63
Carbon reduction cycle and pentose phosphate pathway	Rubisco large subunit	1	7564870	1967	100%	2.33
	Rubisco small subunit	2	105642030	2052; 1953	79%;100%	1.52; 1.52
	Sedoheptulose-1,7-bisphosphatase	3	105646676	1671; 1727; 1913	100%; 100%; 100%	1.58; 1.53; 1.55
	Phosphoribulokinase	2	105631767	1795; 1867	99%; 100%	1.49; 2.25
	Transketolase	2	105643137	4213; 4127	99%; 100%	1.53; 1.48
	Glucose-6-phosphate dehydrogenase	1	105641505	3043	100%	1.09
	NADP dependent glyceraldehyde-3-phosphate dehydrogenase	2	105630249	770; 619	92%; 89%	1.45; 1.58
	Triose phosphate isomerase, chloroplastic	1	105643783	1017	100%	1.51
	Ribose-5-phosphate isomerase	2	105647678; 105636799	1251; 1014	100%; 100%	1.65; 1.87
Starch and sucrose metabolism	Granule bound starch synthase	8	105639084	2286; 2288; 2378; 2278; 2219; 2312; 2314; 2221	99%; 95%; 92%; 94%; 100%; 95%; 91%; 100%	1.67; 1.53; 1.54; 1.48; 1.77; 1.8; 1.64; 1.8

Table 3.7 contd.

Pathway	Description	No. of unigenes	Gene ID	Transcript length	% Similarity (BlastX)	Log2fold change
Starch and sucrose metabolism	Phosphoglucomutase	3	105634080	2178; 2347; 2260	100%; 93%; 97%	2.12; 2.46; 1.93
	Sucrose phosphate synthase 1	2	105637611	3763; 3644	100%; 100%	2.93; 1.93
	Sucrose phosphate synthase 3	1	105633156	3676	100%	-1.95
	Fructose 1,6-bisphosphatase, cytosolic	3	105629115	1109; 1836; 877	94%; 99%; 89%	1.69; 1.59; 1.57
Glycolysis	Hexokinase 1	4	105631535	2178; 1114; 2369; 2204	99%; 99%; 100%; 100%	-1.25; -1.1; -1.02; -1
	Hexokinase 3	1	105648314	2051	100%	1.59
	Phosphofructokinase	2	105632412	812; 1050	89%; 99%	1.56; 1.32
	Aldolase, cytosolic	5	105639139; 105643837	1432; 1316; 2252; 2024; 1807	100%; 100%; 77%; 77%; 55%	1.28; 1.2; 1.02; 1.09; 1.21
	Triose phosphate isomerase, cytosolic	7	105647635	723; 1216; 2184; 1298; 1883; 1687; 1348	94%; 100%; 99%; 100%; 92%; 97%; 100%	1.56; 1.43; 1.48; 1.44; 1.54; 1.56; 1.59
Others	Chloroplastic triose phosphate/phosphate translocator	11	105649590	959; 2158; 1551; 654; 2069; 1761; 2065; 2153; 2127; 2184; 1629	100%; 100%; 100%; 100%; 100%; 100%; 100%; 100%; 100%; 91%; 100%	1.46; 1.42; 1.52; 1.52; 1.53; 1.47; 1.43; 1.38; 1.51; 1.49; 1.51

Table 3.7 contd.

Pathway	Description	No. of unigenes	Gene ID	Transcript length	% Similarity (BlastX)	Log2fold change
Others	Chloroplastic glucose 6-phosphate/phosphate translocator	2	105645263	1694; 1700	98%; 100%	-2.26; -1.44
	Rubisco activase	5	105647331	2587; 1300; 1867; 2586; 2460	47%; 43%; 73%; 45%; 50%	1.66; 1.58; 1.5; 1.51; 1.68
	Carbonic anhydrase	4	105641553; 105640386	4207; 666; 1140; 1042	44%; 97%; 99%; 100%	1.5; 2.81; 2.1; 2.16
Chlorophyll metabolism	Uroporphyrinogen decarboxylase	4	105637012	1942; 1941; 1856; 1538	99%; 99%; 100%; 99%	1.51; 1.66; 1.63; 1.62
	Magnesium-protoporphyrin O-methyltransferase	1	105648719	1923	100%	1.51
	Chlorophyllase-1	3	105649943	1259; 1085; 1012	97%; 97%; 99%	1.67; 1.73; 1.59
Cell wall metabolism and remodelling	Cellulose synthase-like protein D1	1	105650364	1643	99%	-2.92
	Xyloglucan glycosyltransferase 4	3	105642017	3154; 3002; 2693	100%; 99%; 100%	-2.33; -2.53; -2.33
	Xyloglucan glycosyltransferase 12	5	105630800	4097; 2267; 3996; 3360; 3189	100%; 100%; 100%; 92%; 100%	-1.92; -2.47; -2.13; -2.03; -2.03
	Endoglucanase 16	1	105638367	1778	100%	-5.1
	Endoglucanase 17	1	105642560	2031	100%	1.62

Table 3.7 contd.

Pathway	Description	No. of unigenes	Gene ID	Transcript length	% Similarity (BlastX)	Log2fold change
Cell wall metabolism and remodelling	Pectin acetylesterase	1	105643391	1482	100%	-1.82
	Pectinesterase (Pectin methylesterases)	15	105642557; 105643313; 105648599; 105638008; 105638004; 105647823; 105632266; 105642557; 105629307; 105635078	2008; 1136; 1770; 1319; 833; 2276; 2004; 2079; 805; 1409; 733; 1505; 1921; 1227; 872	99%; 100%; 100%; 100%; 100%; 100%; 100%; 100%; 100%; 91%; 98%; 100%; 100%; 100%; 100%	-2.39; -4.91; -8.02; -5.26; -4.45; -2.02; -1.94; -1.45; -3.59; 1.73; 1.92; 1.56; -2.01; 2.42; -1.79
	Polygalacturonases	6	105648836; 105629564; 105644635	1696; 2441; 2075; 1826; 2145; 576	100%; 98%; 99%; 100%; 99%; 100%	-3.04; -2.12; -1.8; -2.65; -1.82; 3.25
	Expansin-A11	2	105635297	1337; 1248	100%; 100%	3.17; 3.14
	Beta-expansin 3	6	105643364; 105643365	1429; 1082; 1236; 705; 1397; 1212	100%; 100%; 99%; 88%; 89%; 99%	1.52; 1.72; 1.49; 1.58; 1.71; 1.91
	Xyloglucan endotransglucosylase/hydrolase protein1	1	105630427	1237	100%	1.79
	Xyloglucan endotransglucosylase/hydrolase protein7	4	105639898	1658; 1539; 1326; 675	99%; 99%; 99%; 100%	1.61; 1.76; 1.59; 1.84
	Xyloglucan endotransglucosylase/hydrolase protein22	2	105635614; 105635621	1363; 658	100%; 82%	-1.51; -3.68

Table 3.7 contd.

Pathway	Description	No. of unigenes	Gene ID	Transcript length	% Similarity (BlastX)	Log2fold change
Cell wall metabolism and remodelling	Xyloglucan endotransglucosylase/hydrolase protein23	20	105635617; 105635620; 105629273; 105629275; 105635618; 105635616; 105635615	2136; 1735; 1607; 1382; 622; 1631; 1295; 771; 766; 1192; 1050; 1357; 1759; 1890; 1358; 1520; 1289; 1033; 1108; 1181	86%; 78%; 97%; 97%; 99%; 98%; 100%; 65%; 79%; 99%; 100%; 99%; 80%; 84%; 96%; 100%; 99%; 100%; 99%; 89%	-3.99; -3.65; -3.92; -3.46; -3.57; -4.49; -2.79; -3.94; -3.87; -3.62; -3.65; -3.72; -3.44; -3.91; -2.77; -2.54; -3.38; -2.25; -3.68; -3.82
	Xyloglucan endotransglucosylase/hydrolase protein30	1	105640962	1218	100%	-3.26

Endo-1,4-β-glucanases, a hemicellulose degradative enzyme was found to be differentially regulated with one unigene sequence putatively encoding for endoglucanase 17 up-regulated by ~1.6 folds and another, endoglucanase 16 down-regulated by ~5.1 folds (**Table 3.7**). Significant differential regulation was found in sequences putatively encoding for pectin modifying enzymes, methylesterases and acetylesterases. Pectin acetylesterase and few pectin methylesterases were found to down-regulated by ~1.8 and ~3.9 folds respectively, in leaves of *Jatropha* grown under elevated CO_2 (**Table 3.7**). Interestingly, some transcripts putatively encoding different members of pectin methylesterases were found to be up-regulated by ~1.9 folds under same conditions (**Table 3.7**). Similarly, differential regulation was observed in sequences putatively encoding for another family of enzymes involved in pectin metabolism, polygalacturonases with transcripts both up- and down-regulated by ~3.2 and ~2.3 folds respectively (**Table 3.7**). Cell wall expansion involves the interplay of cell wall loosening enzymes and other proteins. Two transcripts encoding expansin-A11 and six putatively encoding β-expansin 3 were up-regulated by ~3.1 and ~1.6 folds respectively, in CO_2 enriched conditions (**Table 3.7**). Besides expansins, xyloglucan endotransglucosylase/hydrolases (XTHs) are also suggested to regulate cell wall loosening and expansion. We found 32 unigene sequences putatively encoding for XTHs differentially regulated. Some common XTHs like XTH1 (~1.8 folds) and XTH7 (~1.6 folds) were up-regulated while XTH22 (~1.5 folds), XTH23 (~3.5 folds) and XTH30 (~3.2 folds) were down-regulated (**Table 3.7**).

3.3.3.4 Transcription factors

We identified representations from known TF families reported for plants and also putative uncharacterized TFs in the assembled unigenes from ambient and elevated CO_2-grown *Jatropha* plants (**Fig. 3.12**). The differentially expressed family of TFs identified were MYB (up – 3; down – 14), bHLH (up – 8; down – 10), GATA (up – 5; down – 1), NAC (up – 3; down – 12), nuclear (up – 2; down – 11), MADS (up – 3; down – 7), WRKY (down – 20), AP2/ERF (up – 4; down – 23), zinc finger (up – 2; down – 14) and others (up – 27; down – 50) (**Fig. 3.13**).

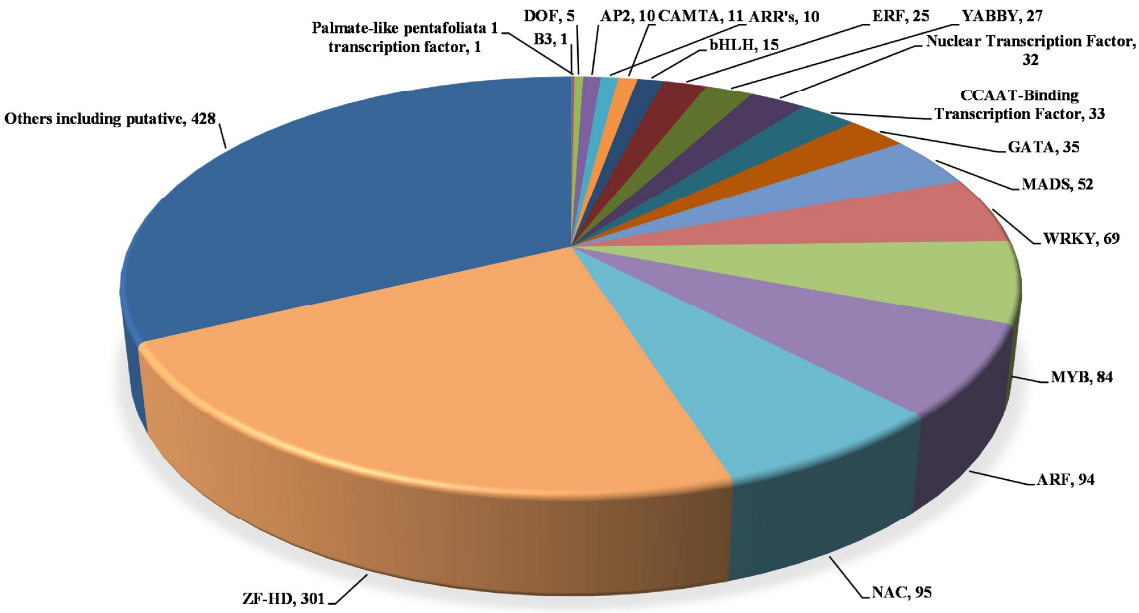


Fig. 3.12. Distribution of unigenes into transcription factor families with the number representing the absolute number identified for that particular family.

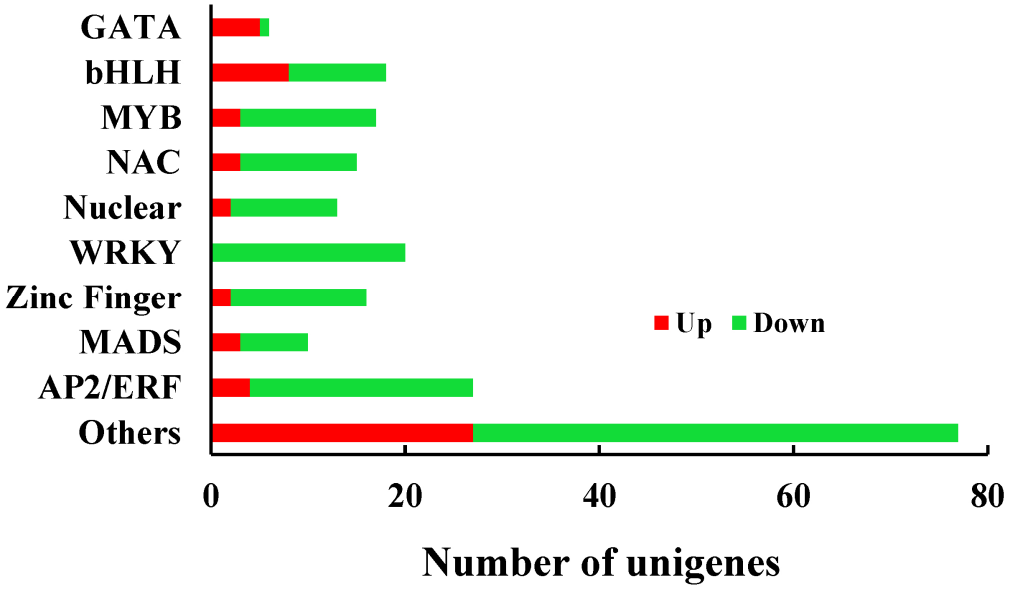


Fig. 3.13. Transcription factors differentially expressed in leaves of elevated CO_2 grown *Jatropha*.

3.3.4 Temporal expression pattern of key regulatory genes of photosynthesis exhibit efficient carbon conversion in elevated CO_2 grown Jatropha

To understand the expression trends of key regulatory genes of photosynthesis and associated metabolism for carbon conversion, we quantified the expression of thirty-five identified DEGs in leaves collected at four different time points [90, 180, 270 and 360 days after treatment (DAT)] during the course of growth of plants in elevated CO_2 through qRT-PCR. The temporal expression of DEGs corresponding to two subunits of PS II (PsbB and PsbP) were found to be up-regulated by 2.1, 2.03, 1.98, 1.83 and 2.82, 2.66, 1.87, 0.98 folds respectively, at all four time points of growth in elevated CO_2 (**Fig. 3.14**). Similarly, PsaA and PsbA were up-regulated at 180, 270, 360 and 90, 180, 360 DAT respectively with no change in expression at 90 (PsaA) and 270 (PsbA) DAT (**Fig. 3.14**). PsbC was found to be up-regulated at 90 and 360 DAT by 1.61 and 1.41 folds respectively. However, no change in expression at 180 DAT and down-regulation of -2.05 folds was recorded for PsbC at 270 DAT (**Fig. 3.14**). Similarly, FNR was up-regulated at 180 and 360 DAT (the two time points corresponding to the reproductive growth) only by 2.29 and 1.77 folds respectively and no change in expression at 90 and 270 DAT in the leaves of elevated CO_2 grown *Jatropha* (**Fig. 3.14**). FTA showed down-regulation at 90 DAT and no change in expression at 180, 270 and 360 DAT (**Fig. 3.14**). Both NADH dehydrogenase subunit B and K recorded significant up-regulation at 90, 270 and 360 DAT with down-regulation at 180 DAT (**Fig. 3.14**).

Similarly, DEGs pertaining to carbon conversion also showed varied temporal expression trends in the leaves of elevated CO_2 grown *Jatropha* (**Fig. 3.15**). Rubisco large subunit (RL) was significantly up-regulated at all four time points of growth by 1.05, 1.16, 1.37 and 1.57 folds respectively. Similarly, rubisco small subunit (RS) was significantly up-regulated at 90 and 270 DAT by 1.64 and 1.11 folds respectively with no change in expression at 180 and 360 DAT respectively (**Fig. 3.15**). RA, an essential regulator of rubisco, also showed co-ordinated expression along with rubisco and was found to be significantly induced in expression at 180, 270 and 360 DAT (**Fig. 3.16**). Similarly, PRK and TKL recorded up-regulation at 90, 180 and 360 DAT by 1.88, 1.65, 1.28 and 1.09, 2.45 and 1.03 folds respectively, with no change in expression at 270 DAT (**Fig. 3.15**).

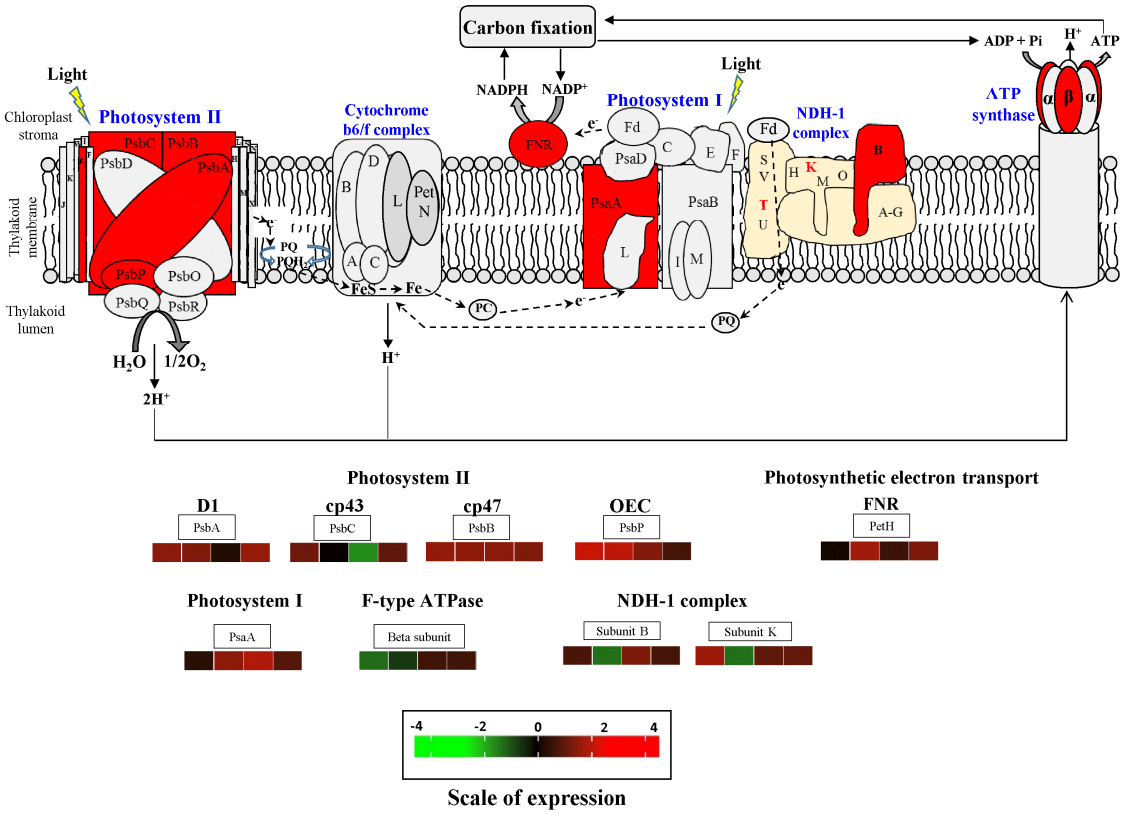


Fig. 3.14. A comprehensive photosynthesis pathway depicting differentially expressed unigenes in multi-subunit complexes (photosystem I and II, NADH dehydrogenase and ATP synthase) participating in light-dependent reactions. The different subunits differentially regulated of each components (identified by differential gene expression analysis) are indicated in red and their expression patterns at different time points (90, 180, 270 and 360 DAT) in elevated CO_2 grown plants with respect to ambient plants are represented. Different colour shades represent level of expression with red representing up-regulation while green as down-regulation. The four different boxes beneath each differentially regulated gene represent the four time points starting from 90 DAT to 360 DAT (direction: left to right).

Chloroplastic TPI also recorded up-regulation at 90, 180 and 270 DAT by 1.92, 1.39 and 2.04 folds respectively in elevated CO_2 growth conditions. SB was also significantly up-regulated at three time points (180, 270 and 360 DAT) of growth while GAPDH and RI recorded up-regulation at 270 DAT only by 2.56 and 1.04 folds respectively (**Fig. 3.15**). CA, which participates in transport of inorganic carbon to actively photosynthesizing cells, was found to be up-regulated at 180, 270 and 360 DAT by 1.92, 1.76 and 3.43 folds respectively (**Fig. 3.16**). G6PD, a key regulatory enzyme of pentose phosphate pathway, was up-regulated at 180 and 360 DAT by 1.77 and 1.63 folds respectively and down-regulated at 270 DAT by -1.79 folds. There was no change in expression recorded for G6PD at 90 DAT. The starch synthesis regulatory enzyme, GBSS under CO_2 enriched environment recorded up-regulation at 180, 270 and 360 DAT by 1.52, 3.27 and 1.89 folds respectively (**Fig. 3.15**). PGM, an important regulatory gene involved in both starch and sucrose metabolism, was up-regulated at 270 and 360 DAT by 1.7 and 2.34 folds respectively, with no change in expression at 90 and 180 DAT (**Fig. 3.15**). Similarly, FB showed significant up regulation at 90, 270 and 360 DAT by 2.19, 1.47 and 2.36 folds respectively (**Fig. 3.15**). Both SPS1 and SPS3 recorded differential regulation at different time points. SPS1 showed up-regulation at 270 and 360 DAT by 3.09 and 3.22 folds respectively with no change in expression at 90 and 180 DAT (**Fig. 3.15**). SPS3 was up-regulated at 90 DAT by 2.75 folds but the expression was significantly reduced at 180, 270 and 360 DAT by -1.79, -3.11 and -1.53 folds respectively (**Fig. 3.15**). The key regulatory enzymes of glycolysis also showed significant down-regulation at the four tome points. PFK recorded significant up-regulation at 360 DAT only with no change in expression recorded at 90, 180 and 270 DAT (**Fig. 3.15**). However, cytosolic aldolase showed up-regulation at all four time points by 1.77, 2.75, 1.53 and 2.57 folds in leaves of *Jatropha* grown under CO_2 enriched atmosphere (**Fig. 3.15**). Cytosolic TPI showed up-regulation during 90 and 180 DAT by 1.54 and 1.3 folds respectively with no significant change in expression recorded for 270 and 360 DAT (**Fig. 3.15**). The most interesting differential regulation was recorded for two isoforms of sugar sensing enzyme, hexokinase, at the four time points. HXK1 was significantly down-regulated at 90 and 270 DAT by -2.14 and -1.35 folds respectively with no change in expression at 180 and 360 DAT (**Fig. 3.15**).

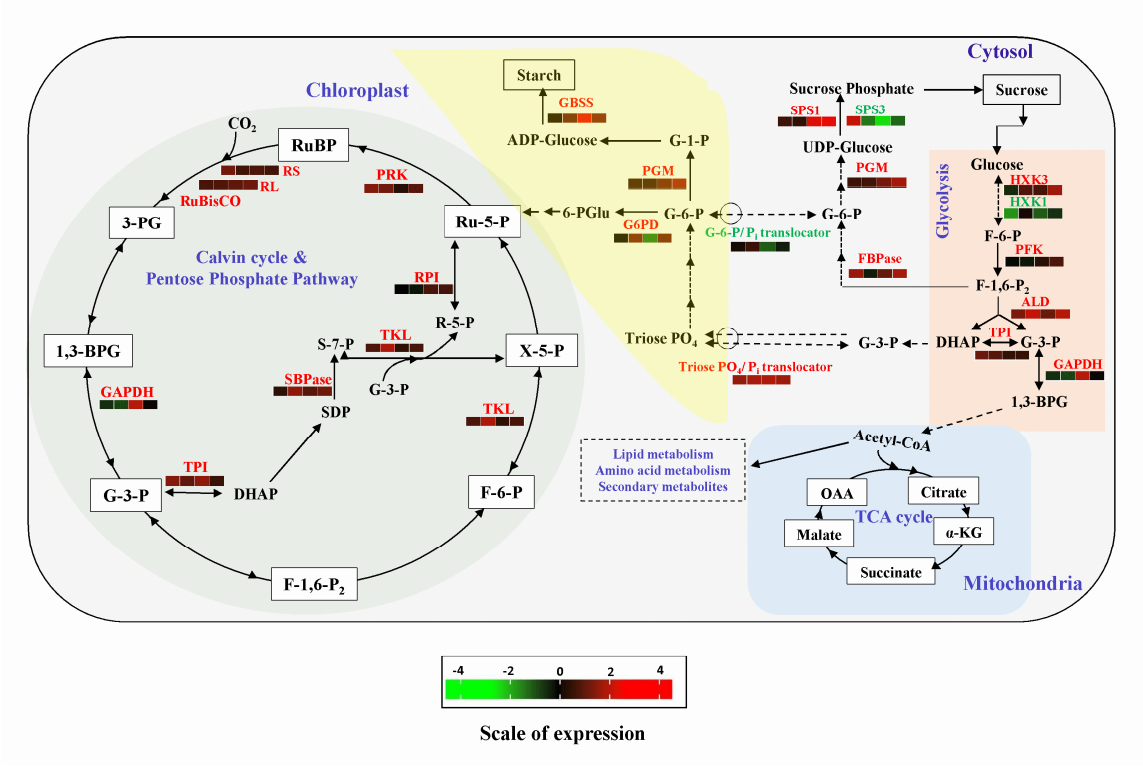


Fig. 3.15. Schematic representation of temporal expression pattern for crucial regulatory genes of carbon metabolic hub that may play critical roles in driving carbon conversion and exchange of intermediates between chloroplast and cytosol involving Calvin-Benson cycle, pentose phosphate pathway, glycolysis, sucrose metabolism and starch biosynthesis in elevated CO_2 grown plants at different time points (90, 180, 270 and 360 DAT) with respect to ambient CO_2 grown plants. Different colour shades represent level of expression with red representing up-regulation while green as down-regulation. The four different boxes beneath each differentially regulated gene represent the four time points starting from 90 DAT to 360 DAT (direction: left to right).

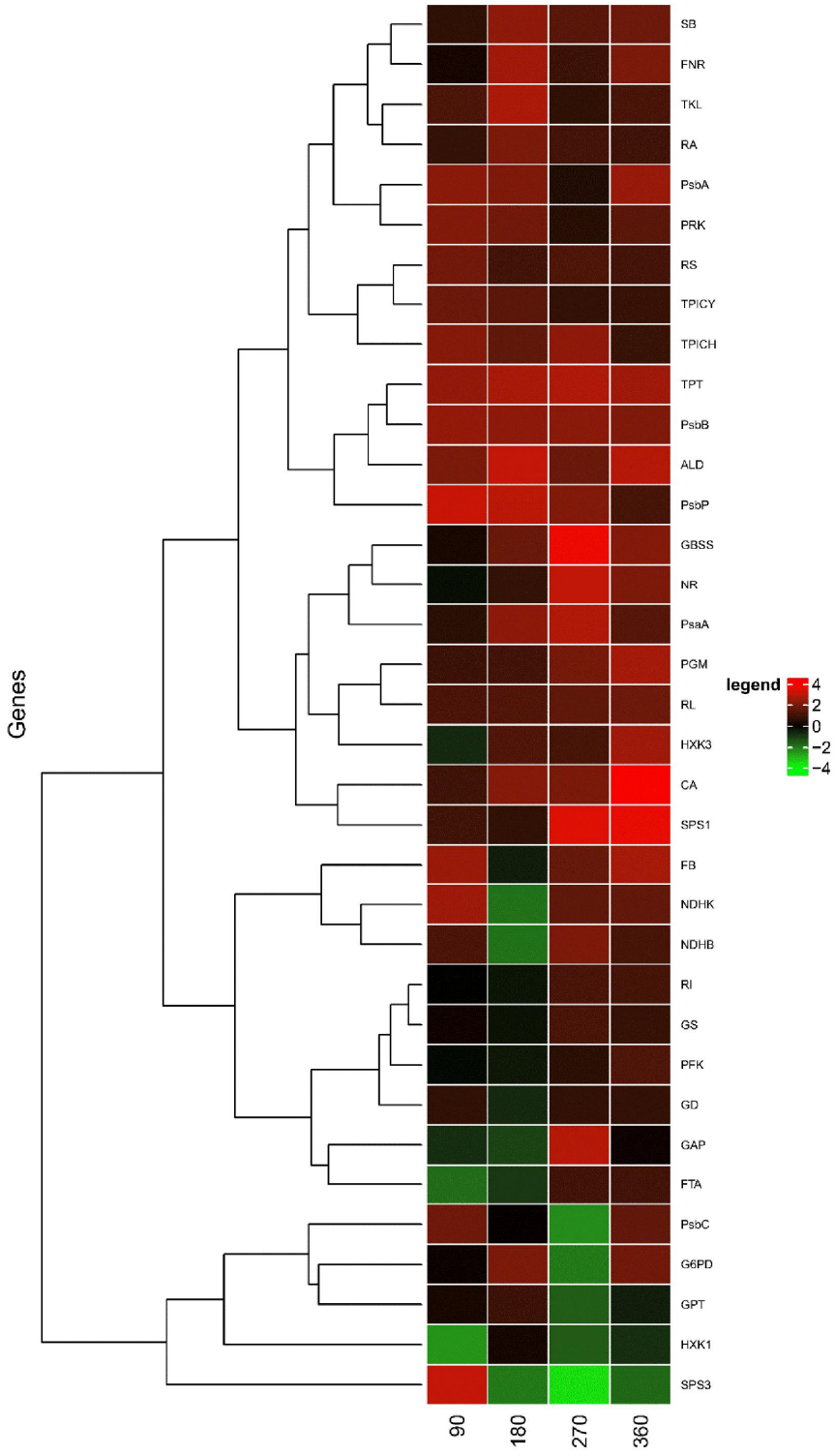


Fig. 3.16. A representative heat map of temporal expression pattern of 35 genes associated with photosynthesis and carbohydrate metabolism at four different time points as recorded in qRT-PCR analysis.

However, HXK3 recorded significant up-regulation at 180 and 360 DAT by 1.14 and 2.27 folds respectively with no change in expression at 90 and 270 DAT (**Fig. 3.15**). The two chloroplastic transporters, GPT and TPT also showed differential regulation at four time points. TPT showed significant up-regulation at all the four time points by 2.09, 2.41, 2.48 and 2.26 folds (**Fig. 3.15**). However, GPT showed significant down-regulation at 270 DAT by -1.38 folds with no significant change in expression recorded at 90, 180 and 360 DAT (**Fig. 3.15**).

Similarly, nitrogen metabolism associated gene nitrate reductase (NR) was also significantly induced under elevated CO_2 at 270 and 360 DAT by 2.71 and 1.79 folds respectively (**Fig. 3.16**). Glutamine synthetase (GS) showed up-regulation at 270 DAT while glutamate dehydrogenase (GD) showed basal level of expression at all the four time points of growth in *Jatropha* under elevated CO_2 (**Fig. 3.16**).

3.3.5 Enzyme activities of certain key regulatory enzymes associated with photosynthetic carbon reduction cycle

The enzyme activities of key regulatory enzymes of photosynthetic carbon reduction cycle positively correlated with the expression levels of the corresponding transcripts at the four time points. Both the initial and final activities of rubisco was recorded to be ~25% significantly higher ($P < 0.05$) in the leaves of elevated CO_2 grown *Jatropha* at all four time points in comparison to ambient grown plants (**Fig. 3.17A**). Similarly, RA, cytosolic FB and SPS demonstrated significantly higher ($P < 0.05$; $P < 0.01$) activities at all four time points in elevated conditions (**Fig. 3.17B, 3.17C, 3.17D**). The activity of HXK at 90 and 270 DAT was recorded to be significantly ($P < 0.05$) higher by ~40% in elevated conditions (**Fig. 3.17E**). However, there was no significant variation at 180 and 360 DAT, the reproductive growth phase of both seasons, recorded in the activity of HXK in elevated CO_2 conditions (**Fig. 3.17E**).

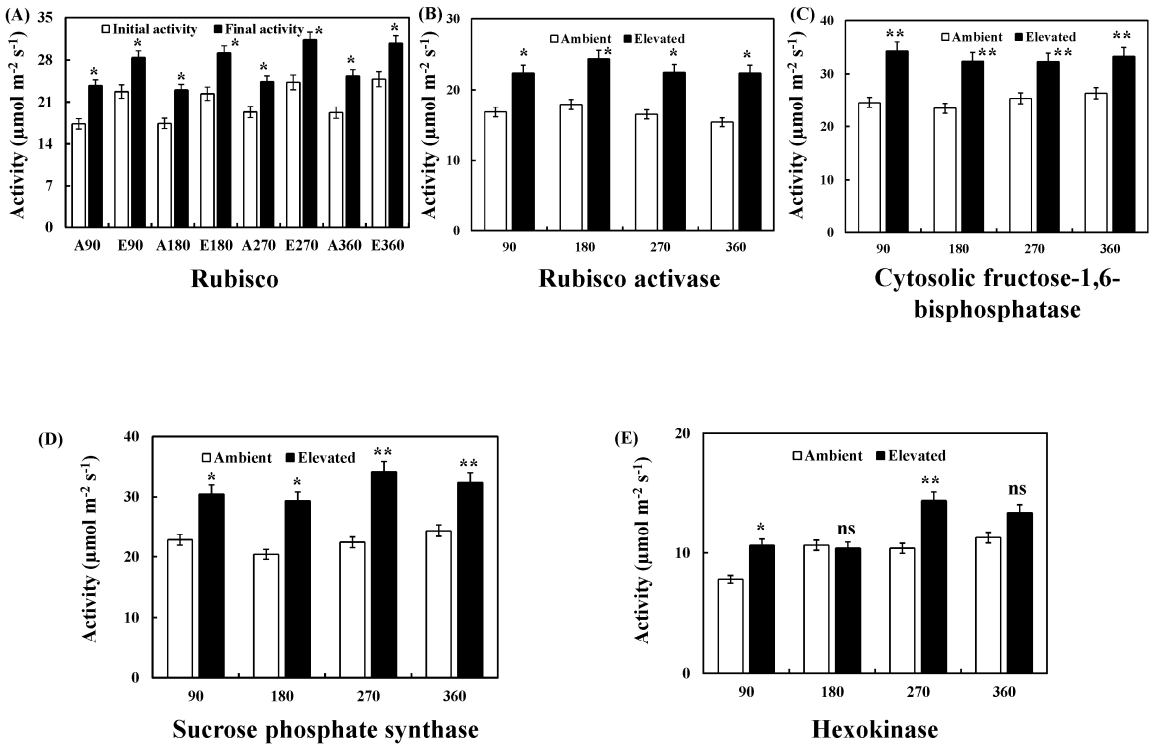


Fig. 3.17. Enzyme activities of key regulatory enzymes associated with photosynthetic carbon reduction cycle in *Jatropha* leaves at four different time points (90, 180, 270 and 360 DAT). The enzyme activities of (A) rubisco, (B) rubisco activase, (C) cytosolic fructose-1,6-bisphosphatase, (D) sucrose phosphate synthase and (E) hexokinase at all the four time points in the leaves of ambient (white bar) and elevated (black bar) CO_2 grown *Jatropha* plants. Values are expressed as mean ± SD. [Note – ns, not significant; **P* < 0.05; ***P* < 0.01].

3.4 Discussion

A better understanding of molecular responses under elevated CO_2 can aid in modifying and managing plants to take maximum advantage of globally increasing CO_2 which is also emphasized in earlier reports (Druart et al., 2006; Leakey et al., 2009c). In the present study, we combined transcriptomics with morphophysiology to investigate further the molecular mechanisms underlying the improved photosynthesis under elevated CO_2 in *Jatropha*. The library preparation in our study was significantly good as demonstrated by the assembly statistics in which N50 value, average contig length, total number of contigs, longest contig length and number of assembled unigenes were higher in both sample A and E libraries in comparison to similar studies on *Jatropha* transcriptome under different conditions (Zhang et al., 2014; Sapeta et al., 2016).

A yearlong growth spread across two growth cycles allowed us to assess the consistency of response of *Jatropha* in CO_2 enriched environment for two different seasons. *Jatropha* exhibited a net increase in photosynthetic activity, fast growth, increased biomass production and was not sink-limited at either growth seasons under elevated CO_2. The growth and development of *Jatropha* shows distinct pattern depending on the environmental conditions (Gour, 2006). A very interesting observation recorded was significant reproductive yield was recorded for *Jatropha* at 180 DAT under elevated CO_2 unlike ambient conditions which showed normal vegetative behaviour with very little or no fruits suggesting elevated CO_2 ameliorated the negative effects of temperature on the fruit formation and development (Gour, 2006; Wang et al., 2008). This clearly indicates the efficiency of *Jatropha* to positively maintain better resource utilization towards sink development in the form of biomass and fruit yield under elevated CO_2 environment irrespective of season thereby sustaining enhanced photosynthesis. These morphophysiological alterations in *Jatropha* under elevated CO_2 involve a transcriptomic reprogramming which includes DEG belonging to diverse GO and KAAS terms as evident in our annotation results. As cellular homeostasis is maintained by metabolic pathways in response to changes in the external environment, exposure of *Jatropha* to elevated CO_2 should have resulted in dynamic shift of metabolic fluxes to sustain increased

photosynthetic capacity and developmental plasticity which is in turn were regulated by gene expression levels. Accordingly, the expression profiles were tailored in *Jatropha* to modulate appropriate metabolic pathways in a coordinated manner to produce a coherent response towards sustaining enhanced photosynthesis in the elevated CO_2 environment.

The physiological data demonstrate that increased atmospheric CO_2 stimulated light-saturated photosynthesis in *Jatropha* through increased absorption of photosynthetically active photon flux density to be used for photochemistry and associated light reactions. These physiological findings were further corroborated by RNA-Seq and qRT-PCR assisted temporal expression analysis. Significant up-regulation in transcripts pertaining to PsbA, PsbB and PsbC in PSII as well as PsaA subunit of PSI under elevated CO_2 at different time points suggest an efficient energy transfer in the form of electrons from antenna complexes to photosystems reaction core for meeting the demands of energy requirement in the carbon reduction cycle (Foyer et al., 2012). Increased F_v/F_0 implies efficient water splitting complex on the donor side of the PS II, resulting improved photosynthetic electron transport capacity. This may be correlated with the up-regulation in transcript of PsbP subunit of oxygen evolving complex suggesting enhanced photooxidation of water and electron transport rates in *Jatropha* under elevated CO_2. However, down-regulation and no change in expression recorded for these genes at some time points indicate possible maintenance of balance in utilizing the light energy for electron transfer at different stages of growth which might involve other PSII and PSI subunits not identified as differentially regulated in this study (Ruban, 2009). FNR demonstrated interesting expression trend with up-regulation during reproductive development and maturation period (180 and 360 DAT) of *Jatropha* under elevated CO_2 suggesting an enhanced electron transport rates in coordination with other subunits to facilitate increase in reducing equivalent production to be utilised in carbon reduction cycle. However, no significant variation in expression during vegetative growth stage suggest possible involvement of other subunits associated with photosynthetic electron transport which has several rate-limiting steps, with FNR catalysing just one of them (Rodriguez et al., 2007). Similarly, differential regulation of both subunits of NDH complex, B and K suggests an optimum balance of electron flow around PSI and PSII in

maintaining increased photosynthesis capacity of *Jatropha* under elevated CO_2 (Yamori et al., 2015; Peltier et al., 2016). There was no correlation in the DGE expression and qRT-PCR expression of FTA indicating probable involvement of other subunits in optimising sustained ATP synthesis to maintain enhanced photosynthesis in elevated CO_2 grown *Jatropha* (Yamori et al., 2011). Our data on photochemical and non-photochemical quenching clearly indicate that most of the absorbed light energy was efficiently used in qP rather than NPQ in elevated CO_2-grown *Jatropha* plants. Altogether, elevated CO_2 increased photosynthesis, photochemistry (overall efficiency of PSII performance) and linear electron flow in *Jatropha* suggesting PSII-adaptive improved growth and productivity performance in elevated CO_2 environment (Ohnishi et al., 2005). Our results also demonstrated no significant changes in the chlorophyll concentrations and chlorophyll a/b ratio in elevated CO_2 conditions at all growth stages regardless of high photosynthetic rates which suggests efficient management of light absorption capacity and radiative energy balance of the photosystems to sustain high photosynthetic rates (Carter et al., 2000). The up-regulation of transcripts associated with both chlorophyll biosynthesis and breakdown in the DGE analysis indicate a possible co-regulation to maintain the chlorophyll concentrations in *Jatropha* under elevated CO_2 conditions.

The most important pathway for any plant is the photosynthetic carbon reduction (PCR) cycle which fixes the atmospheric CO_2 into organic forms to be utilized for growth and development. Most evidently, the DGE and temporal expression analysis showed up-regulation in transcript levels of enzymes of PCR cycle including rubisco (both RL and RS), SB, and PRK in elevated CO_2 grown *Jatropha* plants. The temporal expression pattern of rubisco (both subunits), SB and PRK, especially at 180 and 360 DAT which is the seed setting stage of *Jatropha* under elevated CO_2, indicates enhanced production of carbon intermediates for carbon fixation which correlates with our physiological and biochemical findings of sustained enhanced photosynthetic rates, biomass accumulation, reproductive yields and rubisco activity (Ogawa et al., 2015). The up-regulation of these transcripts possibly ensures enhanced supply of carbon skeletons to manage the demand from newly formed reproductive sink tissues. Further, GAPDH showed up-regulation only at 270 DAT suggesting enhanced photosynthesis and photochemistry of *Jatropha* is

independent of GAPDH expression under elevated CO_2. The upregulation of RA at 180 DAT suggests activation and maintenance of rubisco catalytic activity by promoting the removal of any tightly bound, inhibitory, sugar phosphates from the catalytic site of both the carbamylated and decarbamylated forms of rubisco under elevated CO_2 (Parry et al., 2008). However, there was no variations in expression levels of RA at 90, 270 and 360 DAT indicating balanced ATP/ADP ratios under elevated CO_2 to sustain enhanced photosynthesis in *Jatropha* under elevated CO_2 (Brandner and Salvucci, 2000). Further, the measured RA enzyme activity did not correlate with the transcript levels which may be attributed to the complexity of the relationship between RNA levels, protein levels, and physiological changes (Glanemann et al., 2003). Further, we observed significant enrichment in transcripts of enzymes participating in pentose phosphate pathway (PPP), G6PD, TKL and chloroplastic TPI which generates NADPH and carbon intermediates to meet the demand for carbon skeletons for both vegetative and reproductive sinks in elevated CO_2 grown *Jatropha* during both growth seasons (Krüger and von Schaewen, 2003). Furthermore, the expression analysis of RPI, which maintains pentose and hexose carbon pools in equilibrium with one another as the fluxes through the major metabolic pathways change in response to external environmental conditions did not correlate with DGE analysis with only up-regulation recorded at 270 DAT suggest involvement of other players in the regulatory network in maintaining the pentose and hexose carbon ratio (Xiong et al., 2009). An increase in foliar carbohydrate levels, particularly starch, is a common observation in elevated CO_2 environments which has been predicted to be responsible for photosynthetic acclimation in the absence of sink tissues (Davey et al., 2006). Our biochemical results demonstrated increased starch levels in leaf which correlated with significant upregulation in transcripts encoding granule bound starch synthase (GBSS) in elevated CO_2 conditions (Kötting et al., 2010). We have earlier proposed that *Jatropha* was able to escape photosynthetic downregulation in the later stages of growth due to availability of sufficient sinks like increased tertiary branches, flowers and fruits to sustain enhanced growth (Kumar et al., 2014). This implies efficient source-sink interaction and sustained photosynthetic potential of *Jatropha* under elevated [CO_2]. Moreover, differential regulation of nitrogen metabolism associated gene, NR

indicate improved nitrogen allocation and nitrogen use efficiency in the leaves of elevated CO_2 grown *Jatropha*. However, GS and GD did not demonstrate any differential expression in *Jatropha* under elevated CO_2 environment indicating nitrogen use is not regulated by these two genes at these four growth stages.

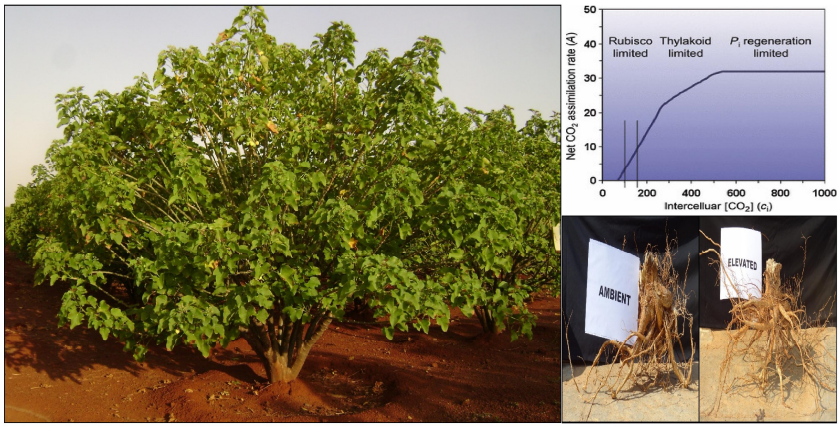
Our foliar biochemical results in this study showed increase in soluble sugar content in elevated CO_2-grown *Jatropha* plants. Starch and sucrose metabolisms are finely co-regulated involving key intermediate metabolites at the cross-road of catabolic and anabolic pathways for the control of carbon flux in the plant cell. There was significant up-regulation of transcripts pertaining to sucrose metabolism and glycolytic pathway. The significant up-regulation of transcripts pertaining to PGM, cytosolic FB, cytosolic TPI and cytosolic ALD imply improved sucrose metabolism in the cytosol (Ogawa et al., 2015). The enzyme activity of cytosolic FB complied with the temporal expression patterns as indicated in our results. The up-regulation of these transcripts also imply increased hexose sugar pools for various carbon skeletons to be provided to the developing sinks under elevated CO_2. However, PFK showed up-regulation at only 360 DAT suggesting its non-regulatory role under elevated CO_2 as it has been implicated in adaptation of plants in non-optimal conditions (Mustroph et al., 2007). Interestingly, we found differential regulation in transcripts encoding isoforms of hexokinase and sucrose phosphate synthase. The differential regulation of both SPS and HXK isoforms may have played a role in exerting regulatory influence on sucrose biosynthesis and hexose sugar pools to accommodate enhanced photosynthesis in *Jatropha* grown in CO_2 enriched atmosphere (Volkert et al., 2014). Also, this suggests the optimum balance in the catalytic and signaling function of HXK and also SPS, which may be one of the adaptive strategy during morning hours for sustained photosynthesis in elevated CO_2-grown *Jatropha* (Granot et al., 2014). The enzyme activity of SPS1 was in agreement with the transcript expression at all stages of growth while the activity of HXK did not alter at the reproductive development stage suggesting regulatory effect of sucrose on hexose sugar pools (Lemoine et al., 2013). The cytosolic glycolytic network may provide an essential metabolic flexibility that facilitates plant growth and development under elevated CO_2. We recorded significant up-regulation of transcripts pertaining to chloroplastic TPT and non-significant down-regulation of

chloroplastic GPT at all stages of growth under elevated CO_2. The up-regulation of TPT indicate a coordinated metabolic interaction between cytosol and chloroplast for high levels of carbon skeletons to be utilized for sustained growth and development of *Jatropha* in elevated CO_2 environment (Flügge et al., 2011). The downregulation of GPT suggests preferential exchange of triose phosphate intermediates between subcellular compartments during morning and may be one of the adaptive strategy for sustained photosynthesis in *Jatropha*.

In conclusion, the present study highlights the importance of differential expression of key regulatory genes of photosynthetic electron transport in chloroplasts and central carbohydrate metabolism in maintaining the improved photosynthetic capacity during long-term growth in *Jatropha* under elevated CO_2.

Chapter IV

Persistent stimulation of growth and photosynthesis in *Jatropha curcas* is associated with photosynthetic nitrogen use efficiency and physiological and biochemical feedbacks during long-term exposure to elevated [CO_2]



Chapter 4

4.1 Introduction

The rise in [CO_2] poses a challenge on ecosystem balance effecting plant's adaptive capacity of photosynthetic CO_2 uptake and release through respiration. A large body of literature has been published on the short- and long-term effects of [CO_2] and other interacting parameters including temperature and drought on growth and productivity of plants which also depends on system, species, functional groups, environmental conditions and the availability of other resources. Majority of crop plants have demonstrated acclimation of photosynthesis after initial stimulation on long-term CO_2 exposure (Kimball, 2016). This acclimation has been correlated synergistically to increased carbohydrate levels, imbalance in C-N stoichiometry and reduced sink strength (Ainsworth and Rogers, 2007). In most cases, the plant productivity declined in the absence of an external N input speeding up the progressive N limitation in these plants. Tree species comprise the bulk of carbon sink in forest ecosystem which contribute up to 70% of the terrestrial carbon fixation and also can partially fulfil the recommendations of Kyoto proto-

-col on the mitigation of excess CO_2 from the atmosphere by providing additional sinks (Waring and Schlesinger, 1985; Melillo et al., 1993; UNFCCC 2008). Tree species with greater sink strength have been implied and also demonstrated no down-regulation in photosynthesis and productivity performance in growth and productivity under long-term elevated [CO_2] studies (Hofmockel et al., 2011; Buitenwerf et al., 2012; Ellsworth et al., 2012; DaMatta et al., 2016). Apart from improved sink strength, the continued growth and productivity of some tree species in elevated [CO_2] was implied to better photosynthetic nitrogen use efficiency (PNUE) thereby delaying progressive N limitation (Peterson et al., 1999; Calfapietra et al., 2007). Hence, it is imperative to understand and predict long-term effects of rising [CO_2] and determine the influence of other resources, such as soil N levels on photosynthesis, and associated growth and productivity responses among different tree species, functional, and ecosystem types. This information is critical to the development of reliable coupled climate-carbon cycle models in the wake of ongoing global climate change.

A meta-analysis on earlier studies of plants grown under long-term elevated [CO_2] suggest that light-saturated photosynthesis increases an average of 26% or 31% across C3 species specifically (Nowak et al., 2004; Ainsworth and Rogers, 2007; Lee et al., 2011). This magnitude of enhancement of photosynthesis reported, is approximately 20–25% less than the predicted range based on theoretical understanding of photosynthetic biochemistry and empirical observations of short-term responses to increasing CO_2 concentrations (Farquhar and von Caemmerer, 1982; Lee et al., 2001). This long-term, leaf photosynthetic acclimation to elevated [CO_2], which results from physiological, morphological and biochemical feedbacks that balance carbon assimilation with growth (i.e. sink) demand at CO_2 enriched atmosphere, has also been found to vary depending on environmental factors such as nutrient and water availability and temperature. These acclimation responses have been mostly reported in studies on tree seedlings or young trees and in some cases mature trees with reduced sink demand (Rey and Jarvis, 1998; Körner et al., 2005). No single factor appears predominant in controlling photosynthetic down-regulation but however, the acclimation of photosynthetic capacity to elevated CO_2 has been shown to be associated with concomitant decreases in leaf N (Ellsworth et al., 2004; Crous et al., 2010). The

results of CO_2 enrichment experiments have been ambiguous with respect to the occurrence of progressive nitrogen limitation in some tree species. CO_2 and N likely interact at multiple levels in affecting plant responses to elevated [CO_2] (Reich et al., 2006a, b). As CO_2 limitations on leaf net photosynthesis are eased under elevated CO_2, lack of available N in systems that are frequently N-limited may lead to greater constraints on the CO_2 response than when N is in ample supply (Hungate et al., 2003; Reich et al., 2006a, b). Also, there is an important modifying role of N in CO_2 response through interactions involving litter quality and decomposition, litter quantity and soil N immobilization, and root production and N uptake or availability (McMurtrie et al. 2000). Mechanistically, N limitation starts with physiological status of plant (N uptake, N-use efficiency, and resorption) and ultimately affects ecosystem response (N deficiency, changes in growth and allocation) (Norby and Iverson, 2006). Hence, detailed and sustained observations on N dynamics in CO_2-enriched trees will aid in understanding the role of N associated feedbacks on continued stimulation of photosynthesis and productivity.

Mature natural forests with closed canopy did not show carbon retention under long term treatment of elevated CO_2 (Körner et al., 2005). A possible alternative to circumvent this situation would be identifying fast growing tree species which could be managed as plantation forests. Short rotation coppice (SRC) is a management practice which is being followed for plantations of fast growing tree species producing repeated biomass on agricultural and degraded forest lands (Mola-Yudego and González-Olabarria, 2010). The biomass produced is being utilized for both paper and pulp industries and also for energy purposes (Aylott et al., 2008). SRC crops such as eucalyptus, poplars and willow are being grown in many countries because of their efficient land use, high photosynthetic capacity and their utility as a renewable energy source. The elevated CO_2 effects on growth and photosynthesis of SRC poplar has been reported extensively during long-term growth under enhanced [CO_2] (Davey et al., 2006; Liberloo et al., 2009; Liberloo et al., 2010). SRC mulberry has also displayed persistent stimulatory photosynthesis and enhanced biomass under elevated CO_2 (Sekhar et al., 2014). In the recent decade, biodiesel has attracted a lot of attention as an alternative to fossil fuel aimed towards the containment of

increasing carbon footprint in our planet. *Jatropha curcas* L. (Family – Euphorbiaceae) is an oil-bearing perennial semi-evergreen tree species which is being targeted for the production of biodiesel (Kumar and Sharma, 2008). It would be noteworthy to understand the N dynamics of this plant under different growth regimes for developing efficient management practices to optimize and maximize the yield potential. Our earlier report demonstrated that *J. curcas* displayed an increased photosynthetic rate and enhanced biomass including seed yields during its growth under elevated CO_2 for two production cycles for a period of one year maintained as coppice plantation (Kumar et al., 2014). In this study, we examined the responses of *J. curcas* grown as SRC for a period of four years with an emphasis on quantification of photosynthetic parameters and consistency of responses over time under elevated CO_2 conditions. The responses of other related variables (such as leaf N, leaf stomatal conductance and water and N-use efficiencies) of plants grown under long-term treatments of CO_2 were also investigated to collectively address the following questions: (1) whether *Jatropha* was able to sustain initial stimulatory photosynthetic response, and showed no effects of seasonal variation on the photosynthetic capacity of upper canopy leaves and plant source-sink relations during its growth for a longer period of time, (2) how leaf N content varies in relation to *in vivo* biochemical capacity of the plant i.e. A_{max} (maximum photosynthetic capacity), V_{cmax} and J_{max} and, (3) does an interaction between CO_2 concentration and soil N availability develop over time (as feedbacks might lead to N limitations)?

4.2 Materials and Methods

4.2.1 Plant material and experimental setup

The study was performed in OTCs constructed in the botanical gardens of University of Hyderabad (UH) [17.3°10′N and 78°23′E at an altitude of 542.6 m above mean sea level (AMSL)], located 20km north of Hyderabad metropolitan in the Telangana region of Andhra Pradesh, India. The design, work flow and maintenance of OTCs has been described in detail in Chapter 2. The average monthly variations in climatic conditions for four years were recorded at the experimental site by an automatic data logger and are presented in **Table 4.1**. Two OTCs each were dedicated towards maintaining elevated CO_2

(550 µmol mol^{-1}) and ambient CO_2 conditions (380 – 400 µmol mol^{-1}, depending on the recordings displayed in official website of NOAA-ESRL), respectively. The duration of the study was four years starting from January 2013 till December 2016. High quality disease free seeds of *Jatropha curcas* L. (variety – Chhattisgarh) were procured from the germplasm centre of Tree Oils India Limited (TOIL, Zaheerabad, Medak district, Telangana, India, latitude 17°36′N; longitude 77°31′E; 622m AMSL). The initialization of *Jatropha* plantation and CO_2 treatment inside OTC are described briefly in Chapter 2. The plants were allowed to complete their full life cycle (i.e. production of mature viable seeds) before coppicing. The full production cycle of *Jatropha* completed in approximately six months. The plants were coppiced on June 2013, December 2013, June 2014, December 2014, June 2015, December 2015 and June 2016, with the final up-rooting on the month of December 2016 so that there were eight experimental growth periods and harvest of seeds. Surface irrigation was provided for four years to maintain soil moisture and prevent drought stress. No additional N was added to the soil during the course of four years of experiment. Important soil physiochemical characteristics including N were measured before the start of study and are presented in **Table 4.2**.

All physiological, biochemical and molecular measurements were performed during the months of March, June, September and December months of all four years (2013-2016). Each experimental growth period consisted of two months of experimental analysis [either March and June (summer months) or September and December (rainy and autumn months)] in which the first measurement month (March or September) depict maximal vegetative growth while the second (June or December) depicts both vegetative and reproductive growth.

4.2.2 Leaf gas exchange measurements

Leaf gas exchange parameters were measured on fully expanded young leaves of upper canopy selected randomly using portable open gas exchange system incorporated with infrared CO_2 and water vapor analyzers (LCpro-32070; ADC Bioscientific Ltd., Hoddesdon, UK). All leaf gas exchange measurements were conducted periodically throughout the course of growth of the plants for the eight growth periods with the

objective to infer changes in the underlying photosynthetic capacity of leaves. Periodic measurements of instantaneous photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (E) and intercellular CO_2 concentration (C_i) were taken between 9:00 and

Table 4.1. Annual weather data at the experimental site of University of Hyderabad.

	Growth months		
Climatic Conditions	**November-February**	**March-June**	**July-October**
Mean max Temperature (°C)	27.9 - 31.8	35.4 - 45.8	30.7 - 34.8
Mean max Temperature (°C)	10.2 - 22.2	22.4 - 30.4	24.7 - 25.8
Relative humidity (%)	49 - 58	39 - 60	63 - 74
Mean rainfall (mm)	8 - 24	10 - 108	95 - 165
Atmospheric CO_2 concentration (μmol m^{-2} s^{-1})	380 - 390	380 - 390	380 - 390
Wind velocity (km h^{-1})	3	2	4-12
Mean PPFD (μmol m^{-2} s^{-1})	1000-1200	1200-1800	1000-1500

Table 4.2. Selected soil physicochemical characteristics recorded at the experimental site prior to the start of experiment.

Soil characteristics (at 0-30 cm soil depth)	
Texture	Sandy loam
pH	4.2
Organic carbon (%)	1.8
Available N (kg ha^{-1})	94
Available P (kg ha^{-1})	12
Available K (kg ha^{-1})	118

10:30 h. The above measurements were performed at the growth [CO_2] (ambient or elevated) and at the temperature, vapor pressure deficit and quantum flux (Q) incident at that point of time. Only the 'steady state' rates of photosynthesis were considered to maximize the accuracy of measurement. This was achieved by avoiding days with variable cloud cover or fluctuations in Q. The C_i was used to compute C_i/C_a (ratio of intercellular to

atmospheric CO_2 concentrations) during all growth periods. Simultaneous with these periodic measurements, response curves of net rate of CO_2 assimilation (A) versus internal leaf CO_2 (A-C_i curves) were also measured. These response curves were performed by recording A and C_i at saturating light intensity (1800 µmol m^{-2} s^{-1}) and CO_2 concentrations ranging from 0 to 1400 ppm and were used for estimating J_{max} and V_{cmax}. The measurements of A were made starting at 390 ppm [CO_2] surrounding the leaf for ambient leaves and 550 ppm for elevated leaves, and [CO_2] was decreased stepwise to 50 ppm. The [CO_2] was then set again to 370 ppm and 550 ppm for ambient and elevated, respectively and increased stepwise to 1400 ppm. The J_{max} and V_{cmax} were estimated by fitting the A-C_i curves according to the photosynthetic model equations described by Sharkey et al. 2007.

$$A = V_{cmax}\frac{C_c - \Gamma^*}{C_c + K_C(1 + O/K_O)} - R_d \quad (1)$$

$$A = J_{max}\frac{C_c - \Gamma^*}{4C_c + 8\Gamma^*} - R_d \quad (2)$$

When A is Rubisco-limited, the response of A to [CO_2] is described by equation (1) and when A is limited by RuBP regeneration, response is described by equation (2) where C_c is the CO_2 partial pressure at Rubisco, K_C is the Michaelis constant of Rubisco for carbon dioxide, O is the partial pressure of oxygen at Rubisco, K_O is the inhibition constant (usually taken to be the Michaelis constant) of Rubisco for oxygen, Γ^* is the [CO_2] at which oxygenation proceeds at twice the rate of carboxylation causing photosynthetic uptake of CO_2 to be exactly compensated by photorespiratory CO_2 release and, R_d is respiratory CO_2 release other than by photorespiration (day respiration) which is presumed to be primarily mitochondrial respiration (**Fig. 4.1**). The maximum CO_2- and light-saturated rate of photosynthesis (A_{max}) at the respective growth [CO_2] (either 390 or 550 ppm) was also determined from A-C_i curves. Light response curves were performed to measure light-saturated rate of photosynthesis (A_{Sat}). These curves were performed by measuring A at decreasing saturated photosynthetic active radiation (PAR) stepwise from 1800 µmol m^{-2} s^{-1} to complete darkness. A_{Sat} was calculated from the asymptote of fitted response functions. R_d, which is presumed to be primarily mitochondrial respiration was

also estimated from these curves according to Wang et al. (2001) by averaging the three CO_2 efflux rates at zero PAR for each plant. The leaf temperature was maintained at 25 ± 1

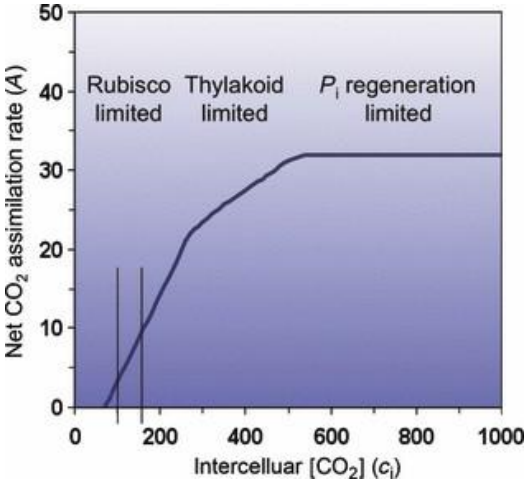


Fig. 4.1. Theoretical A–C_i curve showing the relationship between photosynthetic assimilation of CO_2 (A) and leaf intercellular [CO_2] (C_i).

°C and humidity at 50 ± 10% while performing A-C_i and light response curves.

4.2.3 Chlorophyll a fluorescence measurements

Chl *a* fluorescence parameters were measured simultaneously on the same leaves used for gas exchange studies at 25°C. The Chl *a* fluorescence measurements were performed with the miniaturized pulse-amplitude-modulated photosynthesis yield analyzer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) using a special leaf clip holder (model 2030-B, Walz) as described in chapter 3. The fluorometer was operated via a notebook computer, which also stored the measured fluorescence data. The measurements were taken at two different times of the day, first between 0930 – 1100 h solar time and second between 1230 – 1400 h. The morning measurements corresponded to the optimal time of photosynthesis while the afternoon measurements were undertaken to compare the PSII photochemical behavior of both elevated and ambient CO_2-grown *Jatropha* plants during midday depression of photosynthesis or photoinhibition. Induction curve and recovery analysis of $\Delta F/F_m'$ were obtained by using a default automated program stored in MINI-PAM. A

saturation pulse (SP) of intensity, 3000 µmol m^{-2} s^{-1} was used on the dark-adapted sample to measure F_o and F_m. After a delay of 40 s, actinic light of 120 µmol m^{-2} s^{-1} was switched on along with SP of 3000 µmol m^{-2} s^{-1} for 0.8 s, followed by repetitive application of SPs at every 20 s for 5 min (Vishwakarma et al., 2014). In addition, after last illumination with SP, in the absence of actinic light the recovery of yield in the dark is assessed by six consecutive SPs applied at 10 s, 30 s, 60 s, 2 min, 5 min and 10 min. The relative changes in $\Delta F/F_m'$, photochemical (*qP*) and non-photochemical quenching (*NPQ*) were recorded with each SP. The recovery analysis provides information on recovery of various components of non-photochemical quenching and the extent of photoinhibition. Apparent rate of photosynthetic electron transport of PS II (ETR) was obtained as ETR = $\Delta F/F_m' \times$ PPFD × 0·5 × 0·84, where the factor 0·5 assumes equal excitation of both PS II and PS I and 0.84 is the ETR correction factor which accounts for the fraction of incident light really absorbed by photosystems. Plots of $\Delta F/F_m'$, ETR, *NPQ* and *qP versus* time were constructed from these measurements.

4.2.4 Foliar properties: morphological and biochemical analyses

The thickness of individual fresh upper canopy leaves were measured using digital calipers at different time points for eight experimental growth periods. Leaves were then harvested and placed on ice until they could be returned to the laboratory for processing. Leaf area was determined for each individual leaf using a leaf-area meter (portable laser leaf area meter, CI-202, CID Biosciences, Inc., Camas, WA, USA). Following the measurement of individual leaf areas, all leaves were dried in a 60°C oven for at least 3 days, and then each leaf was weighed individually. These data were used to calculate leaf mass per unit area (LMA). Estimates of leaf volume and density were also calculated, because leaf function is affected by the interaction of leaf morphology (area and thickness) and composition (dry matter, liquid content, and fractional air space) (Niinemets, 1999; Roderick et al., 1999a,b). Usually, the leaves were collected from different trees (especially at the top of the canopy) and randomly selected different branches. Immature and senescent leaves were avoided. Leaf disc samples were taken from the same leaves used for gas exchange and fluorescence measurements for biochemical analysis. Total chlorophyll content, leaf

soluble sugar and starch contents were determined spectrophotometrically as described in Chapter 2. Total non-structural carbohydrate (TNC) was calculated as the sum of soluble sugar and starch. All the above measurements were taken on the same months of gas exchange and fluorescence analysis.

4.2.5 Nitrogen dynamics: leaf and soil N content and C:N estimation

Total foliar crude protein and N content were estimated by combustion method according to AOAC 992.23 (1993) (Leco FP628, Michigan, USA) using EDTA as a standard and soya bean leaf (N concentration of 2.5 g N m^{-2}) as an internal quality check. N content was calculated by using the equation:

$$\text{Crude protein, \%} = N \times 6.25$$

The leaves from both ambient and elevated CO_2 grown *Jatropha* were oven dried and weighed before analysis. The foliar N content was expressed in average N content per unit leaf mass (N_M, in mg g^{-1}) and per unit leaf area (N_A, mg m^{-2}) [$\{N_A = N_M \times LMA\}$]. N content was also analyzed in leaf litter which was also collected on last two months of an experimental growth period as mentioned earlier. After completion of an experimental growth period, the oven-dried leaf litter collected for two months were combined, thoroughly mixed, subsampled, and analyzed for N content. Available soil N content was analyzed after each year of study for four years. The soil samples for analysis were collected at the end of December month for all four years from both ambient and elevated OTCs. The N content in soil were measured by digging soil pits at a depth of 20 cm. Photosynthetic N-use efficiency (PNUE) was calculated by dividing A_{Sat} with N_A content at the end of each year.

Total C from leaves, soil and litter were estimated by dry combustion method (McGeehan and Naylor, 1988) using an elemental analyzer (Leco FP628, Michigan, USA). C:N was estimated in leaf and litter at the end of each experimental growth period and in soil (depth of 0 -30 cm) at the end of December month for all four years. The N fraction of soil C:N ratio was the available soil N for the plant and not total soil N.

4.2.6 Growth, biomass and yield parameters

The growth and development of *Jatropha* was monitored in elevated and ambient CO_2 for eight growth periods till destructive harvest and seed yield estimation was undertaken. Various growth parameters were recorded like plant height, number of leaves plant^{-1}, number of flowers apex^{-1} and number of secondary and tertiary branches plant^{-1} at the end of each growth cycle of plants. Total leaf production during a single experimental growth period was estimated as the sum of existing leaves before destructive harvest and litter fallen. After *Jatropha* fruits were mature and ready to be harvested after the completion of experimental growth period, the plants were coppiced. The above ground biomass (both fresh and dry) was estimated following coppice, which included weight of leaves, stem and fruits at the time of harvest. The canopy productivity index was calculated as annual total shoot biomass increment divided by total leaf area (g m^{-2}) for the 1st, 2nd, 3rd and 4th year of growth. Yield parameters like number of fruits and seeds and weight of 1000 fruits and seeds were also recorded at the end of eight experimental growth periods. At the end of eighth experimental harvest, the *Jatropha* plants from both elevated and ambient conditions were uprooted and below ground biomass was estimated. Carbon sequestration after four years was calculated as described in chapter 2.

4.2.7 Quantitative real time PCR analysis and enzyme activity measurements

The leaves after physiological measurements were harvested and stored in -80°C for qRT-PCR analysis and enzyme activity measurements. These were performed for certain key regulatory enzymes of photosynthesis and carbohydrate metabolism which included RL, cytosolic FB, and SPS1. Gene specific primers for the above genes are described in **Table 3.1**. The procedure followed is described in Chapter 3.

4.2.8 Statistical analysis

The effects of elevated [CO_2] on different variables were evaluated using *t-tests* and two-way analysis of variance (ANOVA) to compare the effects of CO_2 treatment and sampling date. Regression slopes and treatment differences in photosynthesis, chlorophyll content, N content, V_{cmax}, J_{max} and above ground biomass were analyzed for significant difference (at

$P < 0.01$). Individual leaves were considered as experimental units (i.e. pseudo-replication) (df = 25 – 30) instead of chambers (df = 1) for all photosynthesis, chlorophyll content, N content and fluorescence measurements. Similarly, individual trees were considered as experimental unit as opposed to use of chambers as replicates for growth, biomass and yield studies. Whenever there was significant differences in the mean values of the tested variables, the *P* values were Tukey corrected to reduce errors. All statistical analysis were performed using Sigma Plot 11.0. All linear regressions, correlations and curve fitting were done using Sigma Plot and tested for significance at $P < 0.01$.

4.3 Results

4.3.1 Gas exchange measurements

There were significant variations in *p*hotosynthetic rates and the photosynthetic response of upper canopy leaves during the course of growth of *Jatropha* in elevated CO_2 over time. The P_n values recorded in *Jatropha* plants for eight experimental growth periods were found to be significantly higher (~50 – 55%; $P < 0.01$) in elevated CO_2 conditions (~26 – 31 μmol m^{-2} s^{-1}) in comparison to ambient CO_2 conditions (~17 – 20 μmol m^{-2} s^{-1}) (**Fig. 4.2A, Table 4.3**). This increased photosynthesis was maintained during both vegetative and reproductive growth phases of elevated CO_2-grown *Jatropha* plants suggesting no effect of sampling date on instantaneous photosynthesis also evident in the statistical analysis (**Table 4.3**). However, stomatal conductance (g_s) and transpiration rate (E) was found to be decreased (~8 - 20%) in *Jatropha* grown under elevated CO_2 atmosphere in comparison to ambient grown *Jatropha* plants (**Fig. 4.2B, 4.2C**). The values recorded were lower in hot summer months of growth (March) for both ambient and elevated grown plants with the elevated grown plants exhibiting decreased g_s and E. The C_i/C_a values did not show any significant variation between ambient and elevated grown plants (~0.7 for both conditions) across the four years of growth (**Fig. 4.2 D**).

Similarly, A_{Sat} derived from light response curves (A/Q) performed on ambient and elevated CO_2-grown *Jatropha* was also recorded to be significantly higher by ~64% ($P < 0.01$) for elevated CO_2 grown plants during the first three years of experimental growth period (**Fig. 4.3A**). The relative increase in A_{Sat} at the end of fourth year of growth was ~55

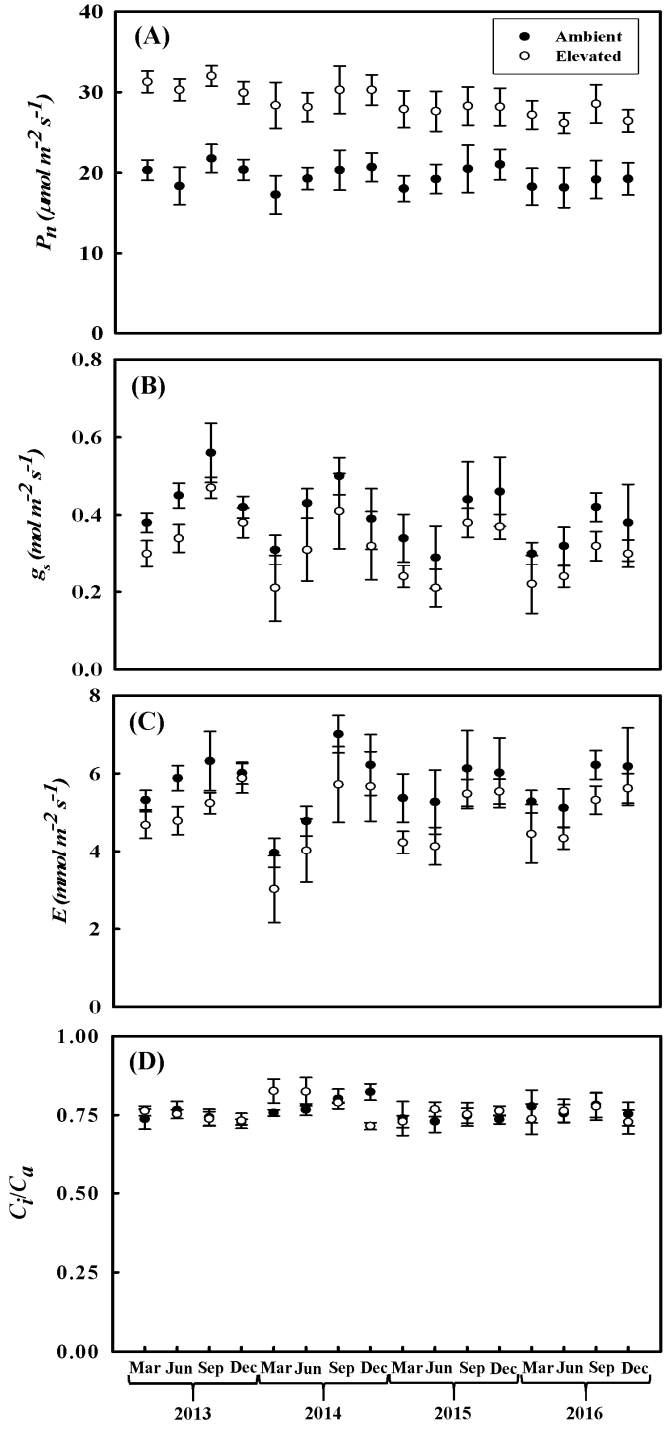


Fig. 4.2. Periodic variations in (A) P_n, (B) g_s, (C) E and (D) C_i/C_a measured for a period of four years during the months of March, June, September and December for each year. All values of P_n are statistically significant at $P < 0.01$. Values are presented as mean ± SD.

% in elevated conditions, which is still significantly ($P < 0.01$) higher when compared to plants grown in ambient conditions (**Fig. 4.3A**). There was no significant CO_2 effect and significant effect of sample date on parameters measured and calculated (V_{cmax} and J_{max}) from A/C_i curves (**Table 4.3, Fig. 4.3B, Fig. 4.3C**). The elevated CO_2 grown *Jatropha* plants did not show photosynthetic acclimation during the course of growth of the plants for four years (**Fig. 4.3B, Fig. 4.3C**).

Table 4.3. ANOVA results for *Jatropha curcas* leaf photosynthetic and biochemical parameters. Significance of treatment effect, as indicated by the *P*-value, is reported for CO_2 concentration, sample date, and the interaction between CO_2 treatment and date of sample collection ([CO_2] × sample date).

Parameter	[CO_2]	Sample date	[CO_2] × Sample date
P_n (µmol m^{-2} s^{-1})	< 0.001	0.034	0.939
A_{Sat} (µmol m^{-2} s^{-1})	< 0.001	0.042	0.957
A_{max} (µmol m^{-2} s^{-1})	0.181	0.153	0.758
V_{cmax} (µmol m^{-2} s^{-1})	0.253	0.01	0.960
J_{max} (µmol m^{-2} s^{-1})	0.217	0.01	0.918
J_{max}/V_{cmax}	0.07	0.04	0.161
LMA (g m^{-2})	0.001	0.12	0.092
Leaf density (g cm^{-3})	0.023	0.024	0.465
N_M (mg g^{-1})	< 0.001	< 0.001	0.905
N_A (g m^{-2})	0.257	0.008	0.144
Chl_M (mg g^{-1})	0.001	0.01	0.554
Chl_A (mg m^{-2})	0.022	0.024	0.756
TNC (mg m^{-2})	< 0.001	0.17	0.616
PNUE (µmol g^{-1} N s^{-1})	< 0.001	0.09	0.267
Above ground biomass (g)	< 0.001	0.28	0.737

There was no significant variations in A_{max} recorded for both ambient and elevated grown plants for the four years of study. However, there was a decline in A_{max} recorded for last two years for both ambient and elevated conditions. The decline was non-significant for the third year of growth in comparison to first year. Further, a significant decrease of ~19% ($P < 0.01$) and non-significant decrease of ~13% was recorded in elevated

conditions for summer and autumn months, respectively in comparison to ambient conditions where it was ~9.5% and ~12%, respectively during the fourth year of growth (**Fig. 4.4A**). Similarly, V_{cmax} and J_{max} calculated though fitting A/C_i curves by using non-linear regression (**Fig. 4.3B**, **Fig. 4.3C**), did not show any significant variation between ambient and elevated CO_2 grown *Jatropha* plants during the course of study (**Fig. 4.4B**, **Fig. 4.4C**). However, a decrease was observed in V_{cmax} and J_{max} for the last two years in both conditions, with the decrease more prominent in the summer months {~8% and ~13% (V_{cmax}), and ~13% and ~18% (J_{max}) for ambient and elevated [CO_2] conditions, respectively} in comparison to rainy and autumn months {~9% and ~12% (V_{cmax}), and ~12% and ~8% (J_{max}) for ambient and elevated [CO_2] conditions, respectively} during the fourth year of growth (**Fig. 4.4B**, **Fig. 4.4C**). A similar trend was observed in the ratio of J_{max} to V_{cmax} with no significant variations during the course of four year study period in both ambient and elevated CO_2 conditions (**Fig. 4.4D**). A decrease in J_{max}/V_{cmax} was recorded for the last two years of study with the decline more prominent during summer month in comparison to rainy and winter months (**Fig. 4.4D**). In addition, there was a linear relationship between J_{max} and V_{cmax} with non-significant correlation for leaves grown in both ambient and elevated [CO_2] (**Fig. 4.5**). The regressions for both treatments were statistically significant ($P < 0.01$ ambient, $P < 0.01$ elevated).

4.3.2 Chlorophyll fluorescence measurements

In congruence with the leaf gas exchange measurements, the Chl *a* fluorescence measurements with mini PAM clearly demonstrated the enhanced photosynthetic ability and enhanced PSII efficiency of *Jatropha* under elevated CO_2, in particular, during morning hours. The regular and periodic induction curve measurements performed at morning hours demonstrated that effective yield ($\Delta F/F_m'$) of PSII was recorded to be significantly higher ($P < 0.01$) in elevated CO_2 grown *Jatropha* plants when compared to ambient plants (**Fig. 4.6A**). However, there was a decline in $\Delta F/F_m'$ during afternoon hours in CO_2 enriched conditions when compared to ambient conditions (**4.6B**). A similar trend was recorded for ETR which was significantly higher in CO_2 enriched conditions during morning hours with decreased values during afternoon hours in comparison to ambient

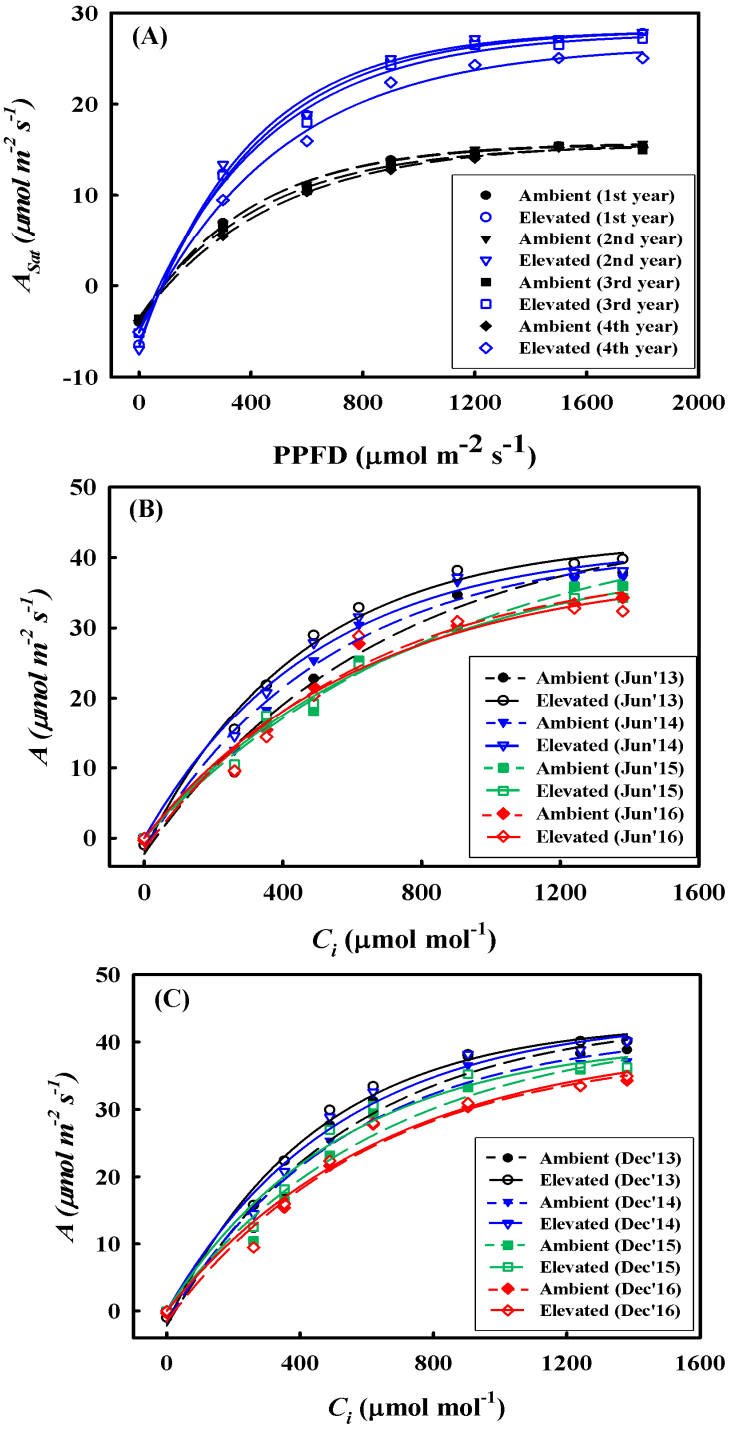


Fig. 4.3. Light response curves (A) depicting relationship between A_{Sat} and photosynthetic photon flux density (PPFD). These curves were performed periodically for four years and a representative analysis of each year with average values of A_{Sat} shown here. Response curves of net *A* to increasing levels of C_i (*A vs* C_i curves) at the end of summer season month (June) (B), and rainy and autumn season (December) (C). The average values of *A* are presented here.

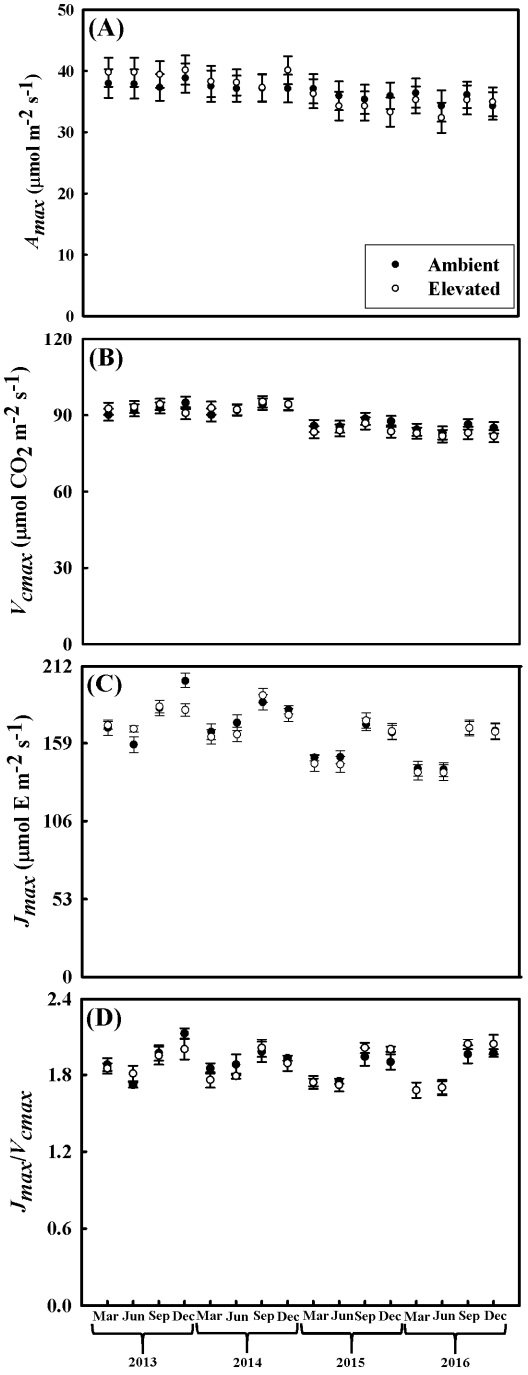


Fig. 4.4. Response of modelled photosynthetic parameters of upper-canopy leaves of *Jatropha* exposed to ambient (~390 ppm) or elevated (~550 ppm) CO_2. *A*-C_i curves were analysed using a C_3 model of photosynthetic biochemistry to determine: (A) A_{max}, (B) V_{cmax}, and (C) J_{max}. (D) The ratio of J_{max}/V_{cmax} was also plotted. Values are presented as mean ± SD.

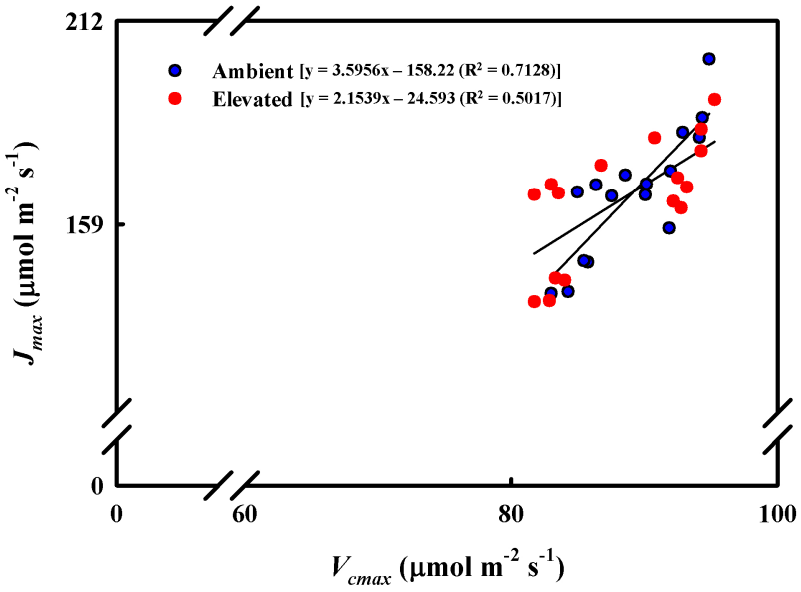


Fig. 4.5. Linear relationship between J_{max} and V_{cmax} of upper-canopy leaves of *Jatropha*. V_{cmax} and J_{max} were derived as best-fit estimates from individual A-C_i curves. The regressions generated for each treatment were statistically significant ($P < 0.01$, n = 30).

CO_2 conditions (**Fig. 4.6C, 4.6D**). A very interesting pattern was observed for both *NPQ* and *qP* in elevated CO_2 grown *Jatropha* during both morning and afternoon hours (**Fig. 4.6E, 4.6F, 4.6G, 4.6H**). *NPQ* was reduced in leaves of elevated CO_2 grown *Jatropha* by ~30% during morning hours and enhanced by ~31% during afternoon hours in comparison to ambient grown *Jatropha* (**Fig. 4.6E, 4.6F**; $P < 0.01$). *qP* showed similar trend like $\Delta F/F_m'$ and ETR with a significant increase during morning hours and decrease during afternoon hours in elevated conditions (**Fig. 4.6G, 4.6H**; $P < 0.01$). This trend was observed during both morning and midday measurements for all the four years of experimental growth period of *Jatropha* under elevated and ambient CO_2 environment.

Recovery analysis performed after induction curve measurements indicate that elevated CO_2 grown *Jatropha* plants were able to recover at a much faster rate from any possible photodamage caused during midday depression of high light intensity during afternoon hours (**Fig. 4.7**). The recovery of $\Delta F/F_m'$ in morning hours was similar in both ambient and elevated CO_2 grown *Jatropha* plants (**Fig. 4.7A**). However, the relative rate of recovery

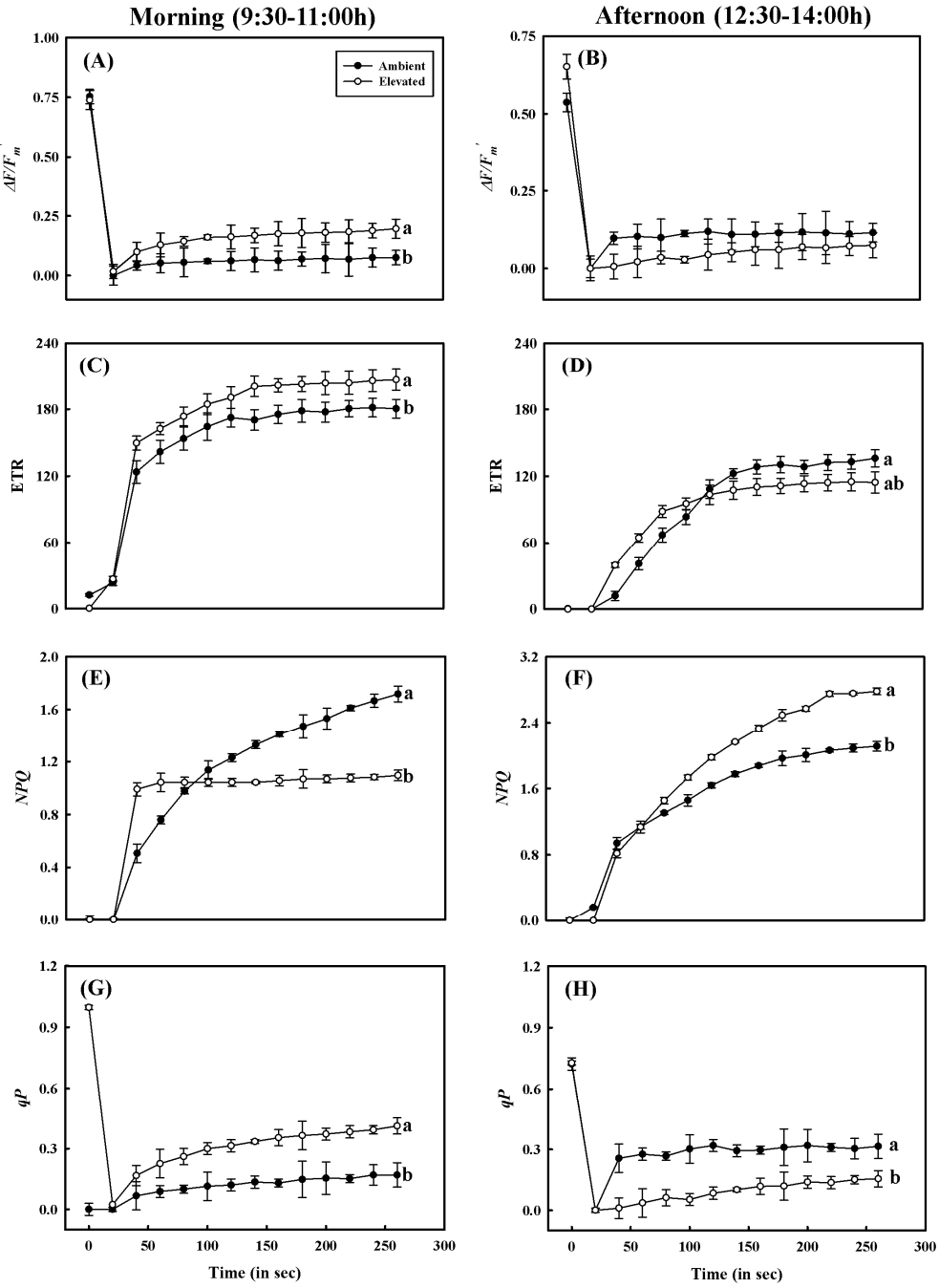


Fig. 4.6. Characteristics of chlorophyll fluorescence parameters of upper-canopy leaves as measured by induction curve in ambient and elevated CO_2 grown *Jatropha* at 25°C: effective quantum yield of PSII ($\Delta F/F_m'$), electron transport rate of PSII [ETR], non-photochemical quenching (*NPQ*) and photochemical quenching (*qP*) during morning, (A, C, E, G) and afternoon, (B, D, F, H) respectively. This analysis was performed periodically for four years and representative curves of different parameters is shown here. Values are mean ± SD. Different lowercase alphabetical letters indicate statistically significant difference ($P < 0.01$).

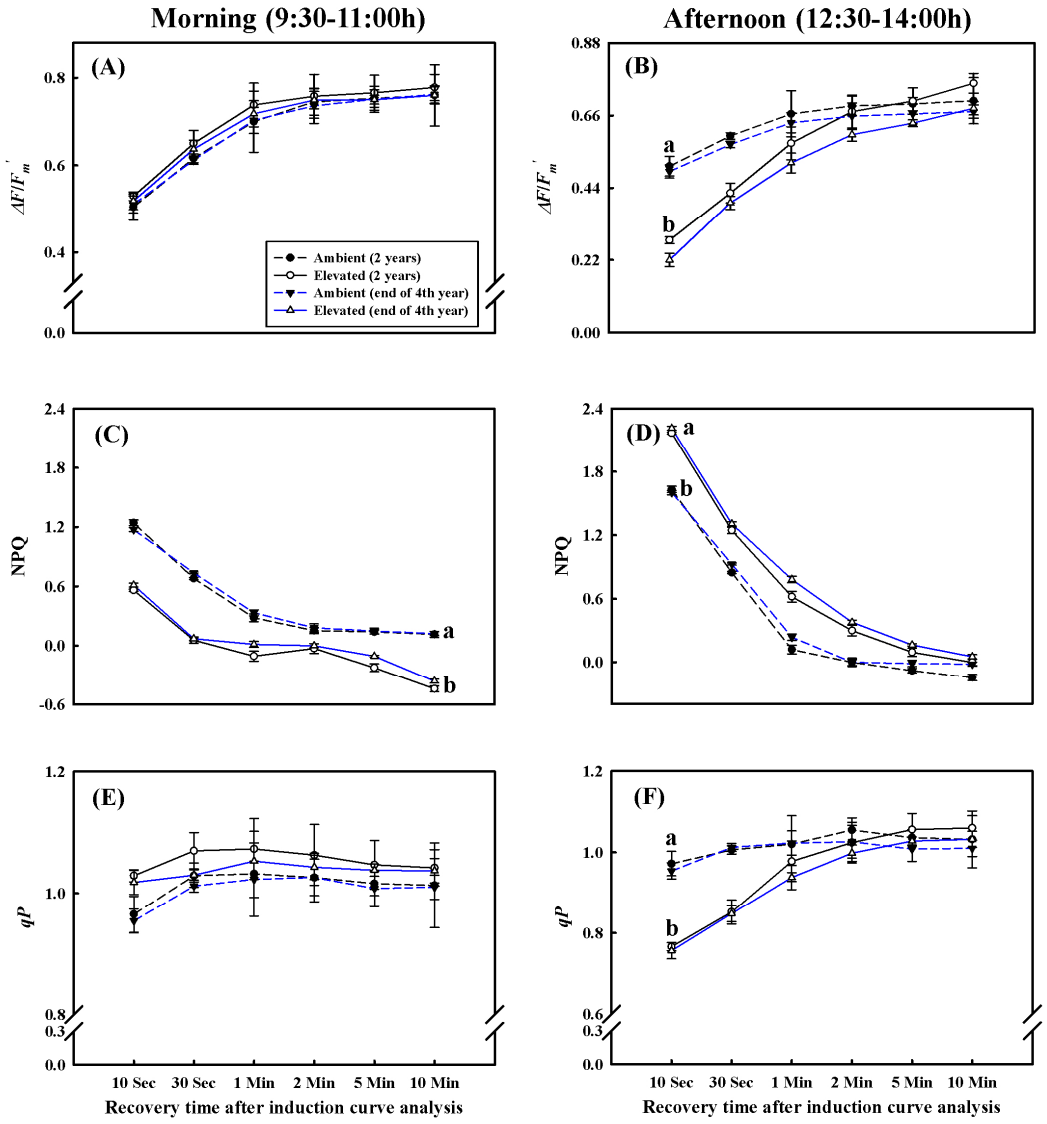


Fig. 4.7. Recovery analysis performed after induction curve measurements of upper-canopy leaves during four years of growth of *Jatropha* in ambient and elevated [CO_2] conditions and the parameters analyzed were $\Delta F/F_m'$, *NPQ* and *qP* during morning, (A, C, E) and afternoon, (B, D, F) respectively. Different lowercase alphabetical letters indicate statistically significant difference ($P < 0.01$).

was significantly ($P < 0.01$) faster in CO_2 enriched conditions during afternoon hours (**Fig. 4.7B**). This trend was observed for all the four years of growth under both conditions. However, there was a slight decrease observed in recovery of $\Delta F/F_m'$ at the end of fourth year during afternoon hours. Further, a relative decrease in *NPQ* was observed in both ambient and elevated CO_2 conditions with the passage of recovery time after induction curve analysis. During morning hours, the decline in *NPQ* was slower in ambient conditions with the progression of time while the same trend was observed in elevated conditions during afternoon hours (**Fig. 4.7C, 4.7D**). Also, increased values of *NPQ* was recorded in elevated CO_2 *Jatropha* plants during the fourth year of growth during afternoon hours (**Fig. 4.7D**). A similar behavior like $\Delta F/F_m'$ was recorded for *qP* with the elevated plants showing higher *qP* as soon as the recovery measurements started during morning hours (**Fig. 4.7E**). However, the relative rate of recovery of *qP* in elevated CO_2 was faster during afternoon hours (**Fig. 4.7F**).

4.3.3 Leaf morphological properties

LMA was recorded to be significantly ($P < 0.01$) higher in leaves of *Jatropha* grown in elevated [CO_2] as compared with ambient [CO_2], with an average range of ~5 – 10% increase over the 4-yr study period (**Fig. 4.8A**). There was no effect of sample date on the measured values of LMA as no differences in LMA on an annual basis was observed (**Table 4.3**). There was no significant CO_2 treatment and sampling date effect on leaf density which was both significantly and non-significantly higher in elevated [CO_2] at different time points (**Table 4.3, Fig. 4.8B**). However, the values of leaf density at the end of rainy and autumn months were higher in comparison to summer months in elevated CO_2 grown *Jatropha* plants (**Table 4.3, Fig. 4.8B**). Furthermore, there was a linear relationship between LMA and leaf density with non-significant correlation for leaves grown in both ambient and elevated [CO_2] (**Fig. 4.8C**). The regressions for both treatments were statistically significant ($P < 0.01$ ambient, $P < 0.01$ elevated).

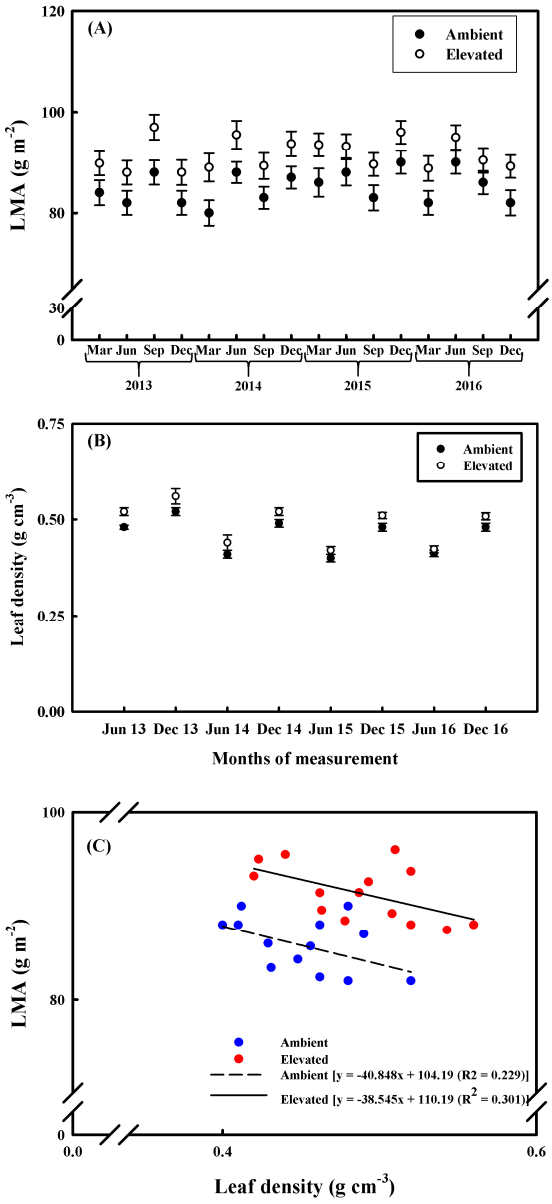


Fig. 4.8. Growth characteristics of young upper-canopy leaves of *J. curcas* exposed to ambient (~390 ppm) or elevated (~550 ppm) CO_2. Growth characteristics were determined for the leaves harvested following the collection of *A*-C_i curves. (A) Leaf mass per unit leaf area (LMA) measurements were collected in all four months as explained in materials and methods for all four years of growth while (B) leaf density measurements were limited to the end of each experimental growth for all four years. Values are presented as means ± SD. (C) Linear regressions of LMA with leaf density of upper-canopy leaves of *Jatropha* for ambient (blue circles; dashed line) and elevated CO_2 conditions (red circles; solid line). The regressions generated for each treatment were statistically significant ($P < 0.01$, n = 30).

4.3.4 Leaf biochemical status

Foliar total chlorophyll concentrations were significantly decreased by growth in enriched [CO_2] when expressed on a mass (Chl_M), but not area (Chl_A) basis (**Table 4.4**). Chl_M was also significantly ($P < 0.01$) affected by sample date with the decrease prominent in the third and fourth year of growth of *Jatropha* in CO_2 enriched atmosphere (**Table 4.3**). The decline in Chl_M was ~8% in leaves of elevated CO_2 grown *Jatropha* in comparison to ambient CO_2 at the end of fourth year (**Table 4.4**). Further, the relative decline in Chl_M under elevated [CO_2] was ~8% between first and fourth year, respectively. There was a relative decline in Chl_A of *Jatropha* leaves between the first and fourth year of both ambient (~13%) and elevated [CO_2] (~15%) (**Table 4.4**). There was no significant effect of sample date on Chl_A (**Table 4.3**). However, there was a non-significant decrease (~5%) in Chl_A recorded at the end of fourth year of growth in elevated [CO_2] when compared with ambient [CO_2] (**Table 4.4**). Area-based TNC content of *J. curcas* leaves were significantly increased in elevated [CO_2] for all the four years of study with no effect of sample date (**Table 4.4**).

4.3.5 Nitrogen dynamics and C:N in leaf, soil and litter

Similar to foliar chlorophyll concentrations, there was a significant decrease recorded for N_M in the third and fourth year of growth of *Jatropha* under elevated [CO_2] in comparison to ambient conditions (**Table 4.5**). The relative reduction was ~9% and ~17% respectively at the end of third and fourth year respectively. There was no significant CO_2 treatment effect on N_A (**Table 4.5**). Sampling date significantly affected only N_M and not N_A (**Table 4.3**). However, a significant decrease in N_A within the treatments was recorded with ~10% (ambient) and ~14% (elevated) between first and fourth year (**Table 4.5**). A significant decrease of ~8% for N_A was recorded in leaves of *Jatropha* under CO_2 enriched conditions when compared to ambient conditions at the end of fourth year. Soil available N content was highest in the year 2013 for both ambient and elevated [CO_2] OTCs and subsequently decreased gradually at the end of second, third and fourth year of experiment. However, the relative decrease in elevated CO_2 OTCs was more pronounced with a decrease of ~7%, ~19% and ~32% at the end of second, third and fourth year respectively (**Table 4.5**). The

Table 4.4. Selected biochemical parameters measured during the course of growth of *Jatropha curcas* under ambient and elevated CO_2. Total chlorophyll content, both mass (Chl_M) and area (Chl_A) based was expressed according to fresh weight while total non-structural carbohydrate (TNC) content in terms of dry weight. Values were mean ± SD. Different lowercase letter indicate values significant between the treatments at $P < 0.01$.

	Ambient															
	2013				**2014**				**2015**				**2016**			
	Mar	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**
Chl_M (in mg g^{-1})	2.16± 0.24	2.34± 0.32	2.35± 0.17	2.23± 0.36	2.12± 0.22	2.08± 0.78	2.34± 0.12	2.22± 0.71	2.08± 0.62[a]	2.04± 0.34	2.29± 0.33[a]	2.10± 0.43[a]	2.10± 0.43[a]	2.05± 0.52[a]	2.26± 0.38[a]	2.15± 0.32[a]
Chl_A (in mg m^{-2})	0.236± 0.02	0.234± 0.02	0.236± 0.01	0.236± 0.03	0.226± 0.02	0.228± 0.01	0.234± 0.02	0.212± 0.01	0.208± 0.02	0.210± 0.03	0.209± 0.03	0.206± 0.03	0.206± 0.03	0.205± 0.02	0.205± 0.03	0.205± 0.02
TNC (in mg m^{-2})	34.16± 2.32[a]	35.56± 3.42[a]	34.76± 4.10[a]	34.04± 3.13[a]	32.22± 3.37[a]	32.18± 4.71[a]	34.47± 5.25[a]	34.22± 2.14[a]	33.88± 2.24[a]	34.21± 2.13[a]	36.29± 3.56[a]	32.36± 3.31[a]	30.96± 5.23[a]	31.25± 2.28[a]	31.58± 3.34[a]	32.05± 2.26[a]
	Elevated															
Chl_M (in mg g^{-1})	2.23± 0.22	2.14± 0.27	2.36± 0.18	2.15± 0.32	2.02± 0.12	2.18± 0.17	2.21± 0.22	2.12± 0.18	1.97± 0.42[b]	1.98± 0.34	2.16± 0.35[b]	1.99± 0.34[b]	2.06± 0.34[b]	1.94± 0.19[b]	2.09± 0.33[b]	1.98± 0.22[b]
Chl_A (in mg m^{-2})	0.230± 0.02	0.224± 0.02	0.226± 0.01	0.226± 0.03	0.216± 0.02	0.218± 0.01	0.224± 0.02	0.212± 0.01	0.202± 0.02	0.198± 0.03	0.200± 0.03	0.203± 0.03	0.196± 0.03	0.195± 0.02	0.196± 0.03	0.195± 0.02
TNC (in mg m^{-2})	51.40± 2.23[b]	52.34± 3.12[b]	51.56± 3.82[b]	50.34± 2.84[b]	52.22± 4.25[b]	52.24± 2.32[b]	50.74± 2.72[b]	52.48± 2.44[b]	52.20± 3.32[b]	52.71± 3.53[b]	53.09± 3.83[b]	52.76± 5.23[b]	53.76± 4.43[b]	51.20± 2.22[b]	53.15± 3.82[b]	52.35± 3.28[b]

Table 4.5. Nitrogen contents measured during the course of growth of *Jatropha curcas* under ambient and elevated CO_2 in leaf, litter and soil. The nitrogen content of leaf, both mass- (N_M) and area-based (N_A), was measured for all the four months of growth in an experimental growth year. The N content in litter was measured in the month of harvest of seeds (June and December) at the end of an experimental growth period while soil available N content was measured at the end of experimental year (December month). All the values are expressed according to dry weight. Values were mean ± SD. Different lowercase letter indicate values significant between the treatments at $P < 0.01$. Also, ** indicates significance at $P < 0.01$ within the treatments.

	Ambient															
	2013				**2014**				**2015**				**2016**			
	Mar	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**
N_M (in mg g^{-1})	25.46± 2.14	25.34± 2.22	25.45± 2.27	25.73± 2.26	24.06± 2.12	23.24± 2.78	25.14± 2.22[a]	25.82± 2.51[a]	24.02± 2.22[a]	23.28± 2.24[a]	24.32± 2.23[a]	25.02± 2.13[a]	23.86± 2.23[a]	23.15± 2.42[a]	24.76± 2.28[a]	24.65± 2.32[a]
N_A (in mg m^{-2})	2.36± 0.02	2.34± 0.02	2.36± 0.01	2.34± 0.03	2.32± 0.02	2.31± 0.01	2.34± 0.02	2.30± 0.01	2.12± 0.02	2.06± 0.03	2.09± 0.03	2.14± 0.03	2.10± 0.03[a]	2.01± 0.02[a]	2.08± 0.03[a]	2.10± 0.02[a, **]
Litter (in mg g^{-1})		7.84± 0.42		6.72± 0.13		7.58± 0.17[a]		6.22± 0.14[a]		6.21± 0.13[a]		6.06± 0.31[a]		5.95± 0.28[a]		5.32± 0.26[a, **]
Soil (in kg ha^{-1})				88± 4.32				82± 6.38				76± 8.23[a]				67± 6.76[a, **]

Table 4.5 contd.

	Elevated															
	2013				2014				2015				2016			
	Mar	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**	**Mar**	**Jun**	**Sep**	**Dec**
N_M (in mg g^{-1})	25.16± 2.24	25.04± 2.32	25.25± 2.17	24.23± 2.36	24.26± 2.22	23.84± 2.78	24.24± 2.12	23.32± 2.71^{b}	21.20± 2.62^{b}	21.08± 2.34^{b}	22.09± 2.33^{b}	22.92± 2.43^{b}	19.76± 2.43^{b}	19.55± 2.52^{b}	20.15± 2.38^{b}	20.56± 2.32^{b}
N_A (in mg m^{-2})	2.3± 0.02	2.23± 0.02	2.26± 0.01	2.20± 0.03	2.16± 0.03	2.12± 0.01	2.14± 0.02	2.12± 0.01	2.01± 0.02	1.98± 0.03	2.02± 0.03	2.06± 0.03	1.96± 0.04^{b}	1.85± 0.02^{b}	1.93± 0.03^{b}	1.92± $0.02^{b,**}$
Litter (in mg g^{-1})		7.56± 0.82		6.24± 0.93		6.58± 0.71^{b}		5.48± 0.44^{b}		5.21± 0.83^{b}		5.02± 0.31^{b}		4.85± 0.28^{b}		4.50± $0.76^{b,**}$
Soil (in kg ha^{-1})				84± 3.25				78± 5.36				68± 7.38^{b}				57± $4.38^{b,**}$

ambient OTCs recorded a relative decrease of ~6%, ~13% and ~23% at the end of second, third and fourth year respectively (**Table 4.5**). A significant ($P < 0.01$) decline of ~15% in available soil N content was observed between the treatments at the end of fourth year (**Table 4.5**). Leaf litter N content significantly ($P < 0.01$) decreased within the treatment and between the treatments (**Table 4.5**). The relative decrease was ~22% and ~32% between the first and fourth year for ambient and elevated CO_2 grown *Jatropha* respectively (**Table 4.5**). However, a decline of ~17% in litter N_M was recorded for CO_2 enriched conditions in comparison to ambient conditions ($P < 0.01$) (**Table 4.5**). The effect of CO_2 on litter N_M increased in magnitude from ~5% in 2013 to ~17% in 2016 (**Fig. 4.9A**). The linear regression of the relative CO_2 response (elevated/ambient) for both leaf and litter N_M vs. time were statistically significant ($R^2_{litter} = 0.77$, $R^2_{leaf} = 0.71$; $P < 0.01$) (**Fig. 4.9A**). PNUE was significantly ($P < 0.01$) higher in elevated [CO_2] grown *Jatropha* for all four years of growth (**Table 4.3, Fig. 4.9B**).

C:N ratio in leaves were significantly affected by CO_2 treatment across the four years. The C:N in leaves of elevated CO_2 grown *Jatropha* was significantly ($P < 0.01$) higher by the end of fourth year of growth in comparison to ambient leaves (**Fig. 4.10A**). However, the litter C:N ratios did not show any significant variations between the two conditions across the four years of study (**Fig. 4.10B**). The soil C:N ratios demonstrated a progressive increase from first year to fourth year which was more significant ($P < 0.05$) in fourth year (**4.10C**).

4.3.6 Growth, biomass and yield parameters

The elevated CO_2 grown *Jatropha* demonstrated superior growth features during all four years of growth in comparison to ambient CO_2 plants (**Table 4.6**). The vegetative features like plant height and number of leaves per plant were always significantly ($P < 0.01$) higher in elevated CO_2 plants during all four years (**Table 4.6**). Further, decreased values were recorded for plant height and number of leaves $plant^{-1}$ during the third and fourth year of growth for both ambient and elevated conditions but still elevated plants demonstrated significantly better performance. The plant height in elevated CO_2 grown plants was ~50% higher during all four years of growth while the relative increase in the number of leaves

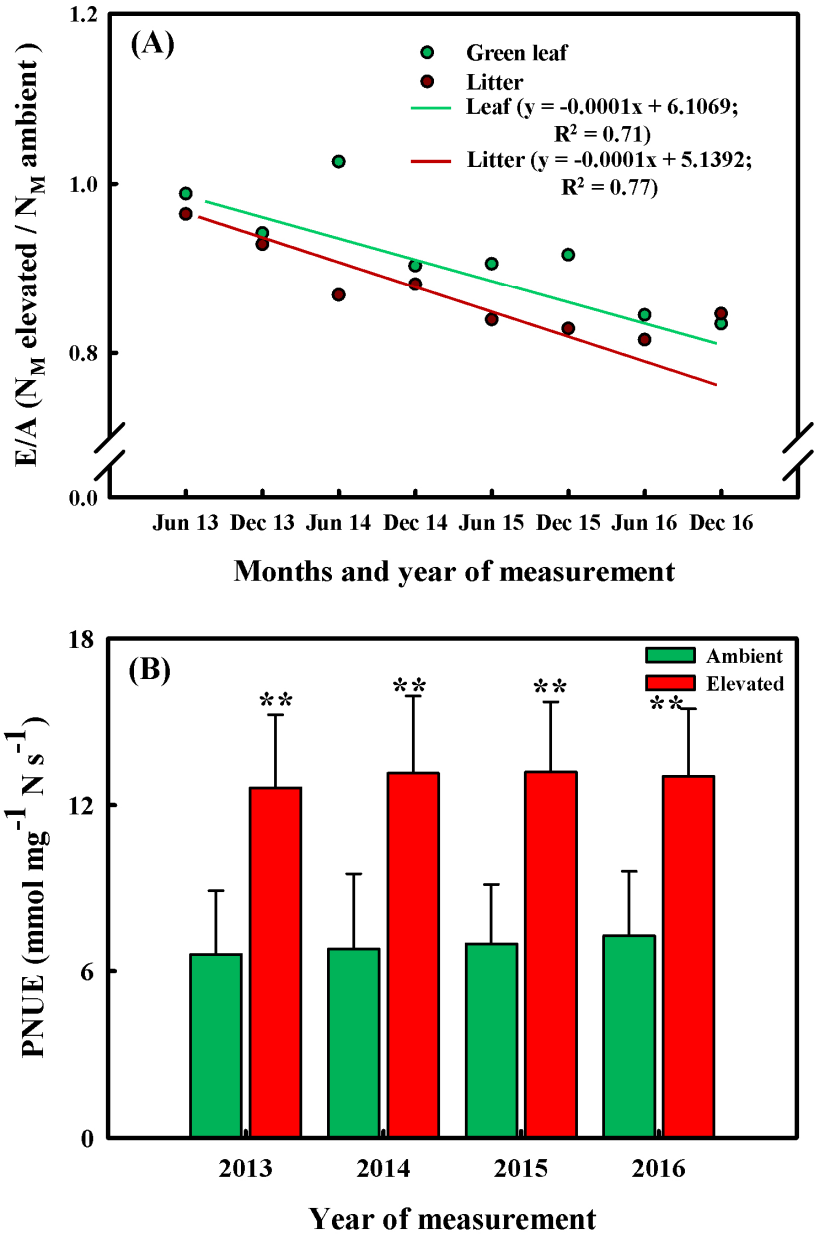


Fig. 4.9. (A) The relative response to CO_2 (E/A) of N_M of leaves (green circles) and N_M of litter (dark red circles) as a linear regression. Both the linear regression were statistically significant (P < 0.01). (B) Photosynthetic nitrogen use efficiency (μmol mg^{-1} N s^{-1}) in ambient and elevated [CO_2] grown *Jatropha* plants. The values are average across the whole year of measurement. Values are mean ± SD (** indicates P < 0.01).

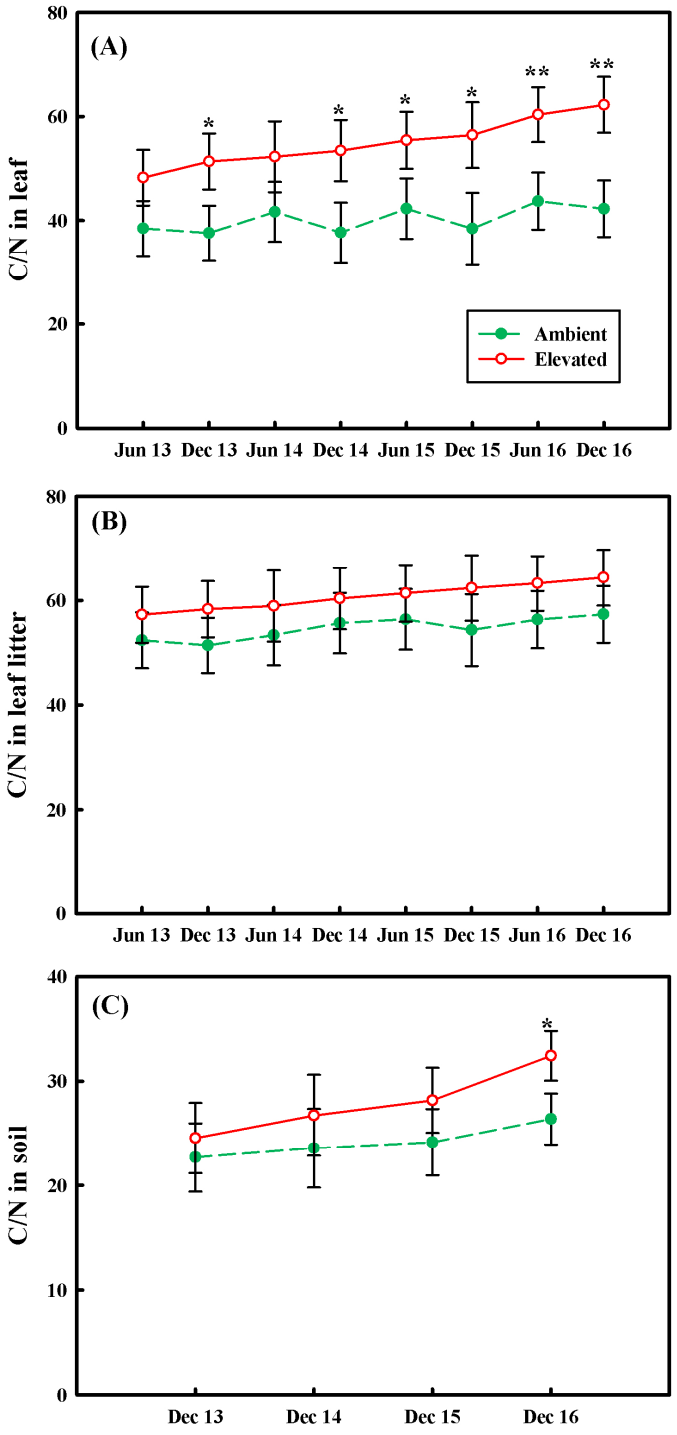


Fig. 4.10. C:N ratios recorded in (A) leaves and (B) leaf litter at the end of each experimental growth period for both ambient and elevated CO_2 grown *Jatropha* during the course of four of years of growth. (C) C:N ratio in soils of ambient and elevated CO_2 OTCs at the end of each year. * and ** indicates significance at $P < 0.05$ and $P < 0.01$ respectively.

Table 4.6. Yield traits of *Jatropha curcas* during long term growth under elevated CO_2. The parameters like plant height, total number of leaves plant^{-1} including leaf litter, number of secondary and tertiary branches, and number of flowers apex^{-1} were estimated before harvesting the plants for each growth period. The parameters like number of fruit plant^{-1}, number of seeds plant^{-1} and 100 seed weight were estimated after harvest of the plants. Values are mean ± SD for each growth period. Different lowercase letters indicate that the values are significantly different between each treatment at $P < 0.01$. [A – Ambient; E – Elevated]

	Plant height (in cm)		**Number of leaves plant^{-1}**		**Number of secondary branches plant^{-1}**		**Number of tertiary branches plant^{-1}**		**Number of flowers apex^{-1}**		**Number of fruits plant^{-1}**		**Number of seeds plant^{-1}**		**100 seed weight (g)**	
	A	**E**	**A**	**E**	**A**	**E**	**A**	**E**	**A**	**E**	**A**	**E**	**A**	**E**	**A**	**E**
June 2013	190.48± 8.36^{a}	282.66± 7.36^{b}	1503.36 ± 272.46^{a}	2243.33 ± 218.53^{b}	10.33± 2.63^{a}	15.33± 1.35^{b}	18.33± 2.16^{a}	22.33± 1.45^{ab}	8.33± 1.23^{a}	18.33± 2.33^{b}	22.25± 2.63^{a}	110.33 ± 7.53^{b}	55.62± 5.28^{a}	204.11± 27.46^{b}	62.56± 5.46^{a}	72.28± 5.35^{ab}
December 2013	217.33± 11.01^{a}	310.33± 8.53^{b}	1519.26 ± 157.43^{a}	2310.45 ± 248.53^{b}	12.66± 1.46^{a}	16.67± 2.26^{b}	21.33± 1.63^{a}	26.33± 2.67^{b}	18.33± 1.36^{a}	27.33± 2.53^{b}	188.62 ± 3.56^{a}	248.54 ± 18.21^{b}	490.41± 34.46^{a}	708.33± 48.38^{b}	70.16± 5.68^{a}	80.28± 4.72^{ab}
June 2014	198.33± 7.63^{a}	290.66± 9.23^{b}	1487.75 ± 192.63^{a}	2334.62 ± 173.35^{b}	9.33± 2.03^{a}	13.33± 1.27^{b}	19.33± 2.33^{a}	23.33± 1.73^{b}	7.33± 0.63^{a}	17.33± 2.13^{b}	18.33± 1.38^{a}	78.72± 8.84^{b}	36.67± 4.48^{a}	154.29± 28.59^{b}	58.89± 4.56^{a}	66.58± 8.53^{a}
December 2014	203.34± 6.23^{a}	310.33± 7.53^{b}	1563.16 ± 202.21^{a}	2285.82 ± 176.33^{b}	11.33± 2.23^{a}	13.33± 1.53^{ab}	22.66± 2.33^{a}	26.33± 1.67^{b}	15.33± 0.98^{a}	25.33± 2.73^{b}	148.72 ± 3.73^{a}	210.33 ± 12.43^{b}	349.49± 17.72^{a}	473.24± 18.73^{b}	69.32± 4.66^{a}	74.33± 6.74^{a}

Table 4.6 contd.

	Plant height		Number of leaves plant^{-1}		Number of secondary branches plant^{-1}		Number of tertiary branches plant^{-1}		Number of flowers apex^{-1}		Number of fruits plant^{-1}		Number of seeds plant^{-1}		100 seed weight (g)	
	A	**E**	**A**	**E**	**A**	**E**	**A**	**E**	**A**	**E**	**A**	**E**	**A**	**E**	**A**	**E**
June 2015	185.32± 5.42^{a}	270.32± 5.13^{b}	1425.78 ± 136.67^{a}	1942.56 ± 218.53^{b}	8.33± 2.13^{a}	9.33± 1.03^{a}	16.33± 1.26^{a}	21.33± 1.33^{b}	2.33± 0.13^{a}	4.33± 1.53^{b}	0.2± 0.03^{a}	2.06± 0.16^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}
December 2015	180.28± 6.32^{a}	280.28± 6.17^{b}	1480.72 ± 178.83^{a}	2178.42 ± 163.42^{b}	9.66± 1.52^{a}	10.33± 8.53^{a}	17.33± 1.17^{a}	20.33± 2.42^{ab}	6.33± 0.53^{a}	10.33± 1.28^{ab}	48.33± 7.36^{a}	88.33± 12.62^{b}	78.5± 4.18^{a}	167.82± 17.33^{b}	53.42± 6.58^{a}	54.73± 5.38^{a}
June 2016	180.56± 8.45^{a}	265.32± 6.92^{b}	1345.23 ± 177.63^{a}	1872.36 ± 188.38^{b}	8.33± 1.76^{a}	9.33± 2.13^{a}	15.66± 2.43^{a}	18.33± 2.34^{ab}	2.33± 1.03^{a}	3.66± 0.73^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}	0.00± 0.00^{a}
December 2016	178.32± 7.45^{a}	270.73± 3.59^{b}	1328.34 ± 213.49^{a}	1732.92 ± 178.53^{b}	8.66± 1.24^{a}	9.33± 0.83^{a}	15.33± 2.03^{a}	18.66± 2.35^{ab}	4.33± 2.13^{a}	8.33± 0.62^{a}	18.22± 1.33^{a}	50.12± 4.76^{b}	14.57± 1.23^{a}	78.26± 4.38^{b}	56.72± 5.34^{a}	52.52± 3.58^{a}

in number of leaves plant^{-1} declined from ~50% to ~30% (**Table 4.6**). Similarly, both number of secondary and tertiary branches, which were significantly higher in *Jatropha* during the first two years of growth under CO_2 enriched conditions, reduced during the last two years of growth (**Table 4.6**). The number of secondary branches was recorded to be more or less similar in number as in ambient growth conditions while the tertiary branches were non-significantly higher in elevated CO_2 conditions (**Table 4.6**). An interesting trend was observed in the reproductive growth and behaviour, especially in the flowering. In the first and second years of growth, early flowering was observed during both experimental growth periods of that particular experimental year. However, flowering was not observed for summer months (June) of third and fourth year. Further, flowering time observed in the rainy and autumn months (September and December) was similar in occurrence with ambient plants. The number of flowers per apex were significantly ($P < 0.01$) higher in elevated CO_2 conditions for first two years with the number significantly reduced in both ambient and elevated conditions for the last two years (**Table 4.6**). However, the number of inflorescence bunch per plant was still higher in elevated CO_2 conditions.

The biomass yields were also significantly ($P < 0.01$) higher in elevated CO_2 grown *Jatropha* plants in comparison to plants grown in ambient conditions (**Fig. 4.11**). The above ground fresh and dry biomass were significantly higher for all the four years of growth under elevated CO_2 (**Fig. 4.11A**). The relative increase recorded in fresh biomass was ~40% for first three years and ~34% for the fourth year while it was ~70% in dry biomass for all the four years (**Fig. 4.11A**). Similarly, the below ground fresh and dry weight (roots) measured after completion of 4 years was significantly higher by ~42% and ~57%, respectively in CO_2 enriched conditions (**Fig. 4.11B**). The canopy productivity index was significantly ($P < 0.01$) higher in elevated CO_2 conditions and was recorded in the range of ~60 g m^{-2} for three years with a slight decrease in the fourth year when compared to ambient *Jatropha* plants, which recorded ~35 g m^{-2} (**Fig. 4.12A**). Since the plants were maintained as coppice plantations, the annual biomass increment was considered as the total biomass gained for a particular experimental year. The carbon sequestered by *J. curcas* after four years of growth in CO_2 enriched environment was in congruence with the biomass increment and canopy productivity index. It was estimated to

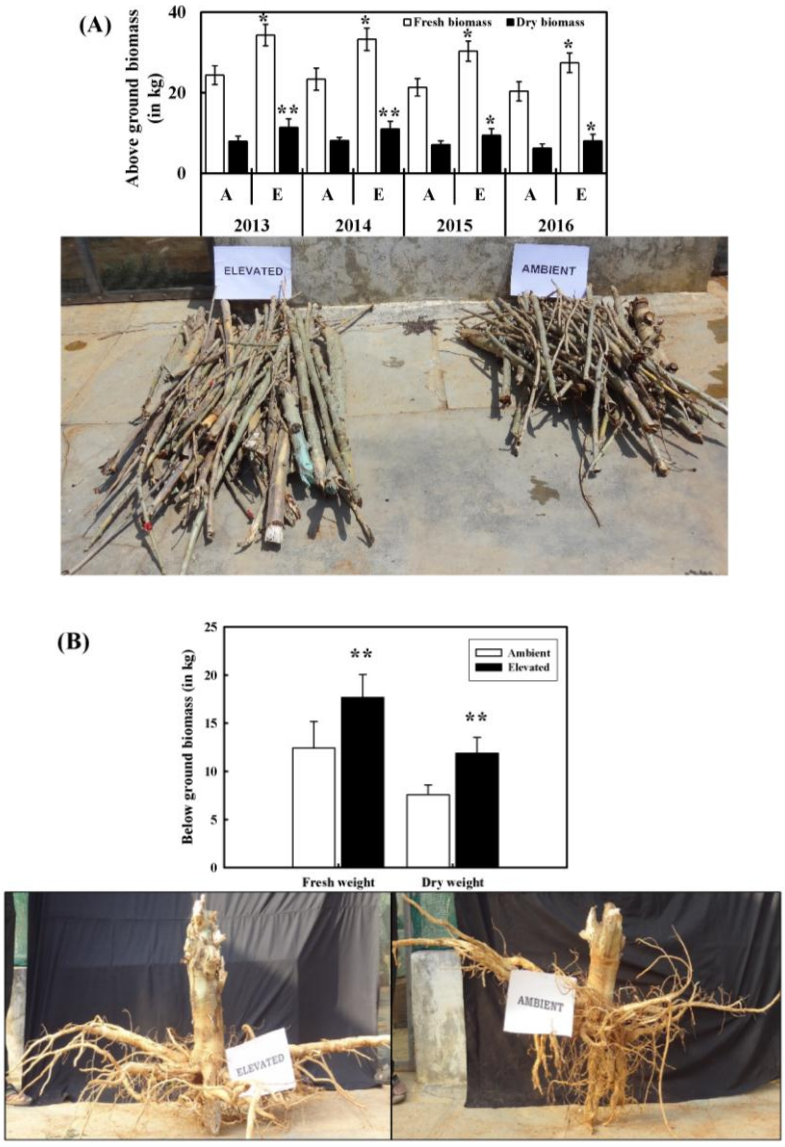


Fig. 4.11. Biomass yields in ambient and elevated CO_2 grown *Jatropha* after four years of growth. (A) Total above ground biomass, both fresh and dry, which includes stem, leaves and fruits. A representational photo showing harvested and dried stems (includes secondary and tertiary branches) of *Jatropha* from elevated and ambient CO_2 conditions after an experimental growth period of six months is presented below the graph. (B) Below ground biomass (roots), both fresh and dry, estimated after harvesting the plants from both ambient and elevated CO_2 conditions after four years of growth. A representational image showing dried root from a single *Jatropha* plant grown in elevated and ambient CO_2 conditions is depicted below the graph. Values are mean ± SD (* - $P < 0.05$ and ** - $P < 0.01$).

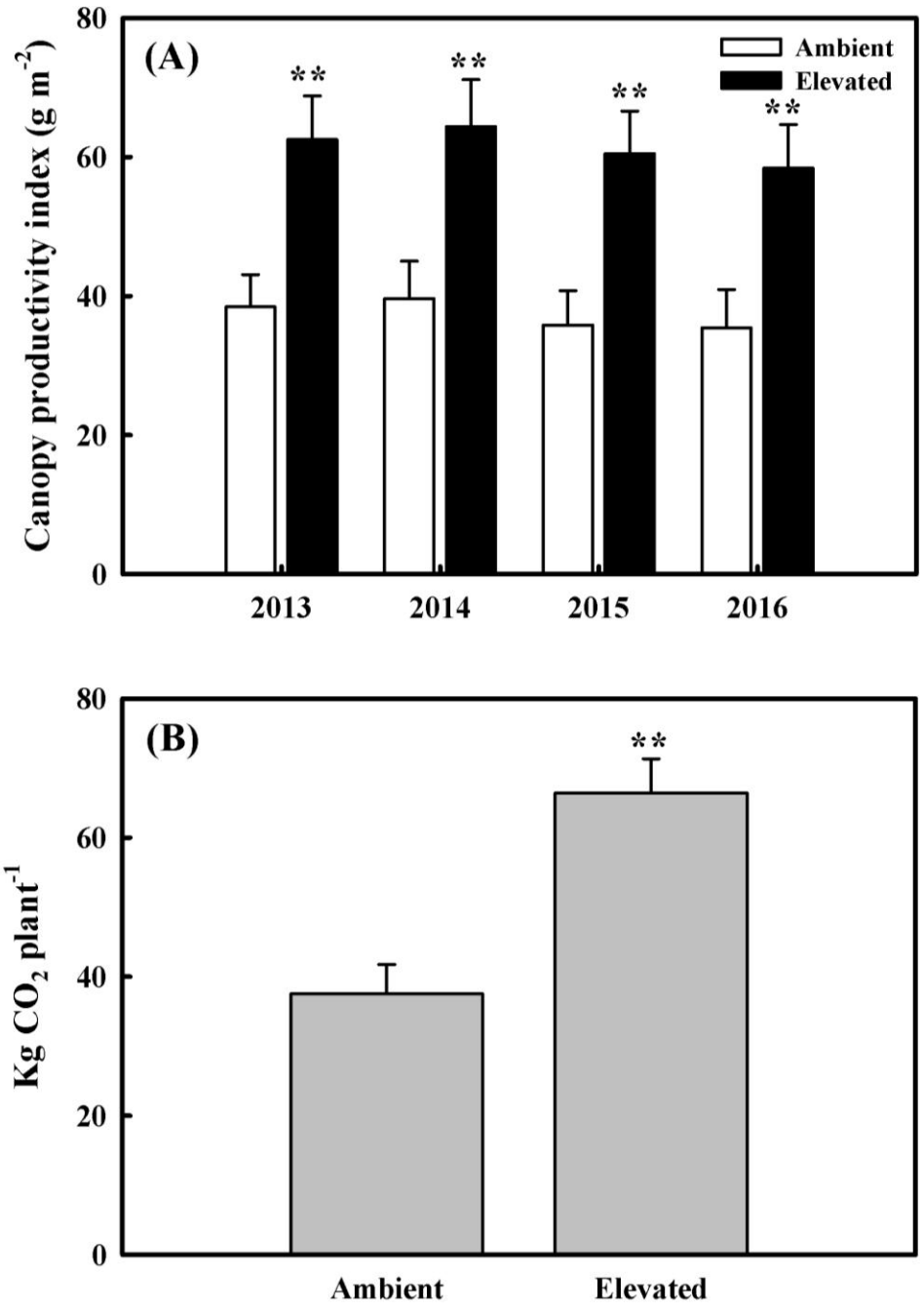


Fig. 4.12. (A) Canopy productivity index in *Jatropha* estimated for all four years of growth under ambient and elevated CO_2 conditions. (B) Amount of CO_2 sequestered by *Jatropha* after four years of growth under both ambient and elevated CO_2 conditions. Values for both parameters is mean ± SD and ** indicates significance at $P < 0.01$.

be ~66 kg CO_2 plant^{-1} in elevated CO_2 grown *Jatropha* plants which was significantly (P < 0.01) higher by ~77% when compared to ambient grown plants (**Fig. 4.12B**).

The reproductive yield data demonstrated interesting and significant variations with the elevated CO_2 grown *Jatropha* recording significantly (P < 0.01) higher yields in comparison to ambient grown plants during all four years of growth. The number of fruits and seeds recorded after harvest of plants were significantly higher for the first two years of growth in elevated CO_2 conditions after both summer (~350% higher) and rainy and autumn (~37% higher) growth seasons when compared to ambient grown plants (**Table 4.6**). As observed in chapter 1 (short term study of one year), a similar trend was observed with very little ambient fruits and seeds in summer month harvest and the yield of ambient plants improving in rainy and autumn months harvest but still lower in comparison to elevated CO_2 grown plants (**Table 4.6**). However, an interesting observation was recorded in third and fourth year of growth. No yields were recorded for both ambient and elevated CO_2 grown *Jatropha* plants in summer month harvest during both third and fourth year of growth. Further, the yields improved in both the conditions during rainy and autumn season harvest for both the years but the number decreased in comparison to first two years in both conditions (**Table 4.6**). There was no significant variations recorded in 100 g seed weight across the four years of harvest (**Table 4.6**).

4.3.7 Expression analysis and enzyme activity of three key regulatory enzymes of carbon metabolism (RL, SPS and FB)

The temporal expression analysis of three regulatory genes demonstrated interesting trends across the four years of study in CO_2 enriched environment. The RL expression were higher in the first two years of growth under elevated CO_2 and subsequently declined in the last two years of growth with the expression more or less similar to ambient conditions in the leaves of *Jatropha* (**Fig. 4.13**). SPS1 showed up-regulation during three years of growth of *Jatropha* in CO_2 enriched environment with the expression recorded as up-regulated in rainy and autumn months and no change in expression pattern during summer months when compared to ambient grown plants. Further, there was no change in expression during last year of growth (**Fig. 4.13**). The temporal expression of cytosolic FB

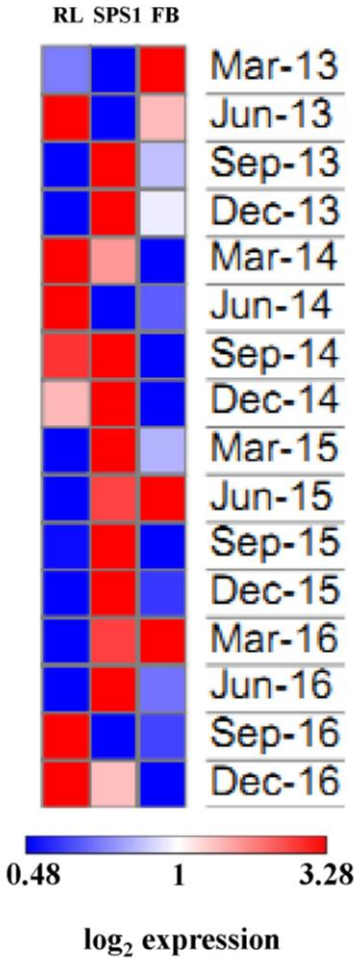


Fig. 4.13. A representative heat map of relative temporal expression patterns of three regulatory genes RL, cytosolic FB and SPS1 associated with carbon conversion and metabolism in *Jatropha* across four years of growth in elevated CO_2 conditions as recorded in qRT-PCR analysis. The values presented here are in log_2 scale with up-regulation ≥ 1.

was significantly higher for the first year of growth of *Jatropha* under elevated CO_2 with no change in expression for the remaining three years when compared to ambient grown plants (**Fig. 4.13**).

The enzyme activities of RL were in accordance with the mRNA expression pattern and showed significant ($P < 0.01$) higher activities for the first two years of growth in *Jatropha* under elevated CO_2 conditions with no change in activities for the last year of growth (**Fig. 4.14A**). The enzyme activity of RL was non-significantly higher in third year under

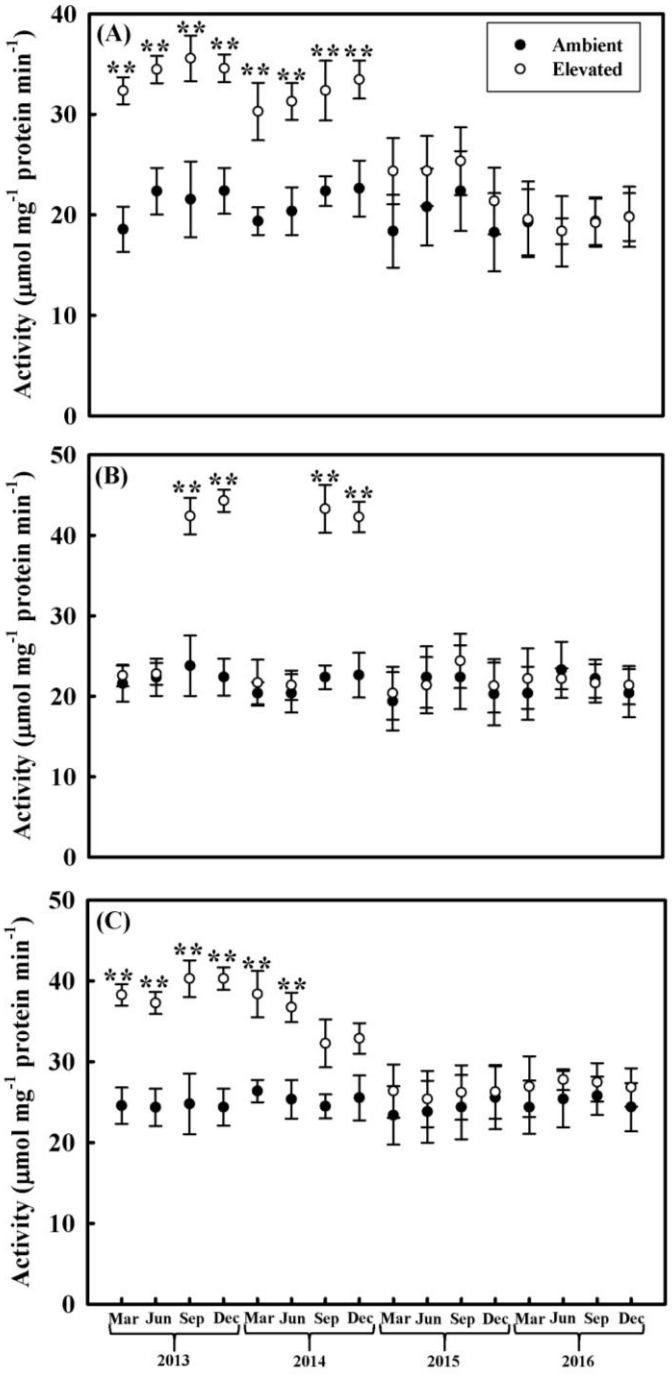


Fig. 4.14. Enzyme activities of three regulatory enzymes (A) RL, (B) SPS and (C) cytosolic FB, associated with carbon conversion and metabolism in *Jatropha* across four years of growth in ambient and elevated CO_2 conditions. Values are mean ± SD and ** indicates significance at $P < 0.01$.

elevated CO_2 conditions (**Fig. 4.14A**). SPS demonstrated increased activities in rainy and autumn months and reduced activities in summer months for the first two years of growth. However, SPS showed no significant change in activity in elevated conditions for the last two years of growth of *Jatropha* in comparison to ambient conditions (**Fig. 4.14B**). Further, enzyme activity of cytosolic FB was not in congruence with the mRNA expression pattern demonstrating increased activity for first two years of growth and subsequent decline in activity under elevated CO_2 for the last two years (**Fig. 4.14C**).

4.4 Discussion

Source-sink relationship and progressive N limitation are the most predominant factors associated with photosynthetic acclimation of plants during long-term growth under elevated CO_2 as highlighted in many studies (Davey et al., 2006; Körner 2006). Furthermore, mature natural forests did not show carbon retention under long-term treatment of elevated CO_2 (Körner et al., 2005). "A possible alternative to circumvent this situation would be identifying fast growing tree species which could be managed as plantation forests thereby aiding in the partial mitigation of rising [CO_2] from the atmosphere at least over a period of decades and buying time for longer-term solutions". We had already reported that *Jatropha*, a fast growing tree species projected for biofuel use, shows better source-sink relationship grown for a duration of one year in CO_2 enriched environment under optimum nutrient conditions (Kumar et al., 2014). It would be interesting to investigate the sustenance of this enhanced source-sink relationship during long-term growth under elevated CO_2. In the present study, morphological, physiological and biochemical feedbacks in relation to source-sink interaction and N dynamics were characterized during long-term growth of *Jatropha* under elevated CO_2 for four years.

Net instantaneous photosynthetic rates (P_n) and A_{Sat} of leaves in the upper canopy of the *J. curcas* did not vary across seasons with consistently higher (50–55%) values in elevated [CO_2] compared with ambient [CO_2] throughout the four years of this study. A similar sustained photosynthetic enhancement (40–55%) in mid- and upper-canopy foliage has been reported in SRC poplar and *Liquidambar styraciflua* during long term growth under

elevated [CO_2] (Bernacchi et al., 2003; Sholtis et al., 2004). "During this period, net biomass productivity was enhanced ~40% by CO_2 enrichment in *Jatropha*, indicating a sustained increase in carbon sinks in elevated [CO_2]". "Analyses of more than 300 *A*-C_i curves collected in this experiment revealed no variations in V_{cmax} and J_{max} between elevated and ambient conditions for first two years and non-significant decrease for last two years for all seasons, further indicates that photosynthetic acclimation to elevated [CO_2] did not occur during the experimental period in this tree species". This lack of photosynthetic down-regulation can be attributed to efficient source-sink balance in terms of fast growth rate as evidenced by plant height and increased biomass yields throughout the experimental period. It also indicates that *Jatropha* demonstrated optimal allocation approach in such a way that V_{cmax} and J_{max} varied with mean environmental and biochemical conditions so as to make optimal use of a finite supply of resources (light, water and nutrients) which maximises the carbon assimilation over a period of time under both ambient and elevated [CO_2] environments (Quebbeman and Ramirez, 2016). The relationship between V_{cmax} and J_{max} represents resource allocation between two photosynthetic processes, electron transport and the Calvin-Benson cycle. The ratio of J_{max} to V_{cmax}, which represents the balance between the above two photosynthetic processes has been theorized to increase in plants grown in elevated [CO_2] which in turn will enhance CO_2 stimulation of photosynthesis (Medlyn, 1996). However, this was not observed in *Jatropha* in our study as they recorded values close to ~1.8 across the four years with non-significant seasonal changes observed during last two years. The change in the J_{max}:V_{cmax} ratio demonstrated in the present experiment occurred within the range of values reported in a study concluding strong correlation between V_{cmax} and J_{max} across a number of species (Wullschleger 1993). The enhanced photosynthetic capacity and biomass yields in *Jatropha* under elevated [CO_2] can be correlated to strong relationship between J_{max} and V_{cmax}, and between these two parameters with other traits resulting from efficient resource investment in J_{max} reflecting effective trade-off between photosynthetic gain and costs of energy dissipation. "Further, the seasonal variations in J_{max}:V_{cmax} ratio suggest that seasonal change in temperature alters the balance between RuBP carboxylation and RuBP regeneration in order to maintain the CO_2 stimulation of photosynthesis (Wilson et al.,

2000)". "Similarly, no shift in the J_{max} to V_{cmax} ratio was observed in a meta-analysis of field-based experiments on European tree species (Medlyn et al., 1999), *Populus tremuloides* and *Acer saccharum* (Kubiske et al., 2002) or in *L. styraciflua* (Herrick and Thomas, 2001; Sholtis et al., 2004)". "Even though stomatal conductance (g_s) was decreased under elevated CO_2 conditions, the leaves maintained constant C_i:C_a ratio across the four years of study suggesting stomatal limitation of photosynthesis was not directly responsible for CO_2 stimulation of photosynthesis (Drake et al., 1997)".

Nitrogen is one of the most important mineral nutrients that limit plant growth in many natural and managed ecosystems. The photosynthetic rate of a leaf is determined by the amount of leaf N and its partitioning between photosynthetic and non-photosynthetic proteins, and among the various photosynthetic components (Hikosaka et al., 2005). Many studies have reported significant correlation between J_{max} and V_{cmax} with leaf N content (Walker et al., 2014). The plant tissue N concentrations are usually decreased in elevated [CO_2], at both the leaf and whole-plant levels (Luo et al., 2006). At the leaf level, such decrease corresponds with metabolic down-regulation of the elevated [CO_2]-induced stimulation of photosynthesis (Stitt and Krapp, 1999; Ellsworth et al., 2004). However, in our study we did not observe any such physiological and metabolic down-regulation under elevated CO_2 environment as exhibited by no significant decrease in A_{max}, V_{cmax} and J_{max} values and increased leaf TNC content even though N_M was significantly reduced for the last two years. This clearly indicates that *Jatropha* exhibited an optimal N allocation strategy that maximizes assimilation over the period of four years for any variation of V_{cmax} and J_{max}. This N reallocation was exhibited in PNUE which was significantly higher in *Jatropha* under elevated [CO_2] indicating efficient investment of N in the N-intensive processes of photosynthesis and photorespiration in leaves and releasing N for venturing in other processes and tissues. The N released from Rubisco and other Calvin-Benson cycle proteins could preferably be allocated to promote the growth of both photosynthetic and non-photosynthetic organs as demonstrated by our data on plant height, number of leaves and tertiary branches (Maire et al., 2012). Further, our temporal data on mRNA expression and enzyme activities of three regulatory enzymes RL, SPS and cytosolic FB showed no significant variation between ambient and elevated CO_2 environments for the last two

years of growth when N_M was limiting. This clearly indicates that elevated CO_2 grown *Jatropha* maintained high photosynthetic rates with lower activities of these enzymes which can be attributed to decreased photorespiration (Woodrow 1994). A lack of congruency between mRNA expression and enzyme activities in *Jatropha* during middle years of growth could be attributed to the complexity of the relationship between RNA levels, protein levels, and physiological changes (Glanemann et al., 2003) Further, in consonance with our data on P_n, plant height and TNC, *Jatropha* exhibited increased LMA during all the four years of study even though leaf density showed no variation for the last two years under elevated [CO_2]. This was in accordance with the reports that LMA positively correlates with photosynthetic and species growth rates. No significant correlation between LMA and leaf density in *Jatropha* was in accordance to reports on many deciduous and evergreen species which also displayed poor correlation between these two traits (de la Riva et al., 2016). "Although changes in leaf characteristics were observed in *Jatropha* grown in elevated [CO_2], this did not affect photosynthetic (A_{max}) and biochemical capacity (V_{cmax} and J_{max})". "Chl_M, but not Chl_A, was significantly reduced in elevated [CO_2] in this study due to reciprocal changes in LMA. We have already demonstrated that photochemistry (overall efficiency of PSII performance) and linear electron flow was enhanced in *Jatropha* grown under elevated [CO_2] for one year suggesting PSII-adaptive improved growth and productivity performance". This trend was maintained by *Jatropha* for four years of growth under elevated [CO_2] as demonstrated by our data on Chl *a* fluorescence parameters measured under light-adapted state indicating no significant effect of decrease of Chl_M on PSII efficiency. The *NPQ* and *qP* showed antagonistic trends demonstrating efficient utilization of light energy to PSII photochemistry associated processes during morning hours which enabled *Jatropha* to maintain increased photosynthesis throughout the four year period (Urban et al., 2001). The more pronounced midday decline in effective PSII quantum yield in elevated CO_2 conditions can be attributed to increasing vapour pressure deficit between leaf and air, decreased g_s and increased accumulation of TNC (Kets et al., 2010). The better recovery rates of $\Delta F/F_m'$ and *qP* during afternoon hours as observed after dark adaptation clearly indicates maintenance of efficiency of PSII photochemistry under elevated [CO_2].

However, a decrease in the recovery process at the end of fourth year reflects on a possible effect of decreased N_M and Chl_M.

"In our study, N_M was generally lower in leaves grown in elevated [CO_2] in all sample periods with more significant reductions during last two years of growth and no significant CO_2 effect on N_A. These results are in agreement with many studies that have shown significant reductions in N_M, but not N_A, because of reciprocal changes in N_M and LMA (Curtis and Wang, 1998; Norby et al., 2002)". This decrease was independent of elevated [CO_2]-induced biomass enhancement which clearly refutes the long-held hypothesis that the decrease in N_M results from growth dilution. "The reductions in N_M may be explained by the presence of increases in TNC as demonstrated in our data which dilutes N_M. Apparently, increased N demand in elevated [CO_2] was met by increased N uptake as indicated by our soil N content data but the relative increase in N acquisition is typically smaller than the relative stimulation of biomass production (Luo et al., 2006)". The quantity of inorganic nitrogen available for plant use often depends on the amount of mineralization occurring and the balance between mineralization and immobilization. It is a very complex process which depends on microorganisms in the soil, external atmosphere, soil type and plant species (Lamb et al., 2014). The soil C:N ratios did not show any significant variation until the fourth year where it was significantly increased under elevated CO_2 conditions. As we had covered the soil in our OTCs with plastic sheet to avoid any absorption of CO_2 by soil, any increase in soil C can be linked to C deposited by plant roots. There is little evidence that elevated [CO_2] alters gross N mineralization and as such no overall significant effect was found via meta-analysis (Reich et al., 2006b). Further detailed studies are needed involving fine roots (produced by *Jatropha* under elevated CO_2 as evident from our photograph of harvested root after four years), climate interaction with soil and many other factors to conclusively assign any reason for the altered C:N ratios of soil (Snowden et al., 2005). Leaf C:N ratios in *Jatropha* were significantly and progressively higher in elevated CO_2 conditions which is in direct relation to increasing TNC and decreasing N_M. A very important aspect of N mineralization in tree species is the nutrient content of leaf litter falling near to the growing plant. "Our data on N_M of leaf litter has been consistently (except for the first year) and

significantly lower (~17%) in elevated [CO_2]". "We cannot yet say whether this effect on litter quality has or will translate into an effect on N mineralization". "The recycling of fine-root N may also affect N mineralization and availability to the above ground parts but more detailed analyses are needed to determine if these responses vary with depth in the soil profile". Furthermore, leaf litter produced in elevated [CO_2] grown *Jatropha* had lower N concentration, potentially making it less readily decomposed, although effects on decomposition rates are highly variable and often small (Norby et al., 2001). It can be posit that the decreased plant N concentration in *Jatropha* under elevated [CO_2] is an indirect consequence of increased biomass production, occurring as C uptake increases more than N uptake (growth dilution) and, over time, as progressively more N becomes locked up in larger pools of litter and biomass with higher C:N ratio under elevated [CO_2] (Taub and Wang, 2008; Feng et al., 2015).

"Many studies have been reported which suggest that even transient reductions in sink capacity may lead to reduced photosynthetic capacity, thereby reducing, but not eliminating, the positive effects of elevated [CO_2] on leaf photosynthesis". But our study clearly demonstrates that sink capacity was efficiently maintained by increased biomass yields thereby not allowing photosynthetic down-regulation to occur. The number of secondary branches was similar to ambient conditions by third year of growth but number of tertiary branches were still higher in elevated conditions. Overall, there was a decrease in numbers in both ambient and elevated conditions for both secondary and tertiary branches but the relative decrease was more in elevated grown *Jatropha* plants suggesting possible effect of N limitation (Finzi et al., 2006). We have earlier reported in our earlier study, elevated [CO_2] ameliorated the negative effects of temperature on the flowering of *Jatropha* in summer season if grown with no nutrient deficiency (Kumar et al., 2014). However, in this study, as the progressive decreases in N_M were recorded, it showed its effect in flowering pattern as well as the flowering time with reduced flowering in third and fourth year of growth during both seasons in *Jatropha* under both ambient and elevated CO_2. This response of no flowering can be manifested to combined negative effects of temperature and reduced N content in leaf. Further, the reduced N uptake or distribution might be a possible reason for this anomaly which otherwise showed marginal

yields in fruits in rainy and autumn season by the end of third and fourth year in both ambient and elevated CO_2 conditions with the elevated CO_2 plants showing superior yields suggesting maintenance of additional sinks for carbon sequestration. Earlier studies have also reported decreased reproductive yield even though vegetative growth has increased (Hikosaka et al., 2005).

In conclusion, photosynthetic enhancement (44–54%) of *J. curcas* was maintained for 4 years with no photosynthetic down-regulation despite decreases in leaf N content and increased TNC accumulation. Elevated [CO_2] also increased PNUE in leaves that are not apparently limited by N in this plant.

Chapter V

Summary and Conclusions

Chapter 5

Rising atmospheric CO_2 concentration and the depletion of fossil fuel stocks have created a demand for secure supplies of carbon-neutral substitute fuels. There is a resurgence of interest in recent years for alternate energy, including biomass-based first generation and oleaginous plant-based second generation biofuels. *Jatropha curcas* has been advocated as promising and potential feed stocks for biofuel production with economical and environmental benefits. If bioenergy is to supply a substantial share of the future energy demand, its potential to mitigate climate change should be evaluated not only under current ambient but also under future elevated atmospheric [CO_2]. Exposure of *Jatropha curcas* to elevated CO_2 atmosphere for one year induced significant physiological, phenological and developmental changes. Our results suggest that *J. curcas* had increased CO_2 assimilation physiology, better source-sink relationship, accelerated flowering and fruit setting time, quantitative enhancement of yield determining growth traits, higher biomass accumulation and enhanced fruit and seed yields in elevated CO_2 growth conditions with no seasonal variations. Net photosynthetic rate was ~50% higher with a subsequent decrease in g_s and E across two seasons of growth in elevated CO_2. This decline was a part of adaptive mechanism in elevated CO_2 grown *Jatropha* which is manifested in more number of closed stomata in comparison to ambient grown plants. Further insights on photosynthetic physiology of *Jatropha* under elevated CO_2 was provided by light response curves and PSII efficiency measured through *Chl a* fluorescence measurements. Elevated CO_2 significantly improved light-saturated rates of

photosynthesis through increased absorption of PAR to be used for improved PSII efficiency and electron transport rates. An increased growth rate in terms of plant height and accelerated flowering time by 8–10 days, leading to early fruit setting and fruit development, and more number of tertiary branches and flowers imply a positive interaction between photosynthesis and morphological components of growth in elevated CO_2 grown *Jatropha*. Furthermore, enhanced biomass and reproductive yields in *Jatropha* under CO_2 enriched conditions can be attributed to efficient coordination between source activity and sink strength suggesting efficient regulation in carbon capture and storage. Interestingly, elevated CO_2 was able to ameliorate the negative effects of temperature on the reproductive yields of *Jatropha* which otherwise is observed in the ambient conditions. Overall, the fast growth and efficient sink strength probably would have allowed *Jatropha* to escape photosynthetic downregulation under elevated CO_2 conditions during its growth for one year.

These morphophysiological findings were corroborated by foliar biochemical and molecular analyses. Starch and soluble sugar content was found to be significantly higher in leaves of elevated CO_2 grown *Jatropha*. Increased sugar levels has been correlated with photosynthetic down-regulation but our data clearly demonstrate that *J. curcas* could sustain enhanced rates of photosynthesis in elevated CO_2 conditions as it had sufficient sink strength suggesting optimized resource use efficiency. We used RNA-seq based approach to understand the molecular mechanisms underlying sustained photosynthesis under elevated CO_2 in *Jatropha*. Genes related to photosynthesis and carbohydrate metabolism were emphasized and the transcript information was further used to understand the temporal expression patterns of these genes in *Jatropha* grown under elevated CO_2. We sequenced the transcriptome of *Jatropha* grown under ambient and elevated (550 ppm) CO_2 levels using Illumina platform. The generated transcriptome consisted approximately 40 million high-quality reads in both conditions. After optimized assembly, a total of 69,581 unigenes with an average length of 1657 bp were generated. The differential gene expression (DGE) analysis showed a total of 3013 differentially regulated transcripts in leaves of elevated CO_2 grown *Jatropha*. The majority of differentially regulated transcripts were associated with metabolism as demonstrated by Gene Ontology (GO) and Kyoto

Encyclopedia of Genes and Genomes (KEGG) analysis. Genes involved in photosynthesis and carbon conversion involving Calvin-Benson cycle, pentose phosphate pathway, starch biosynthesis, sucrose metabolism and glycolysis were differentially expressed implying several venues potentially contributing to elevated CO_2 enhanced growth and photosynthetic capacity including increased reducing power, starch synthesis and sucrose mobilization. Also, the enzyme activities of key regulatory enzymes of photosynthetic reduction cycle positively correlated with the expression levels of the corresponding transcripts.

A pertinent issue to be focused on when assessing elevated [CO_2] responses in C_3 plants, in tree species, is the sustenance of initial photosynthesis stimulation for longer duration. Many factors have been attributed for photosynthetic acclimation under long-term growth under elevated [CO_2] in which two of them, source-sink relations and nutrient limitation in particular N, have been predominantly reported in plants. *Jatropha* showed better source-sink relationship during its growth in elevated [CO_2] for a duration of one year which consisted of two production cycles. We further characterized morphological, physiological and biochemical feedbacks in relation to N dynamics and source-sink interaction in *Jatropha* grown for long-term under elevated [CO_2] for 4 years comprising eight production cycles. Light saturated net photosynthetic rates of upper-canopy leaves were ~55% higher in trees grown in elevated [CO_2] compared with ambient [CO_2] over the 4-yr period, in spite of reduced stomatal conductance and decreased transpiration rates. The C_i/C_a ratio was maintained at ~0.7 throughout the four year period indicating its non-dependence on CO_2 treatment. There were no significant CO_2 treatment effects on photosynthetic or biochemical capacity of *Jatropha* under elevated [CO_2] with either no change (first two years) or non-significant decrease (third and fourth year) in A_{max}, V_{cmax} or J_{max} for the four years. Despite increased area-based leaf sugar and starch content, and reduced mass-based leaf nitrogen concentration, the plants showed better photosynthetic nitrogen use efficiency (PNUE) in elevated CO_2 environment. *Jatropha* consistently showed better source-sink relationship in terms of enhanced biomass yields during its long-term growth under elevated [CO_2]. The soil C:N ratio at 0 – 30 cm depth increased progressively under elevated [CO_2] during its growth for four years. There was seasonal

variation observed in the reproductive behavior of *Jatropha* during long-term growth under elevated [CO_2]. The reproductive yields were better for first two years of growth under elevated [CO_2] as observed during our short term study. The effects of N limitation was observed in reproductive yields during both summer, and rainy and autumn season for last two years of growth. In summer month harvest for the last two years, we did not record any reproductive structure in both ambient and elevated grown *Jatropha* plants. This clearly implies that the elevated CO_2 induced amelioration of negative effects of temperature on reproductive yields were short term and were offset by N limitation. Further, the yields also declined progressively in rainy and autumn season across the four year period in both ambient and elevated grown *Jatropha* plants with the elevated plants showing superior yields for all four years. Rubisco mRNA expression levels declined by the end of second year and there was no variation in Rubisco expression pattern in both ambient and elevated grown *Jatropha*. The enzyme activities correlated with the mRNA expression levels and did not show increased activity for the last two years of growth in leaves of elevated CO_2 grown *Jatropha* plants. The results suggest an optimized Rubisco use and efficient N partitioning between photosynthetic and non-photosynthetic processes to maintain increased growth and photosynthesis under elevated CO_2. Similarly, SPS and cytosolic FB showed decreased expression and enzyme activities during the last two years of growth under elevated CO_2 suggesting efficient sucrose mobilization to maintain source-sink relationships in elevated CO_2 grown *Jatropha*. The carbon sequestration potential in *Jatropha* was estimated to be ~66 kg CO_2 $tree^{-1}$ after four years of growth under elevated [CO_2] which was ~77% higher in comparison to ambient [CO_2].

Our findings depict that the future CO_2-enriched atmosphere would be highly advantageous for superior seed yields in the non-edible oilseed yielding *Jatropha curcas* in non-N limiting conditions, which is projected as a promising feed stock for biofuel production. Even in N limiting conditions, *Jatropha* exhibited water and nitrogen use efficiency producing enhanced biomass thereby sequestering significant amount of C under elevated [CO_2]. Further, *Jatropha* growth under elevated [CO_2] resulted in a limited C debt but the low seed yield attained in N limiting conditions needs to be addressed

through future research focusing on increasing *Jatropha*'s productivity by efficient N management programs.

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