PHYSIOLOGICAL AND MOLECULAR ANALYSES OF FLOWERING RESPONSES IN AMARANTHUS (*Amaranthus* spp.) AND COWPEA (*Vigna* spp.) UNDER ELEVATED CO_2 ENVIRONMENT.

by

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(2015-11-089)

THESIS

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DEPARTMENT OF PLANT PHYSIOLOGY
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DECLARATION

I, hereby declare that this thesis entitled **"Physiological and molecular analyses of flowering responses in amaranthus (*Amaranthus* spp.) and cowpea (*Vigna* spp.) under elevated CO_2 environment."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Rameshwar

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
@	At the rate of
µg	Microgram
µm	Micrometer
°C	Degree Celsius
m^{-2}	Per metre square
CD	Critical difference
cm	Centimeter
ml	Millilitre
M	Molar
EC	Enzyme commission
ppm	Parts per million
o	Degree Celsius
m	Meter
µ	Micro
CRD	Completely Randomized Design
DNA	Deoxyribo nucleic acid
rpm	Rotations per minute
et al.	and other Co workers
OD	Optical density
Fig.	Figure
g	Gram
i.e.	That is
KAU	Kerala Agricultural University
mm	Millimeter
viz.	Namely

IPCC	Inter-governmental panel on climate change
NOAA	National Oceanographic and Atmospheric Administration
mm	Milli meter
ha	Hectare
FACE	Free Air CO_2 enrichment
µmol	Micromoles
mmol	Millimoles
pCO2	Partial pressure of CO_2
µL	Microliter
kDa	Kilo Dalton
µ Enst.	Micro Einstein
mg	Milligram
nm	Nanometer
s	Seconds
A_{663}	Absorbance at 663nm
A_{645}	Absorbance at 645nm
A_{480}	Absorbance at 480nm
A_{510}	Absorbance at 510nm
A_{520}	Absorbance at 520nm
A_{460}	Absorbance at 460nm

INTRODUCTION

1. INTRODUCTION

Climate change is one of the major challenges of our time and adds considerable stress to our societies and to our environment. From shifting weather patterns that threaten food production, to rising sea levels that increase the risk of catastrophic flooding, the impacts of climate change are global in scope and unprecedented in scale. Global climate change has emerged as important environmental challenge due to its potential impact on biological systems on earth. The geographic distribution of species, vegetation types and agricultural cropping patterns demonstrate the very strong control that climate has on plant growth.

Since the beginning of industrial revolution around 1750, human activities have contributed substantially to climate change by adding heat trapping greenhouse gases to the atmosphere which are the most significant drivers of global warming. Heat trapping gases in balanced proportion act like a blanket surrounding earth keeping temperatures within a range that enables life to thrive on a planet with liquid water. However, accumulation of gases in the atmosphere at increasing concentration due to human activities such as burning of fossil fuels, widespread industrialization, clearing of forests for agriculture or development and agricultural practises are leading to thickening of the insulating blanket and overheating of earth.

Environmental Protection Agency considers many molecules such as water vapour (H_2O), CO_2, methane (CH_4) and nitrous oxide (N_2O) as greenhouse gases. Among these CO_2 has contributed the most to climate change (IPCC, 2007) mainly due to its radiative forcing character, longer residence time in the atmosphere and also due to its relative abundance in the atmosphere.

Global concentration of Carbon dioxide in the atmosphere has reached 402 parts per million (ppm) for the first time in the recorded history (NOAA, 2016). Projections suggest that atmospheric CO_2 will reach 700 ppm or more, whereas global temperature will increase by 1.8-4° C by the end of this century. Increase in

global average temperatures would further result in drastic shifts in the annual precipitation with a 20% reduction every year and about 20% loss in soil moisture (Schiermeier, 2008).

CO_2 is actually the food that sustains essentially all plants on the face of the earth as well as those in the sea. As carbon dioxide is a primary substrate for photosynthesis, a rising concentration will have direct effect on plant growth by enhancing the production of assimilates although not proportional. The indirect effects of rising carbon dioxide concentration include changes induced by other environmental variables which occur as a result of the effect of increased CO_2 on global climate. But there exists a spatiotemporal and species (C_3, C_4, and CAM) variation in CO_2 induced responses due to the variation in the availability of other growth resources. This necessitates site specific CO_2 enrichment studies with respect to specific crops.

Earlier researches on plant response to high CO_2 have been conducted under laboratory greenhouse or controlled field condition. Nowadays number of programmes are being carried out all over the world to study the impact of rising CO_2 on agricultural ecosystems. Technologies such as FACE (Free Air CO_2 Enrichment), OTC (Open Top Chamber) and SPAR (Soil Plant Atmosphere Research) have been developed and are being currently used for crop response studies. In India, studies have been reported from IARI New Delhi, CRIDA Hyderabad, IGFRI Jhansi, NPL New Delhi, CRRI Cuttack, BHU, etc. CO_2 enrichment studies in Kerala are being carried out in CPCRI Kasargod and in College of Agriculture Vellayani.

Today's crops and natural vegetation are growing at an elevated CO_2 level that has not been experienced by terrestrial vegetation for 26 million years. Understanding how plants respond and might be adapted to a future increase in CO_2will also help us understand how they are currently responding and how they may have adapted to the increase that has already occurred. The effects of increases in CO_2 on the physiology and development of plants has been the subject of much research over the past 20 years and has been the subject of many

detailed reviews. Elevated $CO_2$and increasing temperature are key climate change factors that could affect plant fitness and flowering related events (Jagadish *et al.*, 2016). The impact of these climate events has already been documented on agricultural crop production, natural species diversity and distribution, and other ecosystem services such as flowering time, pollination etc. (Doney *et al.*, 2012).

Maximal reproductive success of any plant species depends on the time of flowering. There is great diversity of flowering patterns among the different groups of plant species. Still there is an underlying evolutionary conservation of flowering genes and common patterns of flowering regulation. The complexity of flowering regulation is created by an intricate network of signaling pathways which are under developmental regulation as well as under the control of environmental conditions (Valverde, 2002). Being the major determinants of crop growth and development, increased atmospheric CO_2 and temperature can have significant impacts on the phenology and productivity of crop plants. The consequences of atmospheric CO_2 elevation were found to be different in the cases of plants with C_3 and C_4 pathways of CO_2 assimilation

Flowering is a crucial determinant for plant reproductive success and seed set, and changes in the timing of flowering may alter the processes at species, community and ecosystem levels. Besides variable responses of flowering time under elevated CO2 across non-crop species, studies with agricultural crops have shown an overall positive impact of elevated CO2 on growth and yield (Springer and Ward, 2007). Moreover, in an enriched carbon dioxide atmosphere expected in the next century, many species of insects will confront less nutritious host plants that will induce both lengthened larval developmental times and greater mortality (Coviella & Trumble, 1999). Understanding flowering-time responses to global change drivers, such as elevated atmospheric carbon dioxide concentration is necessary to predict the impacts of global change on natural and agricultural ecosystems. The knowledge generated can strengthen modeled projections of future plant evolution.

Amaranth is a C4 dicot which originated in America and is one of the oldest food crops in theworld, with evidence of its cultivation reaching back as far as 6700 BC. Amaranths consist of 60–70 species (Xu and Sun 2001) and include at least 17 species with edible leaves and three grain amaranths grown for their seeds (Jansen *et al.*, 2004). Although several species are often considered weeds, people around the world value amaranths as leafy vegetables, cereals and ornamentals (Trucco and Tranel, 2011). The grain has 12 to 17% protein, and is high in lysine, an essential amino acid in which cereal crops are low. Leaves are also rich in proteins and micronutrients such as iron, calcium, zinc, vitamin C and vitamin A. The grain is high in fibre and low in saturated fats, factors which contribute to its use by the health food market. Recent studies have linked amaranth to reduction in cholesterol in laboratory animals.

Amaranthus has been rediscovered as a promising food crop mainly due to its resistance to heat, drought, diseases and pests, and the high nutritional value of both seeds and leaves. Introduction of amaranth as a human food has been slow, but today it isproduced and used as a grain or leafy vegetable in India, China, Southeast Asia, Mexico, the Andean highlands in South America and the United States.Grain amaranth has been used for food by humans in a number of ways. The most common usage is to grind the grain into flour for use in breads, noodles, pancakes, cereals, granola, cookies, or other flour-based products.

Amaranth is the most common leafy vegetable grown in Kerala and Tamil Nadu. Leaves and succulent stem are good sources of iron, calcium, vitamin A and vitamin C. Both leaf and grain types play a vital role to combat malnutrition of poor people.

Cowpea is one of the most ancient crops known to man. Its origin and domestication occurred in Africa near Ethiopia and subsequently was developed mainly in the farms of the African Savannah. Nowadays it is a legume widely adapted and grown throughout the world. Known for initial fast growth, cowpea can, easily suppress weed growth, therefore, reducing weed-canopy competition. The attributes like, staple fodder, nutritive and medicinal significance, have

established it as the crop of desertic regions world over including Asia, Africa continents and the parts of Southern Europe, USA and Southern America The main cowpea growing countries in Asia are: India, Srilanka, Bangladesh, Myanmar, China, Korea, Thailand, Indonesia, Nepal, Pakistan, Malaysia and. Philippines.

The challenges extended by the changing CO_2 level, make studies on flowering time response of plants under enriched CO_2 highly significant to predict the impacts of global climate change on natural and agricultural ecosystems because modifications in flowering responses will also decide upon the plant fitness to stressful environments. Since flowering time is sensitive to a variety of factors, crops or specific cultivars with more flexibility to adjust flowering time will have successful adaptation under varying environmental conditions. In this context, the current programme, "Physiological and molecular analyses of flowering responses in amaranthus (*Amaranthus* spp.) and cowpea (*Vigna* spp.) under elevated CO_2 environment" was undertaken with the main objective to study the Physiological and molecular basis of elevated CO_2 mediated modifications in the flowering responses of Amaranthus and cowpea.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Changes in Earth's climate have been projected by the end of this century because the effects of rising atmospheric CO_2 concentrations on the global climate have attracted considerable attention. (IPCC, 2001). The notion that humans can influence climate has deep historical roots. This idea appeared briefly in a new form in 1899 when Nils Ekholm, an associate of Arrhenius, pointed out that at present rates, the burning of pit coal could double the concentration of atmospheric CO_2 and could "undoubtedly cause a very obvious rise of the mean temperature of the Earth."

Shifting weather patterns that threaten food production, to rising sea levels that increase the risk of catastrophic flooding, provide some of the most compelling evidences that the impacts of climate change are global in scope and unprecedented in scale. Global climate change has emerged as important environmental challenge due to its potential impact on biological systems on earth. The geographic distribution of species, vegetation types and agricultural cropping patterns demonstrate the very strong control that climate has on plant growth.

After industrial revolution, atmospheric CO_2 concentration has increased significantly, especially during the second half of the last century. Heat trapping gases in balanced proportion act like a blanket surrounding earth keeping temperatures within a range that enables life to thrive on a planet with liquid water but accumulation of gases in the atmosphere at increasing concentration due to human activities such as burning of fossil fuels, clearing of forests for agriculture or development and agricultural practises is leading to thickening of the insulating blanket and overheating of earth.

The intergovernmental Panel on Climate Change Reports (eg. IPCC, 2007, 2013) document evidence of increasing carbon-dioxide concentrations and other greenhouse gases leading to a higher frequency of extreme climate events such as

heat waves and drought events. The impact of these climate events has already been documented on agricultural crop production, natural species diversity and distribution. (Doney *et al.*, 2012). CO_2 has contributed the most to climate change (IPCC, 2007) mainly due to its radiative forcing character, longer residence time in the atmosphere and also due to its relative abundance in the atmosphere.

The rise in atmospheric CO_2 is one of the most pronounced global changes in the past 50 years (Prentice, 2001). According to Intergovernmental Panel on Climate Change (IPCC) Report (2007) the global atmospheric concentration of carbon dioxide (CO2) has increased from pre-industrial level of 280 ppm to the level of 401.62 ppm (NOAA, 2016) and is rising at the rate of 2 ppm per annum and atmospheric CO_2 is expected to reach 700 ppm by the end of 21st century. The existing concentration exceeds by far the natural range over the last 650,000 years (180-300 ppm) as determined by ice cores (Rao *et al.,* 2015). The CO_2 already committed to the atmosphere has warmed the world about 1.8° F since the start of the industrial revolution (Kahn, 2016). Atmospheric CO_2 concentration believed to be increasing at a rate of 0.4-0.5% per year (IPCC, 1995).

CO_2 is actually the food that sustains essentially all plants on the face of the earth as well as those in the sea. Rising CO_2 can influence world ecosystems by direct effects on plant growth and development regardless of changes in global temperature and other climate variables. Many plant and ecosystem attributes will directly or indirectly be influenced by elevated CO_2. Plants respond both physiologically and anatomically to elevated CO_2. Many studies have investigated plant responses to elevated CO_2 on ecosystem, community, population, plant, leaf, physiological, biochemical and molecular scales (Norby *et al.*, 1999)

A potential consequence of the rise in CO_2 concentration with respect to plant biology is its effect on plant process of photosynthesis i.e. biological effect. The higher level of atmospheric CO_2 affects C_3, C_4 and CAM plants differentially (Poorter 1993). CO_2 enhances the growth rate of almost all plants (Kimball, 1983)

but the enhancement was very significant in C_3 species (Sujatha *et al.,* 2008). This necessitates site specific CO_2 enrichment studies with respect to specific crops.

Over the past three decades, a large number of studies have focussed on the effects of increasing atmospheric carbon dioxide concentrations, [CO_2], on the physiology, growth and reproduction of plants. Technologies such as FACE (Free Air CO_2 Enrichment), OTC (Open Top Chamber) and SPAR (Soil Plant Atmosphere Research) have been developed and are being currently used for crop response studies. In India, studies have been reported from IARI New Delhi, CRIDA Hyderabad, IGFRI Jhansi, NPL New Delhi, CRRI Cuttack, BHU, etc. CO_2 enrichment studies in Kerala are being carried out in CPCRI Kasargod and in College of Agriculture Vellayani.

Plants are finely tuned to the seasonality of their environment and shifts in the timing of plant activity (i.e. phenology) provide some of the most compelling evidence that species and ecosystems are being influenced by global environmental change. Phenology is a dominant and often overlooked aspect of plant ecology, from the scale of individuals to whole ecosystems. The timing of the switch between vegetative and reproductive phases that occurs in concert with flowering is crucial to optimum seed set for individuals and populations; variations among species in their phenology is an important mechanism for maintaining species coexistence in diverse plant communities, by reducing competition for pollinators and other resources (Rathcke and Lacey., 1985).

Plants are sessile organisms that have adapted to their habitats to optimize flowering time and thereby guarantee reproductive success and survival. Flowering is a critical phenophase in the life cycle of plants; it determines individual plant fitness, reproductive competence, and synchrony with insect pollinators, successful seed set and propagation. Flowering time is under the control of multiple exogenous cues such as photoperiod and exposure to cold temperatures (vernalization) and indigenous factors like plant size, nutrient availability Changes in the timing of flowering may alter the processes at the

species, community and ecosystem levels. Typically for annual species, the onset of reproduction marks the end of the vegetative growth stage and begins the early stages of senescence. As a result, elevated CO_2 may alter plant fitness both through changes in flowering time and altered plant size at the flowering, which influences the amount of resources available for reproduction. (Springer and Ward, 2007).

During floral transition, the shoot apical meristem switches from the production of leaves from associated secondary shoot meristems to bractless flowers (Parcy *et al.*, 2004). This transition is abrupt and irreversible, suggesting it is regulated by a robust gene regulatory network capable of driving sharp transitions. The moment at which this transition occurs is precisely determined by environmental and endogenous signals. The field of flowering time is organized around these four pathways, with the photoperiod and vernalization pathways mediating the response to environmental cues and the autonomous and gibberellins (GA) pathways acting largely independently from these external signals. A large number of genes acting within these pathways have been cloned and current analyses aim at understanding how they are linked to each other and how the corresponding proteins function (Amasino, 2010). Two genes play a prominent role at the "bottom" of these promotion cascades. The *CONSTANS (CO)* gene is probably the most downstream actor, specific for the photoperiod pathway and both the light and internal circadian clock precisely regulate the *CO* protein accumulation (Valverde *et al.*, 2004). The *FLOWERING LOCUS C (FLC)* gene is the point of convergence of the autonomous and vernalization pathways. Ultimately and in part through *CO* and *FLC*, the flowering signals lead to the induction of floral meristem identity (FMI) genes and responsible for the fate change of the meristems emerging on the flanks of the shoot apex (Long and Barton, 2000). This group of genes includes *LEAFY (LFY), APETALA 1 (AP!)* and *CAULIFLOWER (CAL),* expressed in early floral stages and responsible for their floral fate (Kieffer and Davies, 2001).

Recently, three genes were shown to make the junction between different flowering-time cascades and floral meristem identity *(FMI)* genes. These genes were named floral pathway integrator because they were able to integrate a balance of stimulations originating from the different pathways and convert these heterogeneous inputs into an induction of FMI genes, thereby initiating the production of first floral meristems (Simpson and Dean, 2002).The three genes shown to integrate the influence from different pathways are *LEAFY (LFY), FLOWERING LOCUS T (FT), SUPPRESSOR OF CO OVEREXPRESSION (SOC1).*

Effect of elevated CO_2 on flowering time has been reviewed by Springer and Ward (2007) who summarized 60 studies including 90 different crop and wild species grown under elevated CO_2 in controlled chambers, in the field (using open top chambers and FACE) conditions. Contrasting responses of flowering time to elevated CO_2 among short and long day plants suggested a possible interaction of the photoperiod pathway with elevated CO_2 to regulate floral signaling (Johnston and Reekie, 2008). In *Arabidopsis*, the sustained expression of the floral repressor gene *FLC* was reported to be associated with delayed flowering in the genotype that was selected for high seed yield under elevated CO_2 (Springer and Ward, 2008). Recently, *MOTHER OF FT AND TFL1* (*MFT*), a homolog of *FT* and *TERMINAL FLOWER1* (*TFL1*), has been identified as a candidate gene influencing flowering time with elevated CO_2 (Ward *et al.*, 2012). The involvement of sugars in the regulation of flowering has been reported in *Arabidopsis*, where inadequate sugar levels in the vegetative tissue (leaf) and shoot apical meristem produce TREHALOSE-6-PHOSPHATE(T-6-P) as a proxy signal for floral transition and initiation under inductive environmental conditions (Wahl *et al.*, 2013). *Arabidopsis* plants with mutation in the *TREHALOSE-6-PHOSPHATE SYNTHASE* gene (AT1G78580) failed to flower, showing the essential role of sugar signaling (*T-6-P*) in regulation of flowering time (Jagadish *et al.*, 2016).

Elevated CO_2 can induce floral transition with enhanced substrate supply through increased photosynthesis (Springer and Ward, 2007). However, excess foliar sugar accumulation (beyond a threshold) under elevated CO_2 may delay flowering in several plant species. For instance, elevated CO_2 delayed flowering in *Arabidopsis* plants with a 41 and 105% increase in foliar sucrose and starch content, respectively (Bae and Sicher, 2004), indicating differential response to foliar sugars levels below and above threshold limits. Thus, varying sensitivity to sugar concentration under elevated CO_2 within or across species warrants further investigation to find a links between elevated CO_2 and flowering competency. It is envisioned, in the near future, that the yet to be identified novel regulators involved in the signaling network modulating floral initiation in response to elevated temperature and elevated CO_2 will facilitate understanding and identifying options to develop plants or breed crops to better adapt to changing climate.

Therefore understanding flowering time responses to global change drivers such as elevated atmospheric carbon dioxide (CO_2) is necessary to predict the impacts of global changes on natural and agricultural ecosystems.

Growth Parameters

Plant growth and development depends on a number of endogenous as well as exogenous factors like temperature, relative humidity, light intensity and its duration. Since CO_2 is a major photosynthetic substrate, it has profound impact on the growth rate and development of plant species. The most evident and best studied effect of elevated atmospheric CO_2 is the so called "fertilization effect" (LaMarche *et al.* 1984). Because CO_2 is a substrate for photosynthesis, an increase in atmospheric CO_2 concentration stimulates photosynthetic rates in C_3 plants. The short term responses that are typically measured are different from long term responses (Bazzaz, 1990), however under elevated CO_2 levels, photosynthesis rates increase initially but after a period of time may decrease (Bazzaz, 1990).

Typically for annual species, the onset of reproduction marks the end of the vegetative growth stage and begins the early stages of senescence. As a result, elevated CO2 may alter plant fitness both through changes in flowering time and through altered plant size at flowering, which influences the amount of resources available for reproduction (springer and Ward, 2007).

Number of leaves

Photosynthates acquired by leaves are used for the production of leaves, stems, roots, and reproductive organs. Increase in allocation to the leaf would be beneficial for photosynthesis, but may reduce other functions such as nutrient uptake and reproduction.

An increase in biomass due to increase in the number of leaves or branches has been reported in sweet potato and Japanese honey-suckle under elevated CO_2 environment (Sasek and Strain, 1991). There is a decrease in the number of leaves found in soybean by 23% and 14% under such situations compared with ambient CO_2 (380 $\mu mol\ mol^{-1}$) (Madhu and Hatfield, 2015).

Mousseau and Saugier in 1992 reported that there is considerable increment in the leaf mass per unit area under elevated CO_2 condition and the leaf thickness caused by an increase in the number of palisade cell layers (Thomas and Harvey, 1983).

Specific leaf area

Specific leaf area (SLA) is the one sided area of a fresh leaf, divided by its oven dry mass and it is inverse of specific leaf weight. Specific leaf area is frequently used in growth and analysis because it is often positively related to potential relative growth rate across species.

It is one of the widely accepted key leaf characteristics used during the study of leaf traits (Kraft *et al.,* 2008). Leaf area expansion depends on leaf turgor, temperature, and assimilating supply for growth.

There is a variation in the response of plants to elevated CO_2 in relation to the specific leaf area. For example, Elevated CO_2 enhances leaf size (Kerstiens *et al.,* 1995) and decreases specific leaf area in a number of plant species (Norby and Neill, 1991). Elevated CO_2 resulted in significant increase in leaf area at vegetative and 50% flowering stages in Chickpea, but at pod maturity reverse trend was observed (Saha, *et al.*, 2014)

Days to first flowering

Environmental factors such as photoperiod and temperature that control the timing of the first bud or flowering from a whole-plant physiological perspective has been described in detail by Craufurd and Wheeler (2009). In general, historical records of flowering time, herbaria and aerobiological documents on pollen data indicate advancement in flowering time in perennials (Jagadish *et al.*, 2016). Flowering time in 40 published studies involving both crops and other plant species exposed to elevated CO_2 (from 350 to1000 ppm) showed 28 cases (different species within the same study is considered a case) in which flowering time was earlier (average 8.6 days) and 12 cases in which flowering was delayed (average 5.2 days).

Days to 50% flowering

Pea plants grown under elevated CO_2 during 2014 took less days to 50 per cent flowering (74.31 days), which differed statistically with ambient condition (94.10 days). Highest days to 50 per cent flowering were taken by plants grown under natural condition (Meenakumari *et al.,* 2016). Similarly during 2015, lowest days to 50 per cent flowering were recorded in pea plants grown under elevated CO_2 (73.98 days) and differed statistically from natural condition (91.77 days). Highest days to 50 per cent flowering were taken by plants grown under natural condition.

Root weight

Root system comprise up to half the total tree biomass and below ground net primary production may exceed 50% of total net primary production because

C allocation to roots is often favoured over C allocation to shoots in plants grown under elevated atmospheric CO_2 (Kubiske and Godbold, 2001).

The elevated CO_2 has been reported to promote N allocation in different parts and more particularly to underground part of the plant (Zhou and Shangguan, 2009).

Almost all plant species when exposed to the elevated CO_2 condition shown increased root growth that contributes to root biomass and root dry weight (Rogers *et al.,* 1994, 1996). Cotton plants upon exposure to elevated CO_2 for six weeks exhibited higher dry weights, lengths and volumes of taproots, lateral roots and fine roots. The experiment was carried out in a Free Atmospheric Carbon dioxide Enrichment (FACE) (Prior *et al.*, 1992).

Increased root growth of forest trees under elevated atmospheric CO_2 has been reported by several researchers (Matamala and Schlesinger, 2000, Pretgitzer *et al.*, 2000, Pritchard *et al.*, 2001) Consistent findings show that the production and mortality of fine roots produced by trees growing under elevated CO_2 are significantly increased. (Matamala and Schlesinger, 2000, Pretgitzer *et al.*, 2000, Pritchard *et al.*, 2001).

The temperature and elevated CO_2 concentration found to have combined effect on the growth and development of roots. An increment in fibrous root dry weight was observed in groundnut upon exposure to elevated CO_2 and temperature 15-25° C.

Shoot weight

The measurement of shoot weight is quintessential as it is a measure of the productive investment of the plant dealing with the relative expenditure on potentially photosynthesizing organs.

The average enhancement of photosynthesis for trees exposed to elevated CO_2 has been about 60% (Norby *et al.*, 1999). High CO_2 exposure resulted in more dry matter allocation towards above ground parts compared to roots. The

enhanced photosynthesis has generally been followed by a similar, albeit a somewhat decreased magnitude, enhancement of above ground growth.

Rice plants under elevated CO_2 conditions shown increased shoot dry weight compared to ambient CO_2 and field condition. (Razzaque et al., 2009). Epron *et al.*, 1996 reported that in *Fagus sylvatica,* shoot dry mass was significantly higher (90%) in the elevated CO_2 treatment than in the ambient CO_2 treatment. There were increasing treatments in biomass, above ground biomass, leaf area and below ground biomass in *Larrea tridentate* (Obrist and Arnone, 2003) and Piper nigram L. (Minu *et al.*, 2015).

Root-shoot ratio

Root-shoot ratio is the simple calculation of the ratio of root dry mass to shoot (or stem) dry mass and serve as a measure of the preferential allocation of C to roots or shoots. (Mo *et al.*, 1992). It is one measure which indicates the overall health of plants (Obrist and Arnone, 2003). The partitioning pattern of photosynthate depends on plant developmental stage, plant species, and plant growth conditions along with physiological factors (Van veen *et al.,* 1991). Increase in the root-shoot ratio due to more allocation of carbon to below ground was found in the plants exposed to elevated CO_2 (Oechel and Strain, 1985).

Ellis, 1995 reported that, in tomato, the doubled ambient CO_2 treatment showed significantly lower root-shoot ratio (0.138), than the ambient CO_2 treatment (0.156). Cure, 1985 observed that the range of response in R/S among crop plants to CO_2 elevation ranged from 8.5% decrease to a 6.4% increase, except in sweet potato, in which a 34.9% increase was recorded. Considerable decline in the root-shoot ratio of C_3 and C_4 crops and weeds was found upon exposure to the elevated CO_2 condition (Miri *et al.*, 2012).

Dry matter production

Dry matter is the expression of productivity in terms of the weight of material produced during specific time period. An increase in total dry matter production was reported in soybean (Pan, 1996), dry bean (Prasad, 2002), peanut and cowpea (Ellis, 1995) under elevated CO_2. Madhu and hatfield in 2015 reported considerable increment in the dry matter production in soybean plants and black pepper (Minu *et al.*, 2015). Pilumwong *et al.* in 2007 reported improved dry matter production in mung bean at different growth stages.

The total augmentation in biomass production per plant was reported to be 37.5 g in elevated CO_2 from 27.5 g in ambient CO_2 whereas roots shown largest effect (53% increase) and then on stem (40% increase) with little effect on leaves (11%) (Tezara *et al.*, 2002).

The reduction in the respiratory rates in C_3 plants induced increased biomass production (Reddy *et al.*, 2010). Higher biomass production and seed yields were reported in lentil under elevated CO_2 levels of 700 $\mu mol\ mol^{-1}$ (Nasser, 2008). Upon exposure to the elevated CO_2, total biomass was increased by 12.0% compared with ambient and field grown rice (Weigel *et al.*, 1994). The study conducted by Uprety *et al.*, 2006, showed high grain yield in rice under elevated CO_2. The dry matter production and seed yield were increased by 13.5% and 17.5% respectively in sorghum and 23.8% and 34.7% respectively in Soybean (Reeves *et al.*, 1994). Dry matter accumulation in shoot and root as well as leaf dry weight (81 %) increased over control. Transpiration rates were reduced under CO_2 enrichment by 34 per cent. Increased leaf dry weight accumulation and specific leaf weight under CO_2 enrichment suggested that more carbohydrate may be available to the plant for future growth (Woodrow *et al.*, 1987).

Leaf temperature

Glycine max leaf temperature was found to be increased upon exposure to the elevated CO_2 condition (O'Neill *et al.*, 2011).

Stomatal distribution

Stomata are the pores on a leaf surface controlling gas exchanges, mainly CO_2 and water vapour, between the atmosphere and plants (Woodward, 1987), and thus regulate water cycles in various ecosystems (Franks and Beerling, 2009; Haworth *et al.,* 2010; Taylor *et al.,* 2012) They occupy between 0.5% and 5% of the leaf epidermis and are most abundant on the bottom or abaxial surface.

Sarker and Hara in 2011 conducted study on eggplants reported that stomatal density had reduced when grown under elevated CO_2 environment and water stress. The same phenomenon was observed in both adaxial and abaxial surfaces. Boetsch *et al.* (1996) studied the influence of elevated CO_2 concentration (670 ppm) on the structure, distribution and patterning of stomata in *Tradescantia* leaves in comparisons with plants grown at ambient CO_2. Stomatal frequency in plants grown at elevated temperature (29°C) was not significantly different from that of the control (24°C).

Physiological and biochemical parameters

Pigment composition (Chlorophyll content)

Chlorophyll is quintessential for photosynthetic organisms for trapping light and energy transduction. Chlorophyll is one of the most abundant organic substances on earth.

An increase in the leaf chlorophyll content was observed in rice (Haque *et al.,* 2003). Whereas the leaf chlorophyll content exhibited decline or no change when exposed to the elevated CO_2 (Rao and Tower, 1970). Reeves *et al.,* 1994). reported that total chlorophyll on an area basis was not affected by enriched CO_2 in soybean. However, (Wullschleger *et al.,* 2002 have shown a reduction in the concentration of extractable chlorophyll with elevated CO_2.

There was significant decline in the chlorophyll content of Brassica leaves under elevated CO_2 (Uprety and Mahalaxmi, 2000).

Total soluble protein

Jablonski *et al.,* in 2002 reported that Barley plants when exposed to the elevated CO_2 condition exhibited decline by 20%. Whereas rice or soybean showed no significant difference when exposed to the elevated CO_2 condition. Saravanan and Karthi in 2014 reported the lowest protein content in *Catharanthus roseus* plants under elevated CO_2 concentration of 900 ppm (11.40 mg/ml).

Protein concentrations shown a decline in cereal grains under elevated CO_2 thereby affecting the protein concentration of photosynthetic tissues. It is concluded that the reduction in the Rubisco concentration is causing this decline (Ainsworth and Long, 2005). The protein content was observed to be lowered by 20% under elevated CO_2 condition (Jablonski *et al.,* 2002; Loladze, 2002).

Starch and Reducing sugars

A considerable advancement in the content of reducing sugars and total starch was reported under elevated CO_2 in Black gram (Sathish *et al.*, 2014). Long et al in 2004 reported significant increase in foliar carbohydrate content under elevated CO_2. Observations of increased foliar carbohydrate content in plants grown in elevated CO2 are well documented, including soybean, in which growth at elevated CO2 resulted in a 45% significant increase in total non-structural carbohydrate, although the large increases in starch was also reported (Ainsworth *et al.,* 2005).

High concentration of starch in mature tomato leaves exposed to high CO_2 has been found (Yelle *et al.,* 1989). In Arabidopsis starch content of the shoot was substantially increased upon exposure to elevated CO2, while the soluble sugar content remained unaffected (Kooij and Kok, 1996). Accumulation of

carbohydrates has been observed in many studies during plant growth under CO_2 enrichment (Makino and Mae, 1999). Non-structural carbohydrate concentrations invariably increase within leaves grown at elevated CO_2 (Drake *et al.*, 1997).

Lilley *et al.* (2001) reported that elevated CO_2 conditions produced an average increase in total non-structural carbohydrate contents of 28% for clover and 16% for phalaris. CO_2 enrichement improved the carbohydrate contents of tomato fruit compared to fruits exposed to ambient CO_2 concentration, which might be due to enhanced translocation of photosynthate with elevated enzyme activities (Islam *et al.*, 2006).

Gibberellic acid

The effects of CO_2 enrichment on leaf ultra structure, mineral nutrition and plant hormone concentrations have not been extensively studied in any of the model plants; nevertheless, these aspects, in particular leaf ultra structure and plant hormone concentrations, are very important for an integrative understanding of plant responses to increased atmospheric CO_2 (Rao and Tower, 1970).

Nitrate reductase

CO_2 enrichment also affected leaf nitrate reductase (NR) activity in both the genotypes but no particular trend was observed. In Pusa 1103, NR activity increased significantly during vegetative and flowering stage by 68% and 48%, respectively but declined by 15% at podding stage. NR activity was higher during vegetative stage followed by flowering and podding stage. In Pusa 1105, NR activity declined by 12% and 10% during flowering and podding stage, respectively (Rogers *et al.*, 1996).

Quality parameters

Oxalate and Ascorbic acid

In chickpea, chemical analysis revealed that plantlets grown with elevated CO_2 had somewhat higher levels of oxalates in the leaves and shoots, but levels of

ascorbic acid were low. The higher CO_2 concentration also reduced the rate of ascorbic acid synthesis, which has an important role in key aspects of plant metabolism (Rinallo *et al.*, 2009). Moreover Chickpea plants kept in 750 ppm CO_2 concentration shown greater amount of oxalic acid then those plants kept at 350 ppm CO_2 concentration.

In some cases, the additional carbon fixed by plants during CO_2-enrichment is invested in antioxidative compounds; and one of the most prominent of these products is *ascorbate* or vitamin C. In the early studies of Barbale (1970) and Madsen (1971, 1975), a tripling of the atmospheric CO_2 concentration produced a modest (7%) increase in this antioxidant in the fruit of tomato plants. Kimball and Mitchell (1981), however, could find no effect of a similar CO_2 increase on the same species. Mamata and co-workers in 2014 reported high ascorbic acid content in tomato at both 700 and 550 ppm CO_2 concentration. In bean sprouts, on the other hand, a mere one-hour-per-day doubling of the atmospheric CO_2 concentration actually doubled plant vitamin C contents over a 7-day period (Tajiri, 1985). Highest ascorbic acid content in pods (46.24 mg/100g) were found under elevated CO_2 and least under natural condition (Meena kumari *et al.*, 2017)

An investigation of CO_2 effects on vitamin C production in (sour orange) - was conducted by Idso *et al.* (2002), where a 75% increase in the air's CO_2 content was observed to increase sour orange juice vitamin C concentration by approximately 5%.

Vitamin A

To study the production of Vitamin A through increased CO_2 condition. (Kimball and Mitchell, 1981).

Molecular studies

Protein profiling

As reported by Nie *et al.*, 1995, in spring wheat leaves grown under elevated CO_2 concentration of 550 µmol mol^{-1} the ribulose-1,5- bisphosphate carboxylase/oxygenase (RuBISCO) content declined by 60%. Reduction in total ribulose-1,5- bisphosphate carboxylase/oxygenase (RuBISCO) activity along with plant age was observed lower in the elevated CO_2 compared to the ambient CO_2 - (Hanhong and Richard, 2004). RuBISCO activity and RuBISCO protein in Barley penultimate leaves and wheat flag leaves were decreased under elevated CO_2 concentration of 700 µmol mol^{-1} (Richard and James, 1997). In black gram, enhanced CO_2 concentration was found to decrease the intensity of 52 kDa and 51.4 kDa polypeptide at vegetative and flowering stages (Sathish *et al.*, 2014). Several investigators suggest that most prominent change in leaf photosynthetic apparatus under elevated CO_2 is decrease in the amount of RuBISCO protein (Drake *et al.*, 1997). RuBISCO content of sunflower leaves of well watered plants reduced by 25% by growth in elevated CO_2 compared to ambient CO_2. But in severe water deficit conditions, RuBISCO content decreased more in plants grown in ambient CO_2 then elevated CO_2 (Lawlor and Mitchell, 2000).

Flowering time gene expression using RT-PCR

The transition from the vegetative to the flowering phase of plants is controlled by several genetic pathways that monitor the developmental state of the plant as well as environmental conditions. Despite the cloning of several Arabidopsis genes participating in these pathways, substantial gaps remain in our knowledge of how the signals controlling flowering are transduced and integrated. FLOWERING LOCUS T (FT), which acts in parallel with the meristem-identity gene LEAFY (LFY) to induce flowering of Arabidopsis, was isolated by activation tagging. Like LFY, FT acts partially downstream of CONSTANS (CO), which promotes flowering in response to long days (Kardailsky *et al.,* 1999). Kardailsky and co-workers checked flowering locus T mRNA accumulation was by RT-PCR with UBIQUITIN (UBQ) as control.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiment was undertaken with the main objective to study the Physiological and molecular analyses of flowering time responses of Amaranthus and cowpea under elevated CO_2 condition. For this, plants were raised in pot culture and exposed to ambient and elevated CO_2 environments. The technology used for subjecting the plants to elevated CO_2 environments is the Open Top Chambers (OTC) system. The cowpea seeds were sown in pots inside OTC, and other counterparts in open field condition. In plants exposed to elevated CO_2 concentration, entire crop period was completed in OTC. Experimental plants were maintained for a period of three months. Observations on growth parameters and physiological parameters at the end of CO_2 exposure period and on all the other parameters were taken at the time of harvesting.

3.1 EXPERIMENT DETAILS

3.1.1 Location

The pot culture experiment was conducted in Open Top Chamber located at College of Agriculture Vellayani, situated at 805'N latitude and 7609'E longitude and an altitude of 29 m above mean sea level.

3.1.2 Season

The first set of experiment was conducted from August 2016 to October 2016, second set of experiment was conducted from November 2016 to January 2017 and the third set of experiment in Open Top Chambers.

3.1.3 Planting material

Two varieties of Amaranthus, Arun and CO-1 and Anaswara and Vellayani Jyothika varieties of Cowpea were used for the study. The materials were procured from Department of Olericulture, College of Agriculture, Vellayani.

3.1.4 Layout of the Experiment

The experiment was laid out in CRD with two treatments and four replications for each treatment. Treatment one was chamber A with elevated CO_2 facility and treatment second was open control with ambient CO_2 condition.

3.1.1.1 Techniques for CO_2 enrichment

3.1.1.2 Open top chamber

Open Top Chambers (OTC) are square type chambers constructed to maintain near natural conditions and elevated CO_2 conditions for experimental purposes. The basic structure of OTC was built of metal frame and installed in the experimental field. OTCs were covered with a 200 micron UV poly sheet .The chamber is 3 X 3 X 3 dimension , 450 slope and $1m^2$ opening at the top. Two such chambers were built in the experimental field; one serves to impose CO_2 enrichment and the other serves as control chamber to study the chamber effects. Elevated CO_2 was released into the chamber from a CO_2 cylinder in a controlled manner. Measurements of microclimatic parameters (temperature, humidity and light) were done within and outside the OTCs with the help of sensors on a real time basis. On an average basis, mean temperature of $37.15^{O}C$ relative humidity of 60.96% and solar radiation of 384.65μ Enst were recorded inside the chambers during the experimental period. Potted plants were kept within these chambers for a period of two months and observations were taken.

The elevated CO_2 concentration of 600 ppm was selected on the basis of IPCC (2007) which suggested that atmospheric concentrations of carbon dioxide have been steadily rising with an average annual increase rate of about 2 ppm and continued to rise to 500 - 1000 ppm by the year 2100.

Treatments

These treatments were included:

T1 - OTC with elevated CO_2 concentration (OTC Ec)

T2 – Open field with ambient CO_2 concentration (OTC Ac)

Plate 01: Open Top Chamber for CO_2 enrichment

3.1.1.3 Preparation and Planting

The experiment was conducted in pots filled with potting mixture consisting of farm yard manure, sand and soil in the ratio of 1:1:1. Appropriate pest control measures and nutrients were supplied according to package of practises recommended by Kerala Agricultural University. The experiment was laid out in CRD. The potted plants were kept in OTCs for a period of three months.

3.2 OBSERVATIONS

3.2.1 GROWTH PARAMETERS

3.2.1.1 *Number of Leaves*

Total numbers of leaves in the treatment plants were counted at the time of harvesting in both the treatments.

3.2.1.2 *Specific leaf area ($cm^2 g^{-1}$)*

From each plant, fully expanded third leaf (from main stem apex) was collected. Leaflets were separated, petioles were discarded and area was measured. Leaflets were dried at 800C for 2 days and the dry weight was taken. SLA was calculated using the formula.

$$SLA\ (cm^2/g) = \text{Leaf area/ dry weight}$$

3.2.1.3 *Days to first flowering*

Total number of days from the date of sowing to the occurrence of flowering of cowpea and amaranthus were counted.

3.2.1.4 *Days to 50% flowering*

Total number of days from the date of sowing to the stage where at least 50% of the total plants have exhibited flowering were counted.

3.2.1.5 *Root Weight (g)*

The roots of plants were cut at the base level and washed free of adhering soil with low jet of water. The roots were then oven dried and dry weight was recorded.

3.2.1.6 *Shoot Weight (g)*

Shoot weight was measured by weighing the above ground part of the plants in a weighing balance.

3.2.1.7 *Root Shoot Ratio*

Ratio of weights of dried roots and shoots of sample plants were calculated and mean value arrived.

3.2.1.8 *Dry matter Production (g)*

The sum of root and shoot dry weights were taken as the total dry matter yield.

3.2.1.9 *Leaf temperature (°C)*

Plants often maintain different temperature as compared to their surrounding temperature. Leaf temperature was measured using photosynthetic apparatus.

3.2.1.10 *Stomatal distribution (no cm^{-2})*

Stomatal count refers to the number of stomata per unit area of leaf. A thick mixture of thermocol and xylene was prepared and this was smeared on both the surfaces of leaves and allowed to dry. It was peeled gently after drying and the peel was observed under the microscope and counted using a stage micrometre and stomatal frequency per unit area was calculated using the formula.

$$\text{Stomatal frequency} = \frac{\text{No of stomata}}{\text{Area of the microscopic field}}$$

3.2.2 Physiological and Biochemical parameters

3.2.2.1 *Chlorophyll a (mg g^{-1})*

Chlorophyll content of leaf samples were estimated as per the procedure described by Arnon (1949). A weighed quantity of leaf sample (0.5g) was taken from fully expanded third leaf and cut it into small bits. These bits were put in test tubes and incubated overnight at room temperature, after pouring 10 ml DMSO: 80% acetone mixture (1:1 v/v). The coloured solution was decanted into a Measuring cylinder and made up to 25 ml with the DMSO acetone mixture. The absorbance was measured at 663, 645 nm. The chlorophyll content was measured by substituting absorbance values in following equation.

$$Chla = (12.7 \times A_{663} - 2.69 \times A_{645}) \times \frac{V}{1000} \times \frac{1}{freshweight}$$

3.2.2.2 *Estimation of Total Soluble Protein (mg g^{-1})*

The total soluble protein of leaf samples were estimated by simple protein dye binding assay of Bradford (1976), using bovine serum albumin (BSA) as the standard. One hundred milligram of CBB 250 was dissolved in 50 ml of 95% ethanol. To this 100 ml of 85% (w/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 200 ml with distilled water. 0.1g of leaf samples were taken from third fully opened leaves and was ground to a thin paste and soluble protein was extracted with 10 ml of phosphate buffer (pH 7.8).

The extract was centrifuged at 5000 rpm for 10 minutes. To the 20µl of the supernatant a known volume (5 ml) of diluted dye binding solution was added. The solution was mixed well and allowed to develop a blue colour for at least 5 min but no longer than 30 min and the absorbance was measured at 596 nm. The protein content was calculated using the BSA standard in the range of (10-100µg). The protein content was expressed as mg/g FW

3.2.2.3 *Estimation of Starch (mg g^{-1})*

The estimation of starch in plants was done following the Anthrone method (Mc Cready *et al.*, 1950). A known quantity of plant sample (0.1g) was homogenized in hot 80% ethanol to remove sugars. The homogenate was centrifuged and residue was retained. The residue was washed repeatedly with hot 80% ethanol till the washing does not give any colour with anthrone reagent. Then the residue was dried well over a water bath. The dried residue was mixed with 5ml water and 6.5 ml 52% perchloric acid and was extracted at 0°C for 20 min. This solution was centrifuged and the supernatant was saved. The extraction was repeated using fresh perchloric acid. The supernatants after centrifugation was pooled and made up to 100 ml.

An aliquot of 0.1 ml of the supernatant was taken and again made up to 1 ml using distilled water. The standard was prepared by taking 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard solution and made up the volume to 1 ml in each tube using distilled water. Anthrone reagent (4 ml) was added to both the sample and standard test tubes. These test tubes were heated for eight minutes in a boiling water bath and cooled rapidly. The intensity of colour change from green to dark green was measured at 630 nm. The glucose content in the sample was calculated using the standard curve. This value was multiplied by a factor of 0.9 to arrive at the starch content.

3.2.2.4 *Estimation* of *Reducing Sugars (mg g^{-1})*

The estimation of reducing sugars in plants was done following dinirto Salicylic acid (DNS) method (Somogyi,1952). The sample was weighed (100 mg) and the sugars were extracted with hot 80% ethanol, twice. The supernatant was collected and evaporated by keeping it on a boiling water bath at 80°C. The sugars were dissolved by adding 10 ml water. Aliquots of 0.5 to 3 ml were pipetted out into test tubes and the volume was equalized to 3ml with distilled water in all the test tubes. To this 3 ml of DNS reagent was added. The test tubes were heated in a boiling water bath for 5 minutes.

Rochelle salt solution (40%, w/v) (1 ml) was added to the test tubes when the contents were hot. Then the test tubes were cooled and the intensity of dark

red colour was read at 510 nm. A series of the standard, Glucose, (0 to 500µg) was run and a standard curve was plotted. The amount of reducing sugars in the sample was calculated from the standard graph.

3.2.2.5 Gibberelic acid content *($\mu g\ g^{-1}$)*

Gibberellic acid content from the plant tissue was determined by using the spectrophotometric method. (Sunderberg, 1990 and Kojima, 1995) and expressed in microgram per gram.

3.2.2.6 *Nitrate reductase (NR) activity ($\mu mol\ min^{-1}\ g^{-1}$)*

Nitrate reductase (NR) activity in leaves was estimated in-vitro according to the method of Kaiser and Lewis (1984).

Reagents:

I. Extracting buffer: 0.1 M phosphate buffer pH 7.5, 1mM EDTA, 2 mM dithiothreitol

II. 200 mM Tris, pH 7.6

III. 80 mM Hydroxlamine hydrochloride, pH 7.8 (using KOH)

IV. 100 mM MgCl2.

V. 100 mM ATP.

VI. 1% (W/V) sulphanilamide

VII. 0.02% (W/V) n-l-napthyl-ethylenediamine dihydrochioride

Procedure:

- 1 g leaf material was cut into small pieces and ground in a chilled mortar and pestle in 12 ml of extracting buffer.
- The crude extract was filtered through two layers of cheese cloth and centrifuged at 12000 g for 20 min at 4 °C. The supernatant was stored on ice.
- Each assay mixture tube contained 0.1 ml potassium phosphate buffer, pH 7.5; 0.1 ml NADH (1 mg ml-l), 0.2 ml 0.1 M KNO3 and 0.1 ml leaf extract made up to a final volume of 2 ml with distilled water.

- After 15 min incubation at 28°C the reaction was stopped by the addition of 1 ml of 1% (W/V) sulphanilamide in 1.5 M HCI and 1 ml of 0.02% (W/V) n-l-napthyl ethylenediamine dihydrochioride solution.
- All samples were centrifuged at 10000 rpm for 5 min at 2°C to remove suspended matter.
- Nitrite was determined by measuring absorbance at 540 nm. Triplicate aliquots of crude extract were assayed in each experiment.

3.2.2.7 *Estimation of Ascorbic Acid (mg $100g^{-1}$)*

The ascorbic acid content in plants was estimated volumetrically by the method explained by Sadasivam and Manickam (2008). Working standard solution of 5ml containing 100µg/ml of ascorbic acid was pipetted out into a 100 ml conical flask. 4% oxalic acid was added to it and titrated against 2, 6-dichlorophenol indophenol dye (V1 ml). End point was noted on appearance of pink colour which persisted for a few minutes. The sample (0.5g) was weighed and ground in a mortar with pestle using 15ml 4% oxalic acid.

The homogenate was filtered through a double layered cheese cloth. The filtrate was made up to a known volume and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and made up to 25ml using oxalic acid. 5.0 ml aliquot was pipetted into a conical flask to which 10ml of 4% oxalic acid was added. This was titrated against dichlorophenol indophenol (DCPIP) solution, until the appearance of pink colour (V2 ml). The amount of ascorbic acid is calculated as follows:

$$Ascorbicacid = \frac{0.5mg}{V_1 ml} \times \frac{V_2}{5ml} \times \frac{100}{weightof\ sample}$$

3.2.2.8 *Estimation of Oxalates (mg g^{-1})*

Oxalates content was calculated using titration method (Day and Underwood, 1986). One gram of dried powdered sample was weighed and taken in 100 ml conical flask and 75 ml of sulphuric acid (3M) was added and stirred for 1 h with a magnetic stirrer. The mixture was filtered and 25 ml of the filtrate was

titrated while hot against $KMnO_4$ solution (0.05M) to the end point. The oxalate content was calculated as percent of dry weight.

3.2.2.9 Estimation of *Vitamin A content (mg $100g^{-1}$)*

Vitamin A content was estimated according to the method proposed by Srivastava and Kumar (2003). Five gram of fresh sample was weighed and homogenized with 10-15 ml acetone and few crystals of anhydrous sodium sulphite, in a mortar with pestle. The homogenate was filtered and the supernatant was decanted into a beaker. This was repeated twice and transferred the pooled supernatant to a separating funnel. Petroleum ether (10 ml) was added and mixed thoroughly. Two layers were separated on keeping the separating funnel undisturbed for some time. The lower layer was discarded and upper layer was collected in a 100 ml volumetric flask.

The volume was made up to 100 ml with petroleum ether and the optical density was recorded at 452 nm using petroleum ether as blank.

$$\text{Amount of Vitamin-A} = \frac{(OD\ of\ sample \times 13.9 \times 10^4 \times 100)}{(weight\ of\ sample \times 560 \times 1000)}$$

3.3 Molecular studies

3.3.1 SDS - PAGE

Electrophoresis separation of soluble protein and Rubisco in black pepper leaves were carried out as per the procedure described by Laemelli (1970) One gram of leaf samples were homogenized in 1.5 ml of cold denaturing buffer (Appendix III) at 40C. The extract was centrifuged at 5000 rpm for 15 minutes. The supernatant was mixed with chilled acetone in the ratio 1:1 and the protein was allowed to precipitate by keeping the mixture at 40C for 30 minutes. The sample was centrifuged at 3600 rpm for 10 minutes. The supernatant was removed and the pellet was re suspended in 50 µL of denaturing buffer and vortexed. The homogenate was centrifuged at 5000 rpm for 15 minute .The supernatant was mixed with 10 µL of sample buffer and kept in a boiling water

bath for 3 minutes. These samples were subjected to electrophoresis using SDS-PAGE

Reagents

a) Acrylamide stock (30%)

Acrylamide - 29.2 g

Bis-acrylamide - 0.8 g

Double distilled water - 100 ml

b) Separating (resolving) gel buffer stock (1.5M Tris - HCl, pH 8.8)

Tris base (18.15g) was dissolved in approximately 50 ml of double distilled water. The pH was adjusted to 8.8 with 6 N HCl and made up the volume to 100 ml with double distilled water and stored at 40C.

c) Stacking gel buffer stock (0.5M Tris - HCl pH 6.8)

Tris base (6.0 g) was dissolved in approximately 60 ml of double distilled water and adjusted the pH to 6.8 with 6 N HCl and the volume was made up to 100 ml with double distilled water and stored at 40C.

d) Polymerising agents

Ammonium per sulphate (APS) 10 per cent prepared freshly before use.

TEMED –Fresh from refrigeration.

e) Electrode buffer pH 8.3

Tris base - 6.0 g

Glycine - 28.8 g

SDS - 2.0 g

Double distilled water - 2 L

f) Sample buffer

Double distilled water - 2.6 ml

0.5 M Tris HCl pH 6.8 - 1.0 ml

g) Staining solution

Coomassie brilliant blue R 250 - 0.1 g

Methanol - 40.0 ml

Glacial acetic acid - 10.0ml

Double distilled water - 50.0 ml

h) Destaining solution

As above without Coomassie brilliant blue

Procedure

Separating gel was first casted followed by stacking gel by mixing the various solutions as indicated below

a) Preparation of separating gel (12%)

Double distilled water - 6.7 ml

Tris HCl, pH 8.8 - 5.0 ml

SDS 10% - 0.2 ml

Acrylamide stock - 8.0 ml

Tetra methyl ethylenediamine (TEMED) - 0.01 ml . The separating gel was mixed well and poured immediately between glass plates and a layer of water was added above the polymerising solution to quicken the polymerising process

b) Preparation of stacking gel

Double distilled water - 6.1ml

Tris HCl, pH 6.8 - 2.5 ml

SDS 10% - 0.2 ml

Acrylamide stock - 1.3 ml

The solution was mixed well, degased and the following were added

APS 10% - 0.05 ml

The above solution was mixed well and de gassed for 3 minutes and then the following were added immediately. Freshly prepared 10% ammonium per sulphate (APS) - 0.10 ml.

The water layered over the separating gel was removed and washed with a little electrode buffer and then the stacking gel was poured over the polymerized separating gel, after keeping the comb in position.

After polymerization, the comb was removed and the samples were loaded into the wells. Standards with known molecular weights was also loaded to one well. The electrophoresis was performed at 100 V till the dye reached the separating gel. Then the voltage was increased in 200 V and continued till the dye

reached the bottom of the gel. Immediately after electrophoresis the gel was removed from the glass plates and incubated in the staining solution overnight with uniform shaking. Then the gel was transferred to the destaining solution. The protein appeared as bands and the gel was photographed after placing it on a transilluminator (Appligene Model White/ UV TMW- 20).

3.3.2 Reverse transcriptase PCR for gene expression study

3.3.2.1 *Sequence analysis and primer designing*

From National Centre for Biotechnology Information (NCBI) database genomic and cDNA sequences of FLOWERING LOCUS T (FT) gene of Beta vulgaris and Nicotiana tabacum were downloaded to design primers for Amaranthus and genomic and cDNA sequences of FLOWERING LOCUS T (FT) gene of Vigna radiata, Vigna ungularis and Vigna unguiculata

Were downloaded to design primers for cowpea and subsequently aligned using CLUSTAM OMEGA. Primer sequences were selected manually from the regions which are conserved in both the crops, and the degenerate primers were designed and oligonucleotide properties were analyzed by using PRIMEREXPRESS software (Applied Biosystem).

Some critical considerations in primer designing were Tm, 40-60% GC, avoidance of sequences with potential internal secondary structure formation, avoidance of primer dimmer formation and also avoidance of three G or C in a row near the 3'-ends of the primer. The list of the genes and primers are given below.

Sr. No.	Oligo name	5'<-----Sequence----->3'	Amplicon size	Size (Base pairs)
1	Flowering locus F1 (For cowpea)	GATGTGAATTCAAACCTTCA	238	20 bp
2	Flowering locus R1 (For cowpea)	GAAACAACACAAACACGATA	241	20 bp
3	Flowering locus F2 (For cowpea)	GAATGTTCCATTCCCATGAG	245	20 bp
4	Flowering locus R2 (For cowpea)	ATCATGGGTCGTGGACTCTC	278	20 bp
5	Flowering locus F1 (For Amaranthus)	ATGGTRGATCCAGATGCTCC	247	20 bp
6	Flowering locus R1 (For Amaranthus)	ACAGCRGCAACAGGCAA	235	17 bp
7	Flowering locus F2 (For Amaranthus)	TTGTYAACCAACCTAGGGT	247	19 bp
8	Flowering locus R2 (For Amaranthus)	WYCCAATTGCCGAAACAA	252	18 bp

3.3.2.2 *Total RNA isolation*

Total RNA was isolated from leaves of both the crops five days before flowering. Extreme care was taken to keep all glass wares, plastic wares and solutions free of contaminating RNases. Glass wares and plastic wares to be used for RNA work were treated with 0.1%. Diethyl pyrocarbonate (DEPC) and kept at 37°C overnight. These were autoclaved at 120°C for 30 min to remove any traces of DEPC. Solutions for RNA work were prepared using DEPC treated water.

Total RNA was isolated from the frozen tissue by using Trizol method following the given protocol as described below:

1. Grind 100 mg of tissue into a fine powder in liquid N2
2. Add 1 mL of Trizol reagent to the powdered tissue in mortar and mix gently to homogenize the mixture and incubate at RT for 5 mins. (5min at RT for complete dissociation of nucleoprotein complexes)
3. Transfer the homogenate to a 2 ml pre chilled microfuge tube

4. Add 0.2 mL chloroform and shake vigorously for 15 s and incubate at RT for 5 mins
5. Keep in ice for 10 mins
6. Centrifuge at 12000g for 15 mins at 4°C
7. Transfer aqueous phase to a fresh tube
8. Add 0.5 mL of ice cold isopropanol (100%) to each tube and incubate at RT for 10 mins
9. Mix by inverting the tube
10. Centrifuge at 12000g for 10 mins at 4°C
11. Supernatant removed and pellet washed with 1 ml of 75% alcohol (in DEPC treated water)
12. Sample briefly vortexed and spun at 7500g for 5 mins at 4°C
13. Air dried for 30-40 mins
14. Dissolved in 30 microL RNase free water and incubate at 55 – 60°C for 10 mins

3.3.2.3 ***RNA quantification and Electrophoresis***

The spectrophotometric determination of quality and quantity of total RNA by using NanoDrop Nd-1000 UV/Vis Spectrophotometer (Labtech International Ltd.). Total RNA was quantified by measuring A260 (1 A260 = 40g) and its quality was assessed by A260/A280 ratio. Further, total RNA quality and quantity was assayed by fractionation on agarose gel.

3.3.2.4 ***Agarose gel electrophoresis***

1. Agarose
2. 50X Tris-Borate-EDTA (TBE) Buffer (pH 8.0)

Tris base 54g

Boric acid 27.5 g

EDTA 0.5 M (pH 8.0) 20 g

Final volume 1000 ml with distilled water

Autoclaved and store the buffer at room temperature.

3. Gel loading dye (6X)

- 10mM Tris-HCl (pH 7.6)
- 0.03% bromophenol blue
- 0.03% xylene cyanol FF
- 60% glycerol
- 60mM EDTA

4. Ethidium bromide stock solution: 10mg mL-1 in water

5. Mini gel apparatus and power supply

6. Gel documentation system: Alpha Imager

Procedure

The mini gel apparatus was setup as described by Sambrook and Russell (2001). The appropriate size of comb and slab was selected and both ends of the slab were sealed by a tape to avoid leakage during casting of the gel.

1. An agarose gel (1.2%) was prepared by heat dissolution of 0.60 g of agarose in 50 mL 1X TBE buffer. Ethidium bromide from stock solution (10mg/mL) was added to a final concentration of 0.5 g mL-1.
2. The gel was allowed to cool to almost 50-55°C before pouring to the gel plate.
3. Once the gel is ready, it was placed in the electroporesis tank after removing the comb and tape.
4. Fill the tank with 1X TBE buffer till the gel is completely submerged.
5. The total RNA samples were mixed with appropriate volume of 6X loading dye before loading.
6. The samples were loaded and run at 5 v/cm, with the help of an electric power supply.

7. After one hour of electrophoresis, the agarose gel was viewed using gel documentation system.

3.3.2.5 *RT-PCR*

Two-step RT-PCR analysis was carried out to amplify the gene of interest using heterologous primers (from other related crops).

First strand cDNA synthesis was done by using Revert aid first strand cDNA synthesis kit (Thermofisher) Total RNA isolated from control and treated tissues was used for first strand cDNA synthesis by using reverse-transcriptase.

Protocol:

1. Prepare the RT reaction mix in a PCR-tube as given below:
   10x RT buffer 2 µL
   dNTP mix (10 mM each) 1 µL
   Oligo-dT primer (10 µM) 2 µL
   RNase Inhibitor (10 Units µL-1) 1 µL
   Reverse Transcriptase 1 µL (4U)
   RNase free water 8 µL
   Template RNA 5 µL (0.5 µg)
2. Incubate at 37°C for 1 h
3. Use an aliquot of cDNA for PCR immediately or store at -20°C for later use

PCR amplification of cDNA

PCR reaction mix:

1. Template cDNA 1.0 µL
2. Forward primer 0.5 µL
3. Reverse primer 0.5 µL
4. dNTP mix (10 mM) 1.0 µL
5. 10 X *Taq* buffer containing $MgCl_2$ 2.5 µL
6. *Taq* DNA polymerase (3U µL-1) 0.5 µL
7. Sterile double distilled water 19.0 µL
8. Total reaction volume 25.0 µL

PCR conditions

Step 1	Denaturation	94°C	4 min
Step 2	Denaturation	94°C	1 min.
Step 3	Primer annealing variable according to the primers Tm (55-58°C)		
Step 4	Primer extension	72°C	1 min
Step 5	Repeat steps 2 to 4 35 times		
Step 6	Final extension 72°C 10 min		
Step 7	Hold 4°C		

The PCR products (5 µL) were analyzed by Agarose (1.2%) gel electrophoresis, as described in the earlier section

RESULTS

4. RESULTS

The current programme was undertaken with the main objective of studying the physiological, molecular and biochemical basis of elevated CO_2 mediated modifications in the flowering responses of amaranthus and cowpea. The technology used for subjecting the plants to elevated CO_2 environments was Open Top Chamber. Plants raised and maintained in pots as per POP (KAU) recommendations under elevated CO_2 condition in open top chamber. The control set was kept under open field condition. And the experimental plants were retained till they reach 50% flowering. Growth parameters and observation on physiological and biochemical parameters was taken at the time of harvest. Molecular studies were carried out five days before flowering.

4.1 EFFECT OF ELEVATED CO_2 ON GROWTH PARAMETERS IN AMARANTHUS

4.1.1 Number of leaves

The effect of elevated CO_2 environment on number of leaves in amaranthus is presented in Table 1. Highest mean value (42.44) for number of leaves was recorded in treatment T_1 (elevated CO_2) at the end of exposure period. The mean value of treatment T_2 was found to be less. The varietal difference was observed in two varieties of amaranthus where CO-1 (with varietal mean 39.08) responded more than Arun (with varietal mean 37.37) to elevated CO_2 in terms of number of leaves. Whereas in cowpea, Highest mean value (55.60) for number of leaves was recorded in treatment T_1 (elevated CO_2) at the end of exposure period (Table 20). The mean value of treatment T_2 was found to be less. The varietal difference was observed in two varieties of cowpea where Anaswara (with varietal mean 56.75) responded more than Vellayani Jyothika (with varietal mean 38.62) to elevated CO_2 in terms of number of leaves.

Table 01: Effect of elevated CO_2 on number of leaves in amaranthus.

Treatments	No. of leaves		Mean
	T1	T2	
V1	35.10	39.65	37.37
V2	42.44	35.71	39.08
Mean	38.77	37.68	38.22
CD(0.05): T =0.489, V =0.0.565,T*V = 0.979			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 02: Effect of elevated CO_2 on Specific leaf area ($cm^2 g^{-1}$) in amaranthus.

Treatments	Specific leaf area ($cm^2 g^{-1}$)		Mean
	T1	T2	
V1	172.10	186.49	179.29
V2	219.13	158.10	188.62
Mean	195.61	172.29	183.95
CD(0.05): T =0.376, V =0.453,T*V = 0.742			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 20: Effect of elevated CO_2 on number of leaves in cowpea.

Treatments	No. of leaves		Mean
	T1	T2	
V1	74.25	39.25	56.75
V2	36.75	40.51	38.62
Mean	55.50	39.87	47.68
CD(0.05): T = 0.436, V =0.523, T*V = 0.856			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 21: Effect of elevated CO_2 on Specific leaf area ($cm^2 g^{-1}$) in cowpea.

Treatments	Specific leaf area ($cm^2 g^{-1}$)		Mean
	T1	T2	
V1	454.53	322.13	388.33
V2	242.32	247.62	244.97
Mean	348.43	284.87	316.65
CD(0.05): T = 23.88, V = 24.76 , T*V = 33.76			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

4.1.2 Specific Leaf Area

Table 2 shows the effect of elevated CO_2 on specific leaf area in amaranthus. Specific leaf area was found to be more (219.13 cm^2 g^{-1}) in CO-1 (V_2) under treatment T_1 (elevated CO_2) and 158.10 cm^2 g^{-1} in treatment T_2 i.e. open control. Whereas the variety, Arun recorded the lowest specific leaf area under elevated CO_2 condition 172.10 cm^2 g^{-1} in T_1 (elevated CO_2) and 186.49 cm^2 g^{-1} in T_2 (Open control). Specific leaf area in cowpea was found to be more (454 cm^2 g^{-1}) in Anaswara (V_1) under treatment T_1 (elevated CO_2) and 322.13 cm^2 g^{-1} in treatment T_2 i.e. open control (Table 21). Whereas the variety, Vellayani Jyothika recorded the lowest specific leaf area under elevated CO_2 condition 242.32 cm^2 g^{-1} in T_1 (elevated CO_2) and 247.62 cm^2 g^{-1} in T_2 (Open control).

4.1.3 Days to first flowering

Both the varieties responded to the treatment T_1 (elevated CO_2 condition). The time of flowering was advanced by two days in CO-1(Table 3). Moreover, the time of flowering in Arun exposed to elevated CO_2 was also advanced by one day. In case of cowpea, the time of flowering was advanced by one day in Anaswara. In contrast to Anaswara, the time of flowering in Vellayani Jyothika exposed to Treatment T_1 was delayed by one day (Table 22).

4.1.4 Days to 50% flowering

The response of both the varieties of amaranthus to elevated CO_2 was remarkable. CO-1 plants exposed to the elevated CO_2 condition exhibited advanced 50% flowering by 2 days as compared to the counterparts raised in open control. However the variety Arun exhibited mild difference in days to 50% flowering. The advancement of 50% flowering in Arun exposed to treatment T_1 (elevated CO_2) was by one day (Table 4). Similarly, in the case of cowpea, both the varieties responded to the treatment T_1 (elevated CO_2 condition). The time of flowering was advanced by one day in Anaswara. In contrast to Anaswara, the time of flowering in Vellayani Jyothika exposed to Treatment T_1 was delayed by one day (Table 23).

Table 03: Effect of elevated CO_2 on days to first flowering in amaranthus.

Treatments	Days to first flowering		Mean
	T1	T2	
V1	38.50	39.01	38.75
V2	34.51	36.75	35.63
Mean	36.50	37.88	37.19
CD(0.05): T =0.546, V =0.478,T*V = 0.948			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 04: Effect of elevated CO_2 on days to 50% flowering in amaranthus.

Treatments	Days to 50% flowering		Mean
	T1	T2	
V1	49.75	50.75	50.25
V2	46.00	48.00	47.00
Mean	47.87	49.37	48.62
CD(0.05): T =0.581, V =0.495,T*V = 0.981			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 22: Effect of elevated CO_2 on days to first flowering in cowpea.

Treatments	Days to first flowering		Mean
	T1	T2	
V1	35.00	36.75	35.87
V2	36.25	35.75	36.00
Mean	35.62	36.25	35.93
CD(0.05): T = 0.503, V = 0.439, T*V = 0.711			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 23: Effect of elevated CO_2 on Days to 50% flowering in cowpea.

Treatments	Days to 50% flowering		Mean
	T1	T2	
V1	42.00	45.00	43.50
V2	56.00	57.75	56.85
Mean	49.00	51.375	50.18
CD(0.05): T = 0.735, V = 0.973, T*V = 1.633			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

4.1.5 Root Weight

Both the varieties of amaranthus responded differently under elevated CO_2 condition (T_1). CO-1 registered the highest per cent increase in root weight and have a mean value of 1.45 g in treatment T_1 (elevated CO_2) compared to absolute control (Table 5). Similarly in the case of cowpea, both the varieties responded positively and in a highly significant way under elevated CO_2 condition (T_1) having a mean value of 14.09 g compared to absolute control condition (10.58 g). Under elevated CO_2 (T_1), Anaswara registered the highest per cent increase in root weight and have a mean value of 15.04 g in treatment T_1 (elevated CO_2) compared to absolute control (Table 24).

4.1.6 Shoot Weight

Shoot weights were found to be superior in amaranthus varieties under elevated CO_2 condition (2.43 g) compared to absolute control (1.50 g). Table 6 shows highest per cent increase in shoot weight was registered in CO-1 under elevated CO_2 condition (3.17) Compared to control condition (1.55). Shoot weights in cowpea were found to be superior in varieties under elevated CO_2 condition (62.11 g) compared to absolute control (59.15 g). Table 25 shows highest per cent increase in shoot weight was registered in Vellayani Jyothika under elevated CO_2 condition (61.07) Compared to control condition (55.67).

4.1.7 Root Shoot Ratio

Highest root shoot ratio was exhibited by the variety CO-1 in treatment T_1 (chamber with elevated CO_2) having a value of 0.467 as depicted in Table 07. In contrast to the variety CO-1, Arun showed relatively lower root shoot ratio in elevated CO_2 with the treatment mean value of 0.280 as compared to the open control with the treatment mean value of 0.455. Similarly, highest root shoot ratio was exhibited by the Anaswara in treatment T_1 (chamber with elevated CO_2)

Table 05: Effect of elevated CO_2 on root weight (g) in amaranthus.

Treatments	Root weight (g)		Mean
	T1	T2	
V1	0.32	0.68	0.50
V2	1.45	0.35	0.93
Mean	0.88	0.51	0.72
CD(0.05): T = 0.029, V = 0.028, T*V = 0.041			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 06: Effect of elevated CO_2 on shoot weight (g) in amaranthus.

Treatments	Shoot weight (g)		Mean
	T1	T2	
V1	1.69	1.45	1.57
V2	3.17	1.55	2.36
Mean	2.43	1.50	1.96
CD(0.05): T =0.084, V =0.079,T*V = 0.118			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 07: Effect of elevated CO_2 on root shoot ratio (%) in amaranthus.

Treatments	Root shoot weight (g)		Mean
	T1	T2	
V1	0.280	0.455	0.353
V2	0.467	0.267	0.367
Mean	0.373	0.361	0.365
CD(0.05): T =0.006, V =0.004,T*V = 0.008			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 08: Effect of elevated CO_2 on dry matter (g) in amaranthus.

Treatments	Dry matter (g)		Mean
	T1	T2	
V1	2.30	2.25	2.28
V2	4.93	1.81	3.37
Mean	3.61	2.03	2.82
CD(0.05): T = 0.153, V =0.148,T*V = 0.214			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 24: Effect of elevated CO_2 on root weight (g) in cowpea.

Treatments	Root weight (g)		Mean
	T1	T2	
V1	6.04	4.87	5.45
V2	4.14	3.3	3.72
Mean	5.09	4.085	4.58
CD(0.05): T = 0.423, V = 0.382, T*V = 0.798			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 25: Effect of elevated CO_2 on shoot weight (g) in cowpea.

Treatments	Shoot weight (g)		Mean
	T1	T2	
V1	43.15	35.62	37.88
V2	41.07	35.67	38.37
Mean	42.11	34.14	38.12
CD(0.05): T = 0.443, V = 0.342, T*V = 0.845			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

having a value of 0.213 as depicted in Table 26. The variety Vellayani Jyothika under elevated also showed a significantly higher root shoot ratio (0.204) compared to control condition (0.150).

4.1.8 Dry matter Production

Highest mean value (3.61 g) for total dry matter production was observed in amaranthus varieties under elevated CO_2 condition (T_1). Compared to absolute control condition with the treatment mean value of 2.03. Under elevated CO_2 condition (T_1), the variety CO-1 was noticed for its highest per cent increase in dry matter production (4.93 g) as compared to the open control (1.81). Followed by the variety Arun showing the highest mean value of 2.30 in treatment T_1 (elevated CO_2) as compared to the open control treatment with the mean value of 2.25 (Table 8). Similarly in the case of cowpea, highest mean value (76.17 g) for total dry matter production was observed in varieties under elevated CO_2 condition (T_1). Compared to absolute control condition, under elevated CO_2 condition (T_1), the variety Anaswara was noticed for its highest per cent increase in dry matter production (78.76) as compared to the open control (76.32). Followed by the variety Vellayani Jyothika showing the highest mean value of 73.58 in treatment T_1 (elevated CO_2) as compared to the open control treatment with the mean value of 64.37 (Table 27).

4.1.9 Leaf temperature

Both the varieties of amaranthus exhibited more leaf temperature in treatment T_1 (elevated CO_2) as compared to the treatment T_2 (Table 9). Compared to absolute control condition, under elevated CO_2 condition (T_1), the variety CO-1 was noticed for its highest per cent increase in leaf temperature production (36.16) as compared to the open control (47.58). Similarly in the case of cowpea, table 28 shows that both the varieties exhibited more leaf temperature in treatment T_1 (elevated CO_2) as compared to the treatment T_2 (open field condition). Compared to absolute control condition, under elevated CO_2 condition (T_1), the variety Anaswara was noticed for its highest per cent increase in leaf temperature production (51.73) as compared to the open control (47.58).

Table 09: Effect of elevated CO_2 on Leaf temperature (°C) in amaranthus.

Treatments	Leaf temperature (°C)		Mean
	T1	T2	
V1	35.68	34.84	34.76
V2	36.16	35.15	35.66
Mean	36.42	35.00	35.71
CD(0.05): T =0.547, V =0.483,T*V = 0.784			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 10: Effect of elevated CO_2 on Stomatal frequency (no cm^{-2}) in amaranthus.

Treatments	Stomatal frequency (no cm^{-2})		Mean
	T1	T2	
V1	548.32	529.03	528.67
V2	595.78	549.89	572.83
Mean	572.05	549.46	540.75
CD(0.05): T =8.438, V =8.376,T*V = 11.933			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 26: Effect of elevated CO_2 on root shoot ratio (%) in cowpea

Treatments	Root shoot weight (g)		Mean
	T1	T2	
V1	0.218	0.212	0.215
V2	0.204	0.150	0.177
Mean	0.211	0.181	0.196
CD(0.05): T = 0.028, V = 0.024, T*V = 0.041			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 27: Effect of elevated CO_2 on dry matter (g) in cowpea.

Treatments	Dry matter (g)		Mean
	T1	T2	
V1	49.19	40.46	44.82
V2	45.21	38.97	42.09
Mean	47.20	39.71	43.45
CD(0.05): T = 0.684, V = 0.585, T*V = 1.213			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

4.1.10 Stomatal Frequency

Effect of elevated CO_2 on stomatal frequency in amaranthus at the end of exposure is presented in Table 10. Higher value for stomatal frequency was noticed in the variety CO-1 under elevated CO_2 treatment with the treatment mean of 595.78 compared to absolute control. Stomatal frequency of treatment T_1 (572.05 no cm^{-2}), was found to be more than absolute control T_2 (549.46 no cm^{-2}). Effect of elevated CO_2 on stomatal frequency at the end of exposure is presented in Table 29. Higher value for stomatal frequency was noticed in the variety Vellayani jyothika under elevated CO_2 treatment compared to absolute control. Stomatal frequency of treatment T_1 (2669.2 no cm^{-2}), was found to be more than absolute control T_2 (1714.6 no cm^{-2}).

4.2 PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS

4.2.1 Chlorophyll a content

Both the varieties of amaranthus responded positively to the elevated CO_2 condition. As shown in the table 11, Chlorophyll a content of the variety CO-1 (0.562 mg g^{-1} of fresh weight) was found to be superior in treatment T_1 (elevated CO_2 treatment). In the case of the variety Anaswara (0.470 mg g^{-1} of fresh weight) was found to be superior in treatment T_2 (absolute treatment). However, it is on par in case of the variety Vellayani Jyothika in both the treatments (Table 30).

4.2.2 Total Soluble Protein

Exposure to elevated CO_2 concentration was found to have a positive and significant influence on the total soluble protein content in elevated CO_2 condition. Under elevated CO_2 condition highest per cent increase in total soluble protein was registered in CO-1 (23.02 mg g^{-1}). In contrast to the variety CO-1, the response of Arun was negative with the treatment mean value of 17.71 in elevated CO_2 and 19.21 in open control treatment (Table 12). Similarly, exposure to

Table 28: Effect of elevated CO_2 on leaf temperature (°C) in cowpea.

Treatments	Leaf temperature (°C)		Mean
	T1	T2	
V1	35.67	35.36	35.51
V2	36.70	35.35	36.02
Mean	36.18	35.35	35.77
CD(0.05): T = 0.879, V = 0.713, T*V = 1.235.			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 29: Effect of elevated CO_2 on stomatal frequency (no cm^{-2}) in cowpea.

Treatments	Stomatal frequency (no cm^{-2})		Mean
	T1	T2	
V1	1944.51	1713.14	1828.8
V2	1693.80	1516.22	1605.00
Mean	1819.15	1614.65	1716.91
CD(0.05): T = 5.431, V = 4.327.98, T*V = 8.327			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

elevated CO_2 concentration was found to have a positive and significant influence on the total soluble protein content. The total soluble protein content was found to be highest in treatments T_1 (under elevated CO_2). As shown in table 31, Under elevated CO_2 condition highest per cent increase in total soluble protein was registered in Vellayani Jyothika (1.44 mg g^{-1}) and in absolute control it was found to be less in Vellayani Jyothika 1 (1.32).

4.2.3 Starch

Starch content was found to be highest in amaranthus in treatment T_1 (under elevated CO_2) (3.07 mg g^{-1}) as compared to the absolute control (2.35 mg g^{-1}). Both the varieties under elevated CO_2 hold its significantly higher mean values for starch content. In treatment T_1, CO-1 recorded the highest mean value for starch content (3.61 mg g^{-1}) (Table 13). In the case of cowpea, starch content was found to be highest in treatment T_1 (under elevated CO_2) (7.69 mg g^{-1}) as compared to the absolute control (4.29 mg g^{-1}). Both the varieties under elevated CO_2 hold its significantly higher mean values for starch content. In treatment T_1, Anaswara recorded the highest mean value for starch content (9.16 mg g^{-1}) (Table 32).

4.2.4 Reducing Sugar

In amaranthus, the treatment T_1 (elevated CO_2) holds the highest mean value for reducing sugar content (17.51 mg g^{-1}) whereas mild variation in reducing sugar content was noticed in the variety Arun in between treatment T_1 and T_2 (absolute control). In treatment T_1 reducing sugar content was found to be highest in CO-1 compared to absolute control with a value of 18.46 mg g^{-1}. In case of cowpea, the treatment T_1 (elevated CO_2) holds the highest mean value for reducing sugar content (13.16 mg g^{-1}) whereas no significant variation in reducing sugar content was noticed in Vellayani Jyothika in between treatment T_1 and T_2 (absolute control). In treatment T_1 reducing sugar content was found to be highest

Table 11: Effect of elevated CO_2 on chlorophyll a content (mg g^{-1}) in amaranthus.

Treatments	Chlorophyll a content (mg g^{-1})		Mean
	T1	T2	
V1	0.457	0.376	0.417
V2	0.562	0.313	0.435
Mean	0.509	0.344	0.427
CD(0.05): T =0.014, V =0.018,T*V = 0.026			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 12: Effect of elevated CO_2 on total soluble protein content (mg/g) in amaranthus.

Treatments	Total soluble protein content (mg/g)		Mean
	T1	T2	
V1	17.71	19.21	18.46
V2	23.02	17.66	20.34
Mean	20.37	18.43	19.40
CD(0.05): T =0.640, V =0.554,T*V = 1.108			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 13: Effect of elevated CO_2 on starch content (mg/g) in amaranthus.

Treatments	Starch content (mg/g)		Mean
	T1	T2	
V1	2.52	2.37	2.40
V2	3.61	2.24	2.92
Mean	3.07	2.35	2.71
CD(0.05): T =0.153, V =0.142,T*V = 0.213			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 14: Effect of elevated CO_2 on reducing sugar content (mg/g) in amaranthus.

Treatments	Reducing sugar content (mg/g)		Mean
	T1	T2	
V1	16.55	15.27	15.92
V2	18.46	13.33	15.90
Mean	17.51	14.30	15.91
CD(0.05): T =0.423, V =0.341,T*V = 0.873			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 30: Effect of elevated CO_2 on chlorophyll a content (mg g^{-1}) in cowpea.

Treatments	Pigment composition (mg g^{-1})		Mean
	T1	T2	
V1	0.456	0.470	0.463
V2	0.520	0.519	0.519
Mean	0.487	0.494	0.491
CD(0.05): T = 0.013, V = 0.016, T*V = 0.036			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 31: Effect of elevated CO_2 on total soluble protein content (mg/g) in cowpea.

Treatments	Total soluble protein content (mg/g)		Mean
	T1	T2	
V1	1.42	1.17	1.29
V2	1.44	1.32	1.38
Mean	1.43	1.24	1.33
CD(0.05): T = 0.077, V = 0.063, T*V = 0.108			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 32: Effect of elevated CO_2 on starch content (mg/g) in cowpea.

Treatments	Starch content (mg/g)		Mean
	T1	T2	
V1	9.16	5.24	7.20
V2	6.23	4.60	5.41
Mean	7.69	4.92	6.31
CD(0.05): T = 0.054, V = 0.067, T*V = 0.117			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 33: Effect of elevated CO_2 on reducing sugar content (mg/g) in cowpea.

Treatments	Reducing sugar content (mg/g)		Mean
	T1	T2	
V1	15.36	12.63	14.00
V2	10.95	10.60	10.77
Mean	13.16	11.61	12.38
CD(0.05): T = 0.423, V = 0.341, T*V = 0.873			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

in Anaswara compared to absolute control and have a value of 15.36 mg g^{-1} (Table 33).

4.2.5 Gibberellic acid

Both the varieties of amaranthus exhibited significant variation in gibberellic acid content under elevated CO_2 condition. CO-1 exhibited more Gibberellic acid content in treatment T_1 (elevated CO_2) with a mean value of 0.198 µg g^{-1} (Table 15). In case of cowpea, anaswara exhibited more Gibberellic acid content in treatment T_1 (elevated CO_2) with a mean value of 0.615 µg g^{-1}. In contrast to Anaswara, the variety Vellayani Jyothika shown lesser mean value of 0.541 µg g^{-1} as compared to the absolute control with a mean value of 0.554 µg g^{-1}. (Table 34)

4.2.6 Nitrate reductase

Both the varieties of amaranthus exhibited significant variation in the content of nitrate reductase. Under elevated CO_2 condition (Treatment T_1), the variety CO-1 found to have more amount of nitrate reductase with a mean value of 0.654 µg g^{-1} as compared to the absolute control condition with a mean value of 0.159 µg g^{-1}. In contrast to the variety CO-1, the variety Arun exhibited lesser amount of nitrate reductase in treatment T_1 with a mean value of 0.340 µg g^{-1} as compared to the absolute control condition with a mean value of 0.265 µg g^{-1} (Table 16). Similarly in the case of cowpea, both the varieties exhibited significant variation in the content of nitrate reductase. Under elevated CO_2 condition (Treatment T_1), the variety Anaswara found to have more amount of nitrate reductase with a mean value of 0.543 µg g^{-1} as compared to the absolute control condition with a mean value of 0.354 µg g^{-1}. In contrast to the variety Anaswara, the variety Vellayani Jyothika exhibited lesser amount of nitrate reductase in treatment T_1 with a mean value of 0.360 µg g^{-1} as compared to the absolute control condition with a mean value of 0.395 µg g^{-1} (Table 35).

4.3 Quality parameters of Amaranthus

Table 15: Effect of elevated CO_2 on Gibberellic acid (µg g^{-1}) in amaranthus.

Treatments	Gibberellic acid (µg g^{-1})		Mean
	T1	T2	
V1	0.176	0.157	0.166
V2	0.198	0.135	0.168
Mean	0.187	0.146	0.166
CD(0.05): T =0.008, V =0.007,T*V = 0.012			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 16: Effect of elevated CO_2 on Nitrate reductase (µg g^{-1}) in amaranthus.

Treatments	Nitrate reductase (µg g^{-1})		Mean
	T1	T2	
V1	0.340	0.265	0.302
V2	0.654	0.159	0.407
Mean	0.497	0.212	0.354
CD(0.05): T = 0.020, V = 0.023, T*V = 0.033			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 34: Effect of elevated CO_2 on gibberellic acid (µg g^{-1}) in cowpea.

Treatments	Gibberellic acid (µg g^{-1})		Mean
	T1	T2	
V1	0.615	0.552	0.584
V2	0.541	0.554	0.547
Mean	0.578	0.553	0.565
CD(0.05): T = 0.034, V = 0.043, T*V = 0.072			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

Table 35: Effect of elevated CO_2 on Nitrate reductase (µg g^{-1}) in cowpea.

Treatments	Nitrate reductase (µg g^{-1})		Mean
	T1	T2	
V1	0.543	0.354	0.448
V2	0.360	0.395	0.377
Mean	0.451	0.374	0.413
CD(0.05): T = 0.023, V = 0.036, T*V = 0.054			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Anaswara
V2- Vellayani Jyothika

4.3.1 Ascorbic acid

In amaranthus, the treatment T_1 (elevated CO_2) holds the highest mean value for ascorbic acid (40.58 mg g^{-1}) whereas no significant variation in ascorbic content was noticed in Arun between treatment T_1 and T_1 (absolute control). In treatment T_1 ascorbic content was found to be highest in CO-1 compared to absolute control and have a value of 28.77 mg g^{-1} (Table 17).

4.3.2 Oxalate content

Both the varieties exhibited significant variation in oxalate content under elevated CO_2 condition. Arun exhibited more oxalate content in treatment T_1 (elevated CO_2) with a mean value of 10.04 mg g^{-1}.

4.3.3 Vitamin-A content

Vitamin-A content was found to be highest in treatment T_1 (under elevated CO_2) (2.241 mg g^{-1}) as compared to the absolute control (1.1877 mg g^{-1}). Both the varieties under elevated CO_2 hold its significantly higher mean values for starch content. In treatment T_1, CO-1 recorded the highest mean value for starch content (31.893 mg g^{-1}).

4.6 MOLECULAR STUDIES IN CO-1 AND ANASWARA

4.6.1 PAGE

No conclusive result could be obtained for protein profiling using PAGE.

4.6.2 FLOWERING TIME GENE EXPRESSION USING RT-PCR

Molecular study was conducted in selected varieties which shown remarkable response to the elevated CO_2 condition. The varieties Anaswara and CO-1 of cowpea and amaranthus respectively were selected for molecular studies. Flowering time gene *(FLOWERING LOCUS T)* expression analysis using RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) was done as a part of molecular work. Two treatments, T_1 (elevated CO_2) and T_2 (Open field) were selected for studying the expression of the gene *FLOWERING LOCUS T*, the key

Table 17: Effect of elevated CO_2 on ascorbic acid (mg/100g) in amaranthus.

Treatments	Ascorbic acid (mg/100g)		Mean
	T1	T2	
V1	16.97	21.90	19.43
V2	40.58	19.46	30.02
Mean	28.77	20.68	24.73
CD(0.05): T =0.546, V =0.473,T*V = 0.948			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 18: Effect of elevated CO_2 on oxalate content (mg/g) in amaranthus.

Treatments	Oxalate content (mg/g)		Mean
	T1	T2	
V1	11.01	8.86	9.94
V2	9.06	8.33	8.69
Mean	10.04	8.59	9.31
CD(0.05): T =0.662, V =0.578,T*V = 0.936			

V-variety, T- treatments
T1- Elevated CO_2 Chamber
T2- Control

V1-Arun
V2- CO-1

Table 19: Effect of elevated CO_2 on Vitamin-A content (mg/g) in amaranthus.

Treatments	Vitamin-A content (mg/g)		Mean
	T1	T2	
V1	1.545	1.877	1.711
V2	2.241	1.148	1.694
Mean	1.893	1.512	1.703
CD(0.05): T =0.079, V =0.64,T*V = 0.122			

V-variety, T- treatments

T1- Elevated CO_2 Chamber

T2- Control

V1-Arun

V2- CO-1

gene for floral induction in plants. There was distinct variation in the expression levels of *FLOWERING LOCUS T* among the treatments as evidenced by RT-PCR. Anaswara plants grown under elevated CO_2 condition had highest gene expression with the primer combination of F_2R_2, whereas CO-1 variety of Amaranthus shown highest expression in treatment T_1 (elevated CO_2) with the primer combination of F_2R_2. The designed primers are sown in the following table

Table 42: Designed primers for gene expression study in amaranthus and cowpea.

Sr. No.	Crop	Primer	Sequence
1	Amaranthus	F1	ATGGTRGATCCAGATGCTCC
		R1	ACAGCRGCAACAGGCAA
		F2	TTGTYAACCAACCTAGGGT
		R2	WYCCAATTGCCGAAACAA
2	Cowpea	F1	GATGTGAATTCAAACCTTCA
		R1	GAAACAACACAAACACGATA
		F2	GAATGTTCCATTCCCATGAG
		R2	ATCATGGGTCGTGGACTCTC

4.6.3 Salient findings:

Differential expression was observed in both Anaswara and CO-1

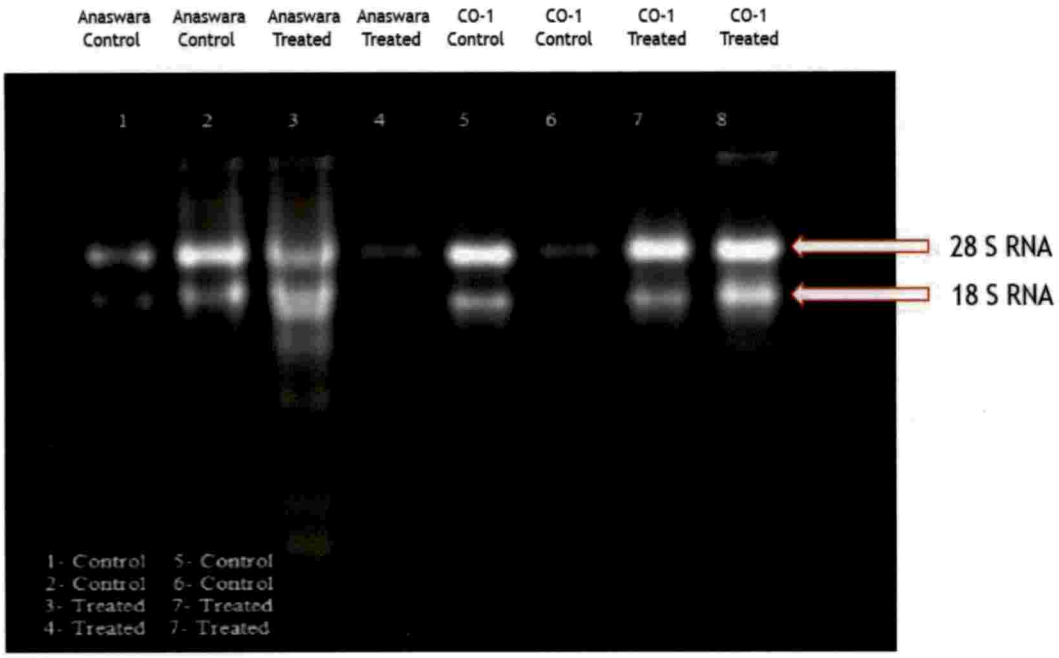

Plate 06: Total RNA isolation in CO-1 and Anaswara

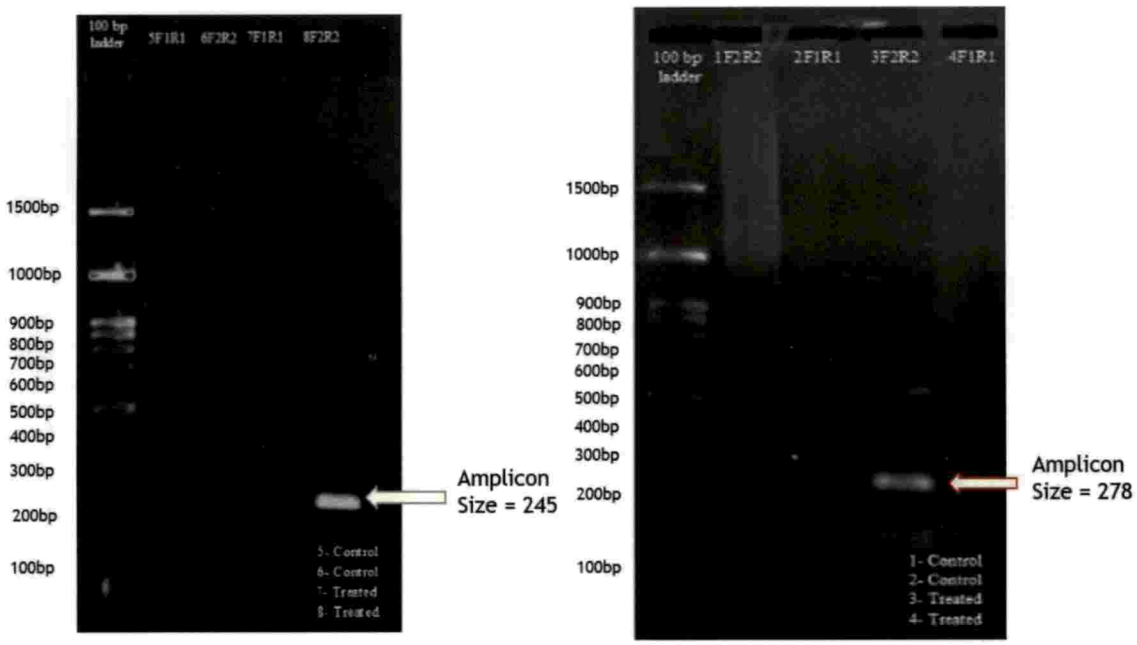

Plate 07: RT-PCR product of the gene FLOWERING LOCUS T.

DISCUSSION

5. DISCUSSION

Atmospheric CO_2 concentration has risen at an accelerating pace since the start of the Industrial Revolution. For the 1000 years prior to the Industrial Revolution, CO_2 was stable at about 270 ppm. Today CO_2 concentration in the atmosphere is approximately at 406 ppm and by the middle of this century it is predicted to reach 550ppm and to surpass 700 ppm by the end of the century (Long *et al.*, 2004).

CO_2 plays a pivotal role in the functioning of both natural plant communities and agro eco-systems. Response of crops to climate change is closely related to the local climate variability rather than to the global climate patterns and therefore, crop responses to climate change vary with region and plant species (IPCC, 2007). This necessitates site specific CO_2 enrichment studies with respect to specific crops. Technologies such as Free Air CO_2 enrichment (FACE), Open Top Chamber (OTC), Soil Plant Atmosphere Research (SPAR) are currently being used for crop response studies.

India is the second largest producer of green leaves next to china with an estimated production of 96 million tons. Amaranthus is the most popular leafy vegetable of Kerala playing an important role in food and nutritional security. It is less expensive and easily available source of protective nutrients, also called as 'Poor man's spinach'. Since it is an intrinsic part of daily nutritious diet, it is important to assess the impact of changing climatic conditions on different aspects of amaranthus. There is no research report available about the response of amaranthus under elevated CO_2. Hence an experiment was proposed to analyze the physiological, molecular and biochemical basis of flowering responses in amaranthus under elevated carbon dioxide conditions.

Cowpea is a legume widely adapted and grown throughout the world. It is a twining annual herbaceous plant known for initial fast growth; cowpea can easily suppress weed growth, therefore, reducing weed-canopy competition. Cowpea can be grown throughout the year under Kerala conditions. The attributes like, staple

fodder, nutritive and medicinal significance, make cowpea one of the most important crops in Kerala conditions. Therefore it is quintessential to conduct studies on the impact of changing climate scenario on the growth, physiological aspects and phenological aspects like flowering time in cowpea to help us understand the future performance of the crop in altered climatic conditions due to foreseen increase in CO_2 in the atmosphere.

Amaranthus varieties viz. Arun and CO-1 and cowpea varieties Anaswara and Vellayani Jyothika were used for the study. Plants were raised in pots as per the POP recommendations in Open Top Chambers for exposing them to elevated CO_2 concentrations for a period of two months. Observations on growth Parameters, biochemical and physiological parameters and quality parameters of both the crops were taken at the time of harvesting. Molecular studies (gene expression study using RT-PCR and protein profiling using PAGE) were conducted five days before the date of first flowering. The influence of elevated CO_2 on different growth parameters, physiological and biochemical parameters and quality parameters of amaranthus and cowpea are discussed in this section.

5.1 EFFECT OF ELEVATED CO_2 ON GROWTH PARAMETERS

Historical climate change has had a profound effect on current biogeography, Climate change has important implications for nearly every aspect of life on Earth, and effects are already being felt. Plant morphogenesis is governed by the effects of environmental conditions super imposed upon genetic constraints. Thus genetically identical plants can exhibit very different structural features when subjected to different environmental conditions. Carbon dioxide links the atmosphere to the biosphere and is an essential substrate for photosynthesis. Today's crops and natural vegetation are growing at an elevated CO_2 level that has not been experienced by terrestrial vegetation for 26 million years. The direct effect of elevated CO_2 concentration on plant growth is of particular interest because of the possibility of increasing crop yields in the future once the substrate of photosynthesis and gradient of concentration between atmosphere and leaf will increase. For C_3 plants the positive responses are mainly

attributed by the competitive inhibition of photorespiration by CO_2 (Amthor and Loomis, 1996). The various growth parameters considered under this study includes, number of leaves, specific leaf area, days to first flowering, days to 50% flowering, root weight, shoot weight, root shoot ratio and dry matter production, leaf temperature and stomatal distribution.

Flowering is a crucial determinant for plant reproductive success and seed set, and changes in the timing of flowering may alter the processes at species, community and ecosystem levels.

Of all plant organs, leaves are most morphologically diverse exhibiting great structural plasticity in response to disparate environmental conditions. The structural adaptations shown by leaves clearly play a central role in adaptation of plants to changing environments. Number of leaves, leaf size and anatomy are often altered by growth in elevated CO_2, but the magnitude of these changes often decreases as leaves mature and hinges upon plant genetic plasticity, nutrient availability, temperature and phenology (Pritchard *et al.*, 1999).

In this experiment it was found that total number of leaves in plants increased up to 15.86 % under elevated CO_2. This result was in congruent with increased leaf number reported in Japanese honey-suckle (Sasek and Strain, 1991), sweet potato (Bhattacharya, 1985) and berseem (Pal, 2004) grown under elevated CO_2 condition. Exposure of plants to elevated CO_2 stimulates cell division at the shoot apical meristem either directly or indirectly. Undifferentiated cells produced at the shoot apex undergo transition to a more specialized state in which they either become components of organ primordia or contribute to internodes between organs (Clark, 1997).

Specific leaf area was found to be 27.86 % more in varieties exposed to elevated CO_2.The result was in contrast with a recent study in soybean where 22.2% reduction in specific leaf area was reported at 29 DAP (Madhu and Hatfield, 2015). Specific leaf area (SLA) is an indicator of leaf thickness. Exposure to elevated CO_2 can cause an increase in leaf thickness due to increased number of palisade cells, which contributed to leaf thickness (Thomas, 1983). The

reduction in specific leaf area under elevated CO_2 can also be due to the high accumulation of starch and lower rate of leaf expansion (Fig.3).

Days to first flowering In general, elevated CO_2 favors higher photosynthate (sugars and starch) accumulation in plants (Springer and Ward, 2007). A sugar signaling metabolite trehalose-6- phosphate (T6P) showed a strong correlation ($r2 = 0.94$) with vegetative and shoot-apical meristem tissue sucrose levels in *Arabidopsis* (Wahl *et al.*, 2013). T6P has been suggested to relay information about tissue carbohydrate availability and act as key signal for floral induction (Jagadish *et al.*, 2016).

Evidence is accumulating that the effect of CO_2 on time of flowering involves interactions with photoperiod, but the basis for this interaction is unclear. Here, which components of the photoperiod flowering pathway account for this interaction in *Arabidopsis thaliana* were examined. Elevated CO_2 interacted with both the photoreceptors and the subsequent transduction reactions in the photoperiod pathway. The direction and magnitude of the effects varied with photoperiod. Elevated CO_2 also affected flowering by increasing rate of leaf production. The net effect of elevated CO2 on time of flowering varies because CO_2 has a complex array of effects on different elements of the developmental pathway leading to flower induction that may either hasten or delay flowering depending upon the influence of other environmental factors such as photoperiod.

In present study, the flowering was advanced by an average of 2 days in amaranthus (Arun) plants exposed to the elevated CO_2 conditions. Whereas in cowpea, the flowering time as advanced by 1.75 days in cowpea (Anaswara). The results were in congruence with the available literature.

Pea plants grown under elevated CO_2 during 2014 took less days to 50 per cent flowering (74.31 days), which differed statistically with ambient condition (94.10 days). Highest days to 50 per cent flowering were taken by plants grown under natural condition (Meenakumari et al., 2016). Similarly during 2015, lowest days to 50 per cent flowering were recorded in pea plants grown under elevated CO_2 (73.98 days) and differed statistically from natural condition (91.77 days). In present

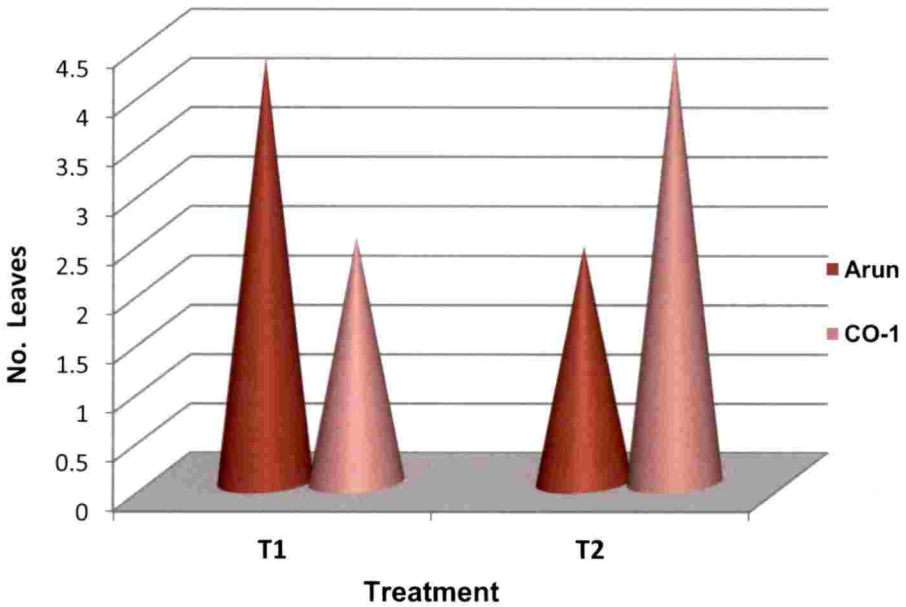

Fig 01. Effect of elevated CO_2 on number of leaves in amaranthus.

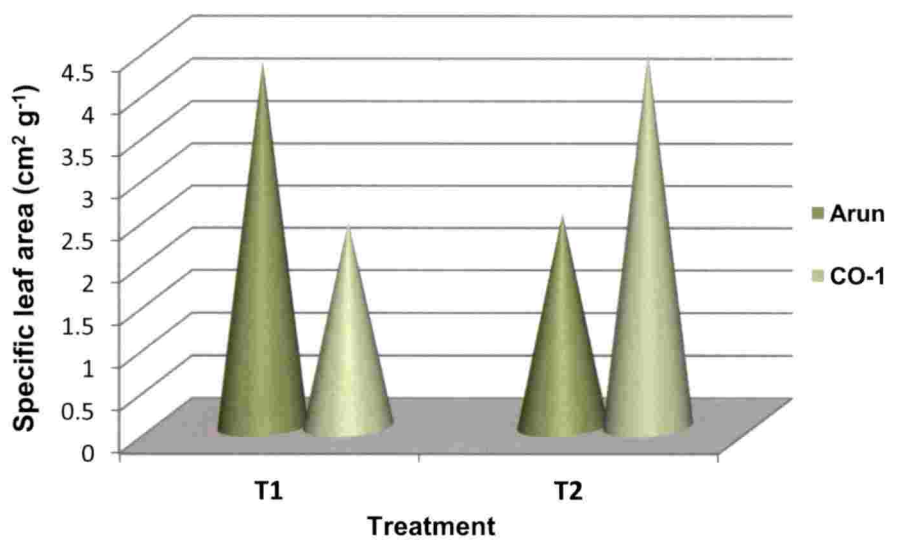

Fig 02. Effect of elevated CO_2 on specific leaf area ($cm^2\ g^{-1}$) in amaranthus.

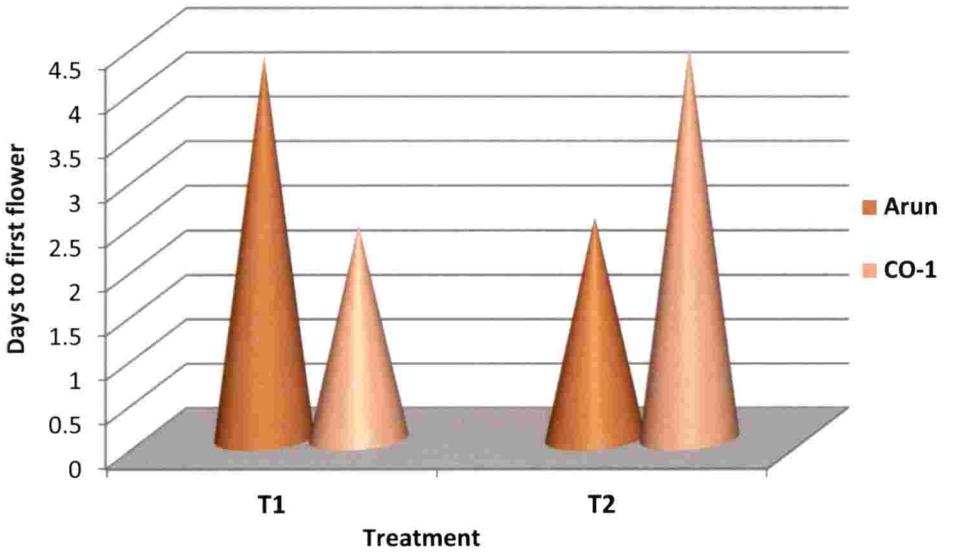

Fig 03. Effect of elevated CO_2 on days to first flower in amaranthus

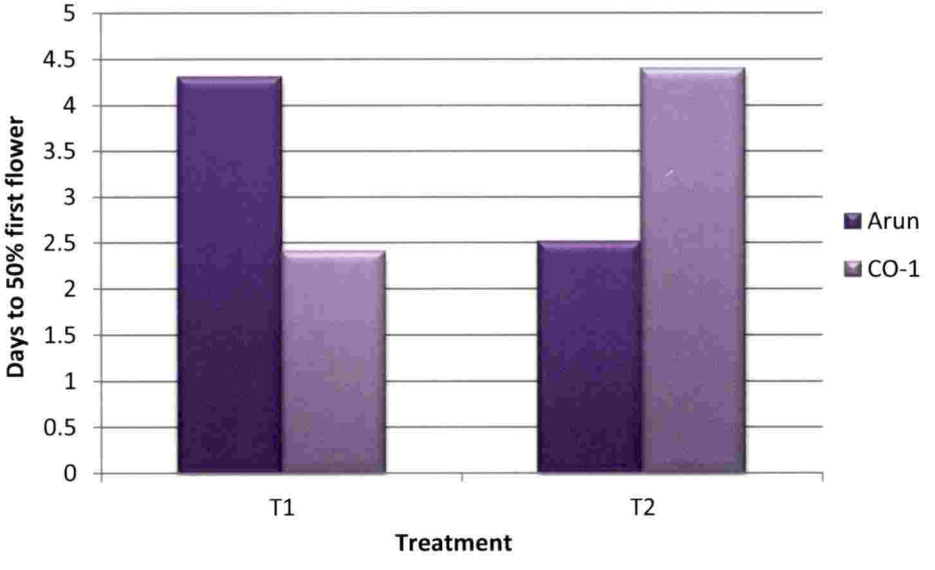

Fig 04. Effect of elevated CO_2 on days to 50% flowering in amaranthus.

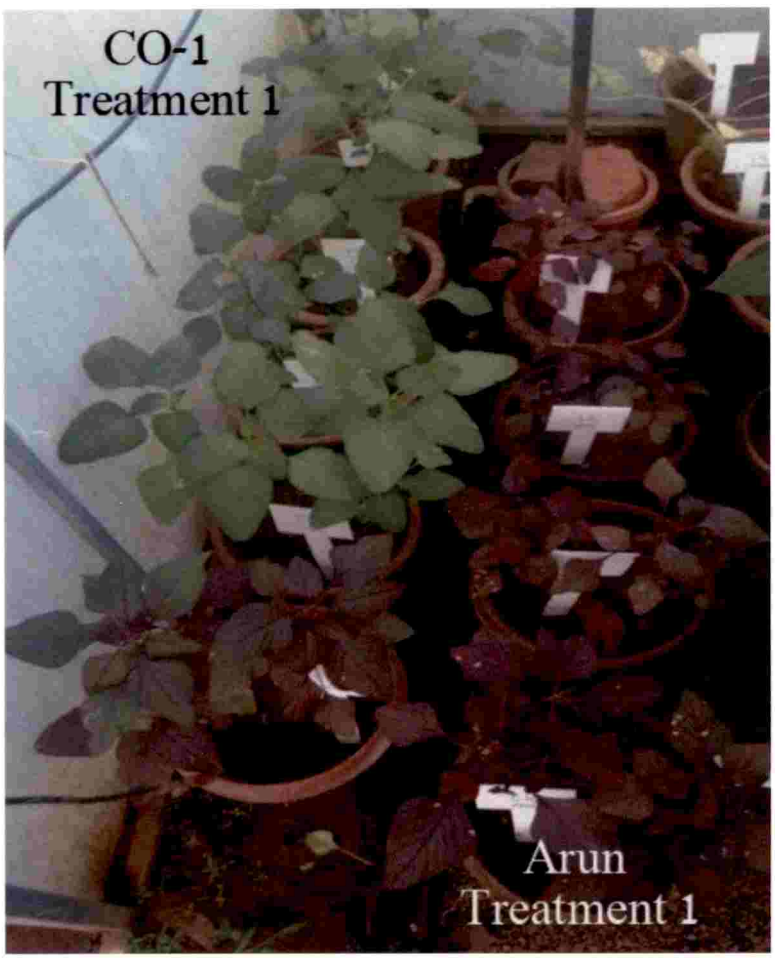

Plate 03: Study of flowering time responses in amaranthus under elevated CO_2 condition in open top chamber.

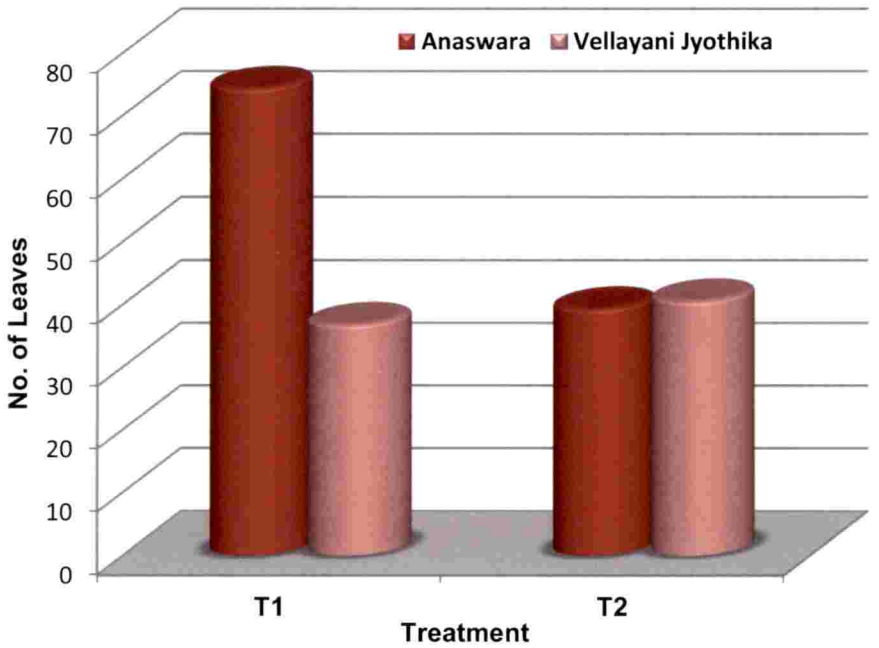

Fig 20: Effect of elevated CO_2 on number of leaves in cowpea.

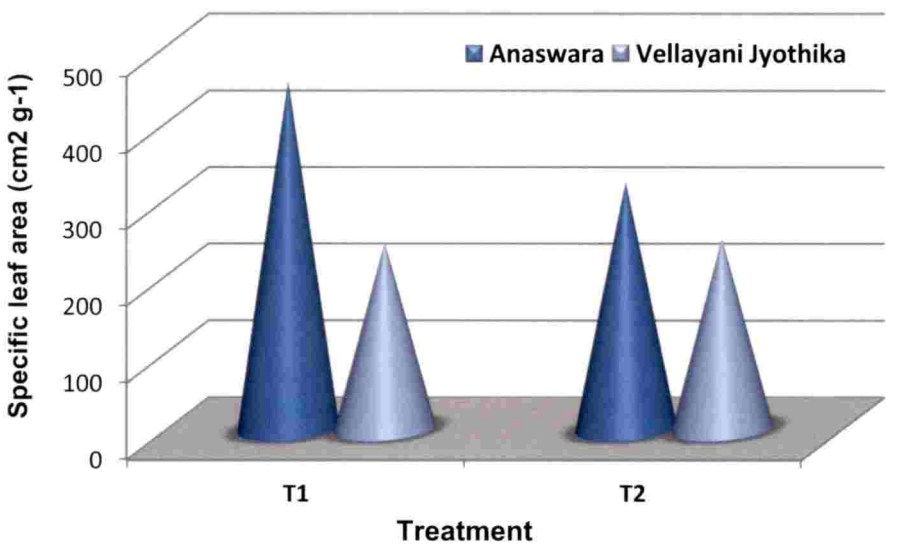

Fig 21: Effect of elevated CO_2 on Specific leaf area ($cm^2\ g^{-1}$) in cowpea.

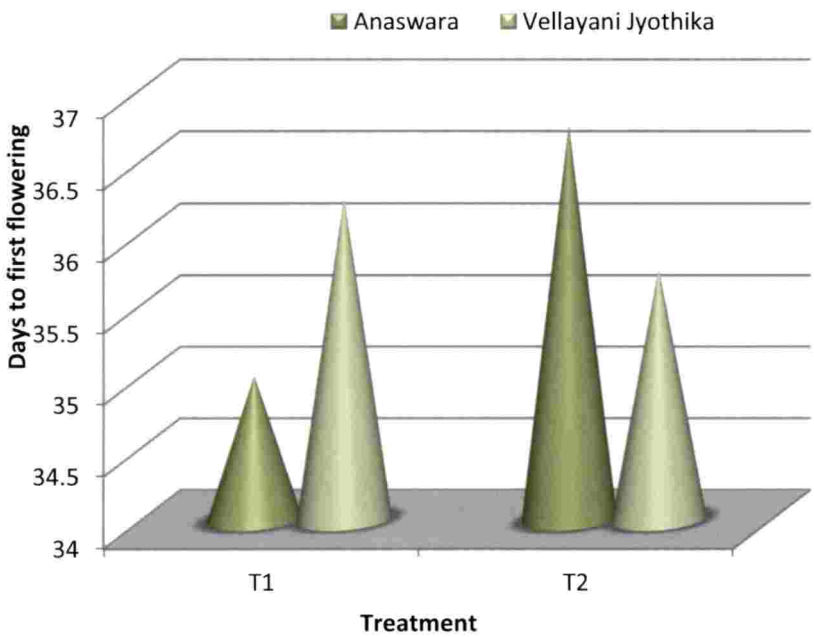

Fig 22: Effect of elevated CO_2 on days to first flowering in cowpea.

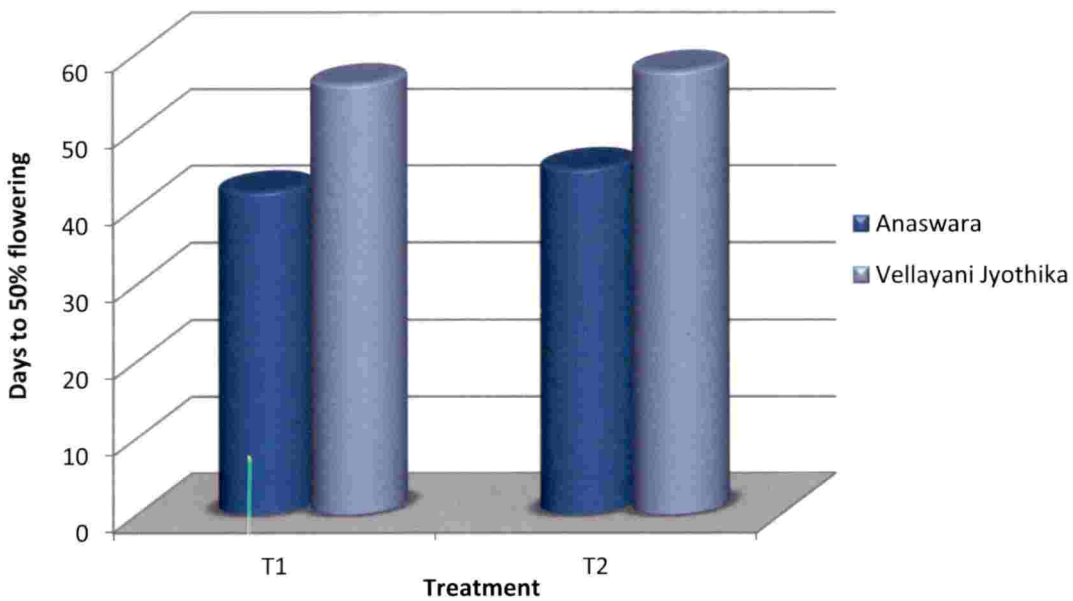

Fig 23: Effect of elevated CO_2 on Days to 50% flowering in cowpea.

Plate 04: Study of flowering time responses in cowpea under elevated CO_2 condition in open top chamber.

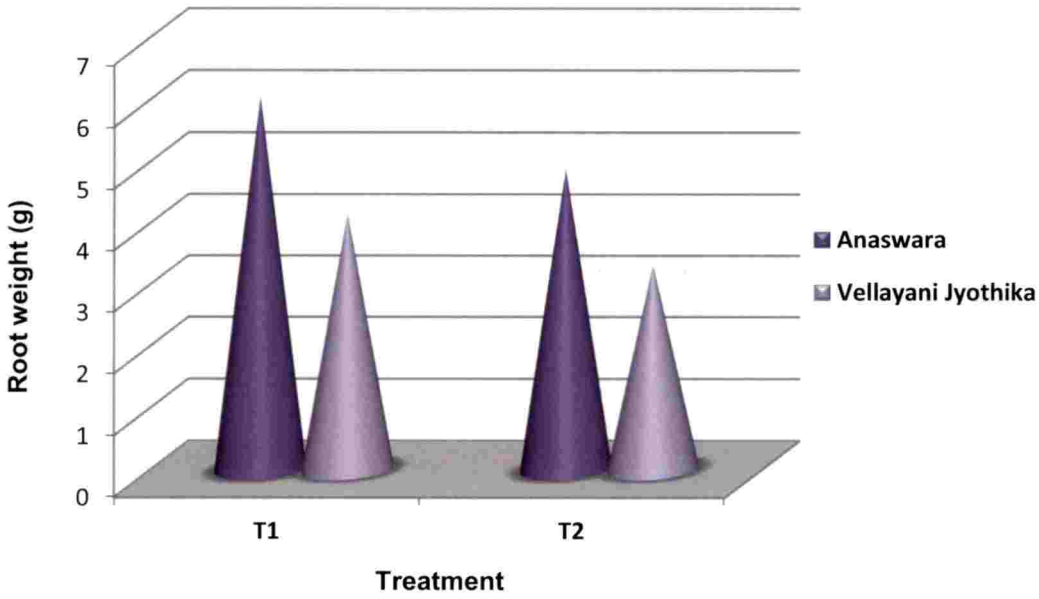

Fig 24: Effect of elevated CO_2 on root weight (g) in cowpea.

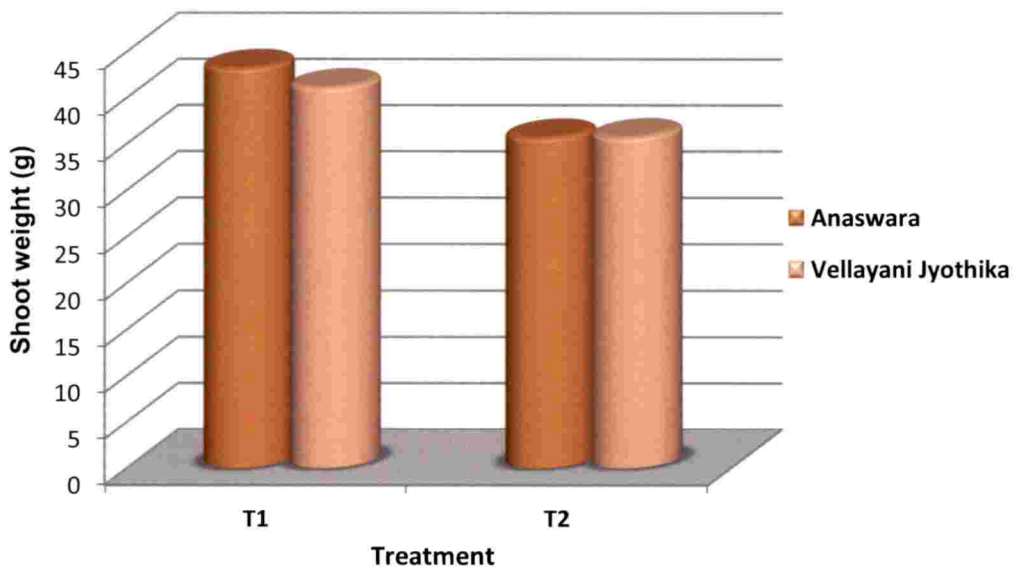

Fig 25: Effect of elevated CO_2 on Shoot weight (g) in cowpea.

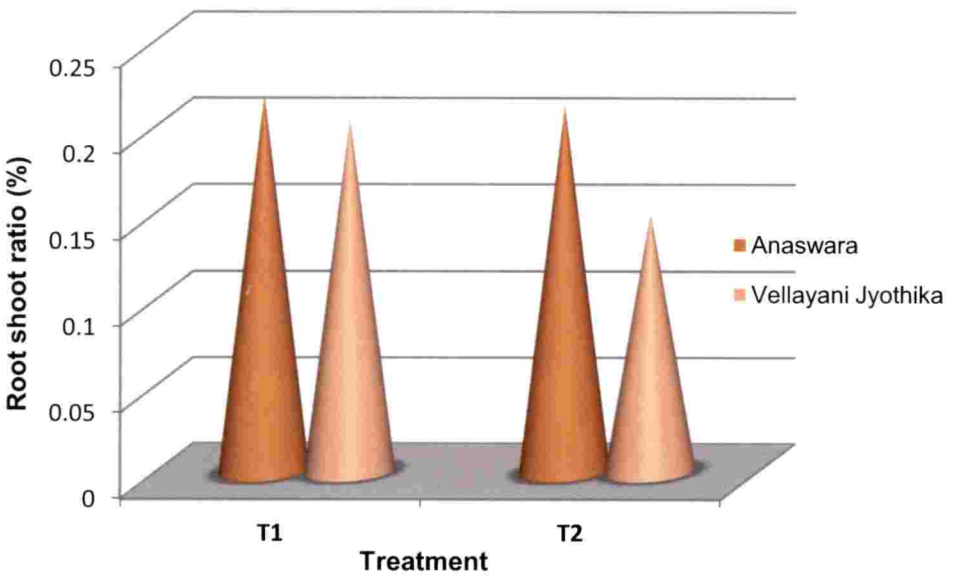

Fig 26: Effect of elevated CO_2 on root shoot ratio (%) in cowpea.

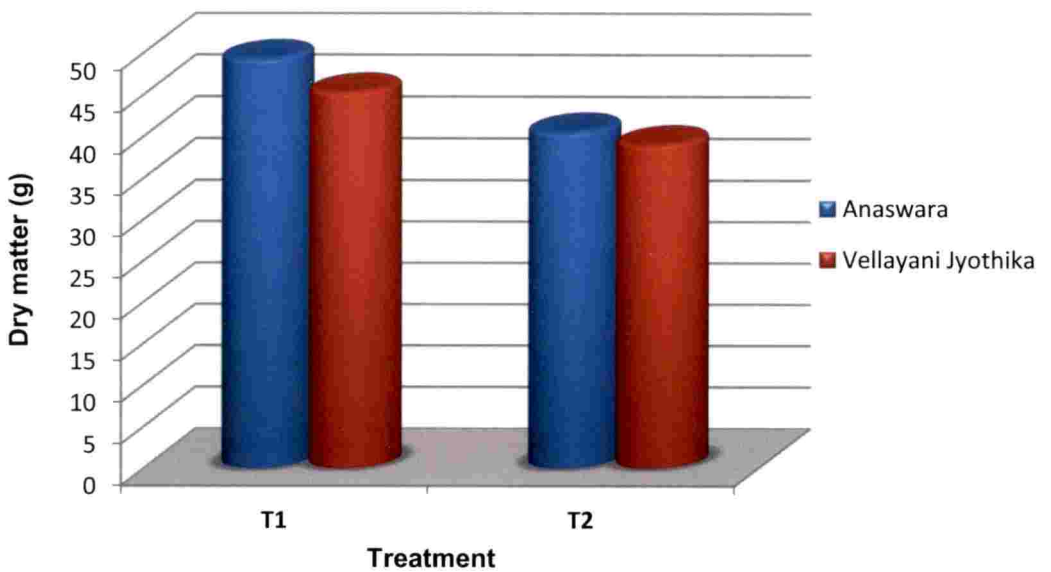

Fig 27: Effect of elevated CO_2 on dry matter (g) in cowpea.

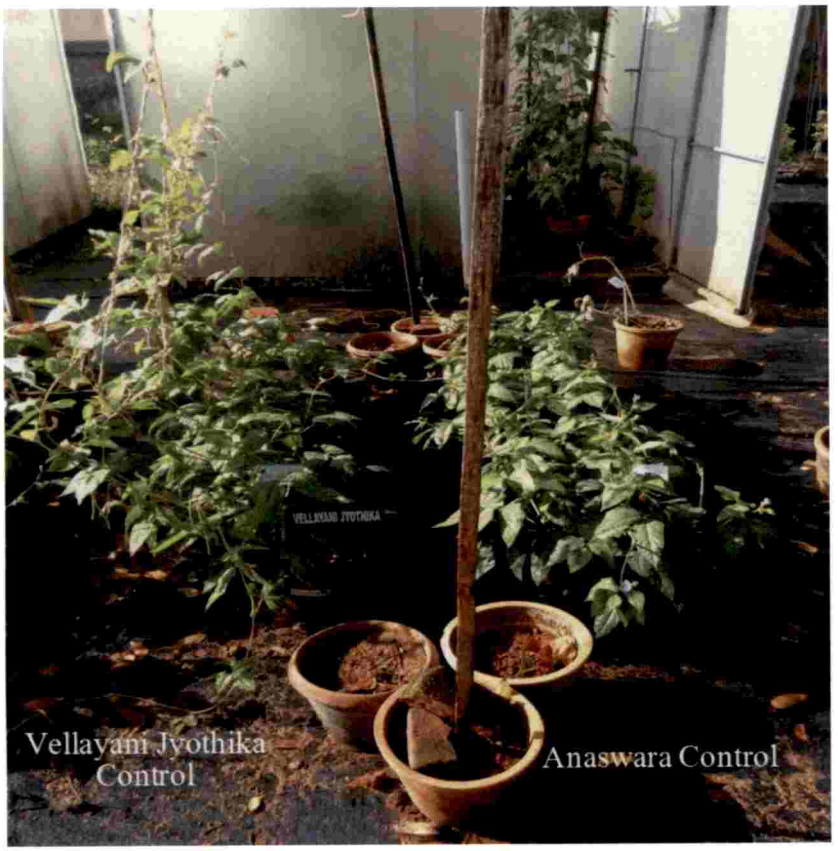

Plate 05: Study of flowering time responses in cowpea in open condition.

study, congruent results were obtained where CO-1 variety of amaranthus took on an average 46 days to 50% flowering as compared to the open field condition with the treatment mean of 48 days and Anaswara variety of cowpea took 42 days to 50% flowering.

Increasing atmospheric CO_2 significantly increased the final plant biomass, aboveground biomass, and belowground biomass (Obrist and Arnone, 2003). Root systems comprise up to half the total tree biomass and below-ground net primary production may exceed 50% of total net primary production (Kubiske and Godbold, 2001). Because C allocation to roots is often favoured over C allocation to shoots in plants grown under elevated atmospheric CO_2, below-ground function of forest ecosystems may change significantly (Pritchard *et al.*, 2001).

Extend of root branching has major implications for the efficiency of water and mineral extraction from the soil. Increased root growth contributes to root biomass and root dry weight under elevated atmospheric CO_2 regardless of species (Rogers *et al.*, 1994,1996).

In the present study 24.13 % increase in root biomass was observed under elevated CO_2 (Fig.5). This agrees with many results of previous studies in black gram (Vanaja *et al.*, 2007), soybean (Rogers *et al.*, 1994,) wheat (Chaudhuri *et al.*, 1990) etc. Stimulation of root growth was as a result of cell expansion caused by cell wall loosening in concert with higher cell turgor pressure and increased cell division. The average enhancement of photosynthesis for trees exposed to elevated CO_2 has been about 60% (Norby *et al.*, 1999).The enhanced photosynthesis has generally been followed by a similar, albeit a somewhat decreased magnitude, enhancement of above-ground growth. Exposure of plant canopies to high CO_2 concentration often stimulates growth of shoots and roots. The general consensus is that photosynthesis and C allocation to plant roots increases as atmospheric CO_2 rises which leads to an increase in above and below biomass (Del Castillo *et al.*, 1989). The plants exposed to elevated CO_2 resulted in 48.89 % increase in shoot dry weight.

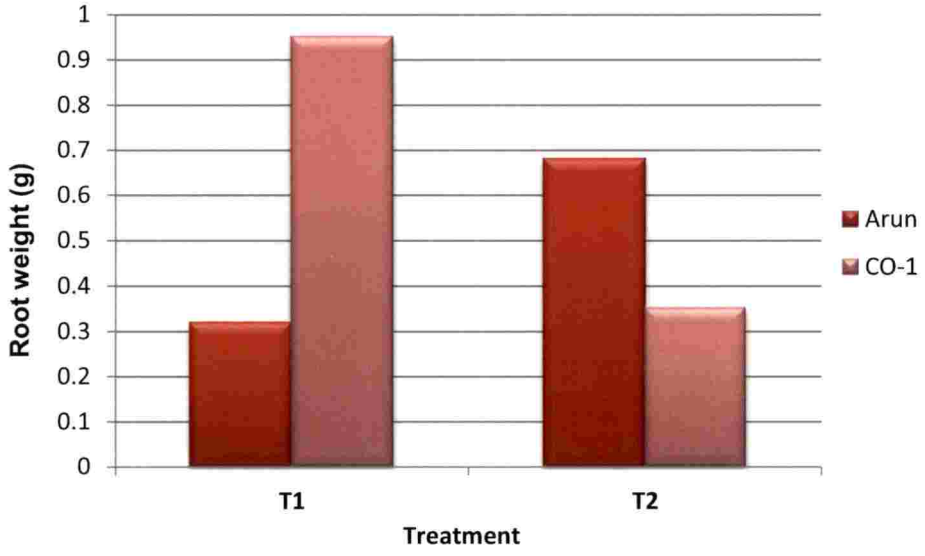

Fig 05. Effect of elevated CO_2 on root weight (g) in amaranthus.

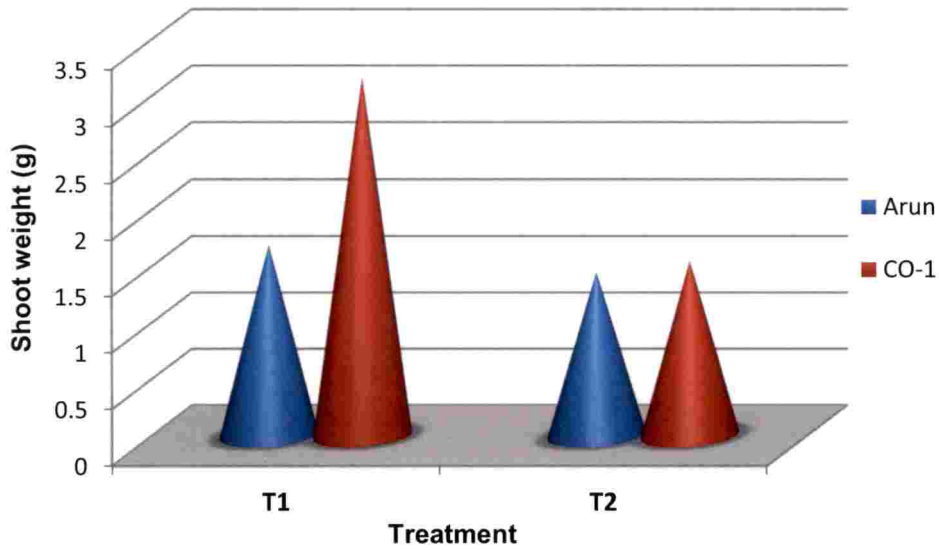

Fig 06: Effect of elevated CO_2 on shoot weight (g) in amaranthus.

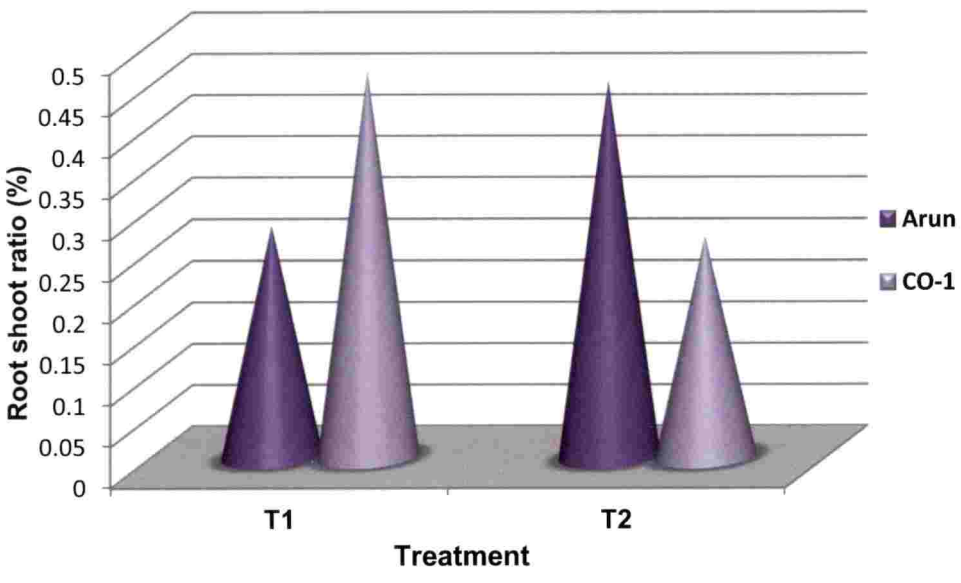

Fig 07. Effect of elevated CO_2 on root shoot ratio (%) in amaranthus.

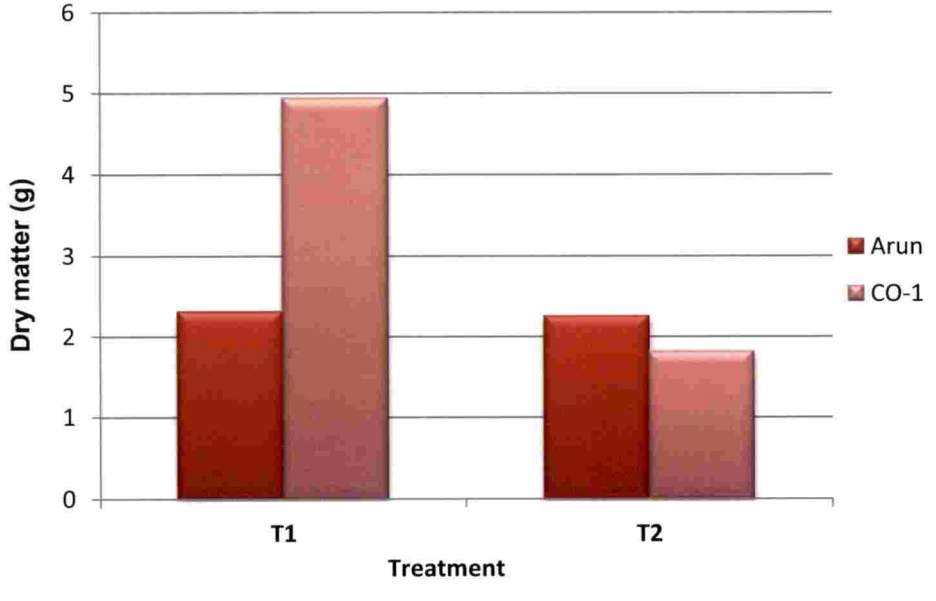

Fig 08. Effect of elevated CO_2 on dry matter (g) in amaranthus.

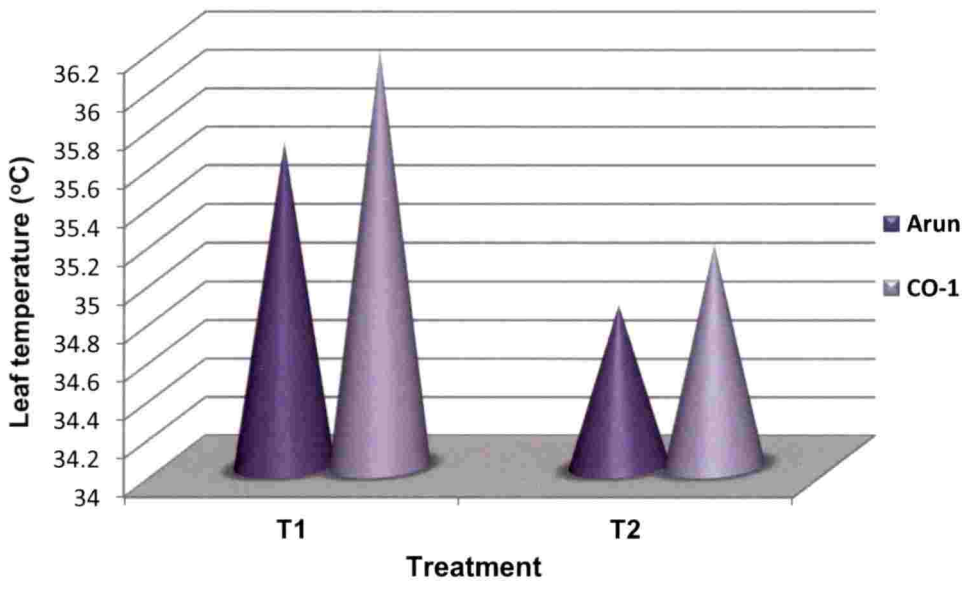

Fig 09: Effect of elevated CO_2 on leaf temperature (°C) in amaranthus.

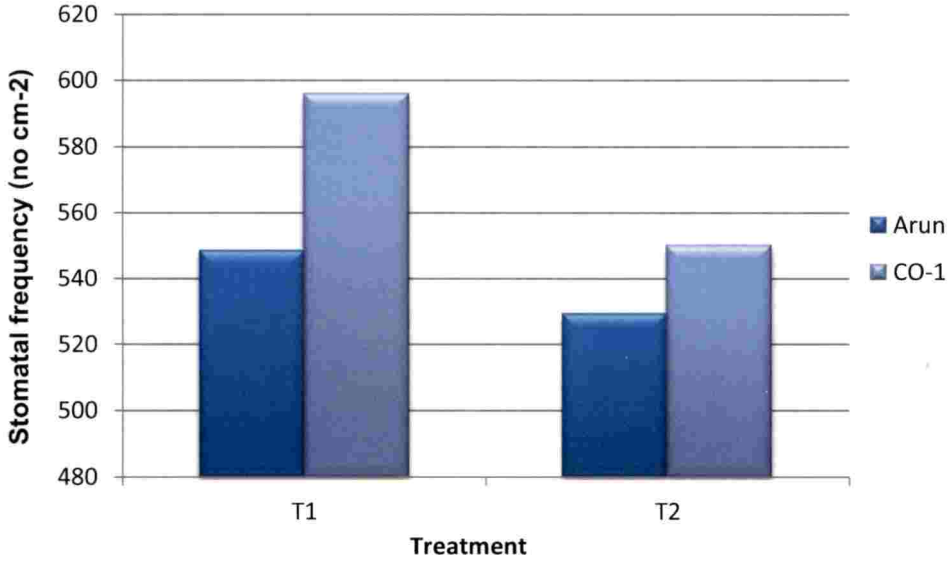

Fig 10: Effect of elevated CO_2 on stomatal frequency (no cm^{-2}) in amaranthus.

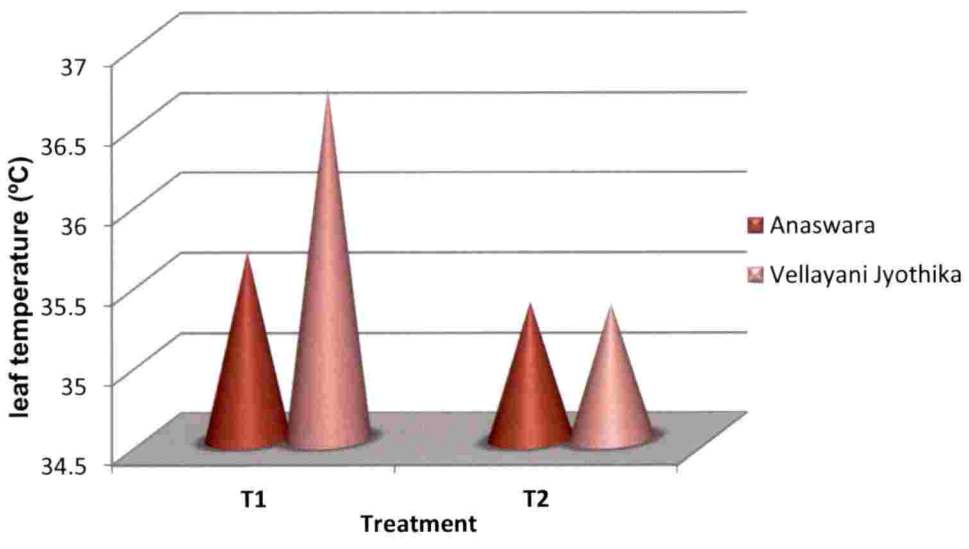

Fig 28: Effect of elevated CO_2 on leaf temperature (°C) in cowpea.

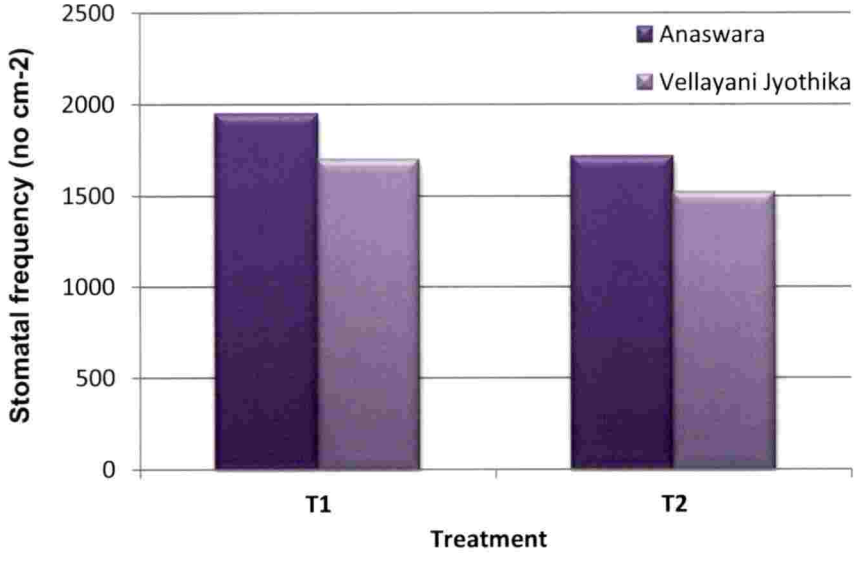

Fig 29: Effect of elevated CO_2 on Stomatal frequency (no cm^{-2}) in cowpea.

Root/shoot ratio is the simple calculation of the ratio of root dry mass to shoot dry mass and should serve as a measure of the preferential allocation of C to roots or shoots (Madhu and Hatfield, 2013). Allen *et al.* (1988) reported that soybean grown under increased CO_2 maintained a similar partitioning of C into their respective components. The partitioning pattern of photosynthate depends on plant development stage, plant species, and plant growth conditions along with physiological factors (Van Veen *et al.*, 1991). If plants allocate proportionately more C belowground, resulting an increase in R/S ratio.

In this experiment, an increase in root shoot ratio of 57.17% was reported under elevated CO_2condition. The result was in agreement with former reports by Cure, (1985) in sweet potato, Baxter *et al.* (1994) in montane grass species, Norby *et al.*, (1999) in yellow poplar trees. Zheng *et al.* (2010) reported significantly higher ratio of below ground to above ground biomass in *Caragana korshinskii* a desert herb, under elevated CO_2.

Elevated CO_2 stimulates photosynthesis in various intensities during different phenological phases and its direct consequence is increased dry matter production (Ziska *et al.*, 1998). An increase in dry matter production was seen in the present study; Under elevated CO_2 conditions 36.71% increase in dry matter production was observed. This was in agreement with the findings of Chaturvedi *et al.* (2009) in *Podophyllum Hexandrum*. Total biomass accumulation in *Hevea brasiliensis* increased under conditions of elevated CO_2 (Devakumar *et al.*, 1998). An increasein total dry matter production was also reported in soybean (Pan, 1996), dry bean (Prasad, 2002), peanut (Clifford *et al.*, 2000) and cowpea (Ellis, 1995) under CO_2 enrichment.

In the FACE experiments (Free Air Carbon dioxide Enrichment) in Arizona, Kimball *et al.* (1995) measured an average rise in canopy temperature of 0.56°C over the growing season. Such a higher leaf temperature may also have important consequences for the longevity and photosynthetic capacity of the individual leaves and at the canopy level, as ageing may be accelerated (Kimball *et al.*, 1995; Ellis *et al.*, 1990). In the present study, about 3.89 % increase in the leaf

temperature was observed in amaranthus. Whereas, 2.29 % increase in the leaf temperature was observed in cowpea.

Stomata are the integrators of all environmental factors affecting plant growth (Morison, 1998). A reduction in stomatal density with increasing CO_2 concentration is a general response in plants (Woodward, 1987). According to Casson and Gray, (2008) elevated CO_2 can alter stomatal density by affecting cell cycle machinery, including the number of initial divisions of meristemoid mother cell (stomatal entry), or spacing and amplifying divisions. Wax composition and accumulation can also affect the stomatal development by modulating the perception of diverse environmental signals (Holroyd *et al.*, 2002; Casson and Gray, 2008).

In the present study, about 7.71% increase in stomatal density was observed under elevated CO_2 and in Trench system. Similar results were obtained in *Betula pendula* and *Fraxinus ornus* (Rey and Jarvis, 1997) under elevated CO_2. Ferris *et al.* (2002) also reported a reduction in stomatal density when populus clones were exposed to elevated CO_2 condition.

5.2 EFFECT OF ELEVATED CO_2 ON BIOCHEMICAL AND PHYSIOLOGICALPARAMETERS

The effect of high CO_2 level on various biochemical and physiological parameters like pigment composition, total soluble proteins, reducing sugars, starch, gibberellic acid and nitrate reductase were analyzed. The results of these parameters are discussed below. Plant productivity is a unique process that depends greatly on the amount of chlorophyll present in the chloroplast. Chlorophyll is the pigment that gives plant their characteristic green colour, it plays a unique role in the physiology, productivity and economy of green plants. The amount of chlorophyll in leaf tissues is influenced by nutrient availability and environmental stresses (Palta, 1990). The function of the vast majority of chlorophyll is to absorb light and transfer it by resonance to a specific chlorophyll pair in the reaction centre of the photosystems. Leaf chlorophyll content is a good indicator of photosynthesis activity, mutations, stress condition and nutritional

status of plants (Ghasemi *et al.*, 2011).

The higher chlorophyll in elevated CO_2 grown plants could be explained by the larger size and number of chloroplasts present in the tissues exposed to high CO_2 levels (Robertson and Leech, 1995). Moreover, the better water use efficiency observed at high CO_2 (Bazzaz, 1990) could have limited chlorophyll degradation.

In the present study, even though chlorophyll a content was found to have no significant difference during the first month, the continued exposure to elevated CO_2 caused 44.31 % increase in chlorophyll a content.

Similar increase in chlorophyll content under elevated CO_2 was reported in several studies. Sgherri *et al.* (1998) reported an increase in chlorophyll content of Alfalfa plants grown under 600ppm of CO_2. Orchid plants subjected to elevated CO_2 showed a 64% increase in chlorophyll content (Gouk *et al.*, 1999).

Several contradictory results were also reported in the case of chlorophyll content under elevated CO_2. No change in leaf chlorophyll content was reported in potato (Sicher and Bunce, 1999), wheat (Bugbee, 1998), sugar maple (Li *et al.*, 2000) under elevated CO_2. A decrease in chlorophyll content was reported in pineapple by Zhu *et al.* (1997). Zhao and Running, (2010) reported a decrease in leaf chlorophyll and photosynthetic rate in sorghum plants at elevated CO_2 grown in N-deficient soil.

In this experiment 23.29 % increase in total soluble protein was observed under elevated CO_2. The result was in agreement with increase in the soluble protein recorded in the leaves of *Stylosanthes hamata* grown under 600ppm CO_2 (Baig *et al.*, 2012). Increased carbon entering the belowground system (increased root biomass) under elevated CO_2 can result in greater N uptake, including in N-limited ecosystems (Finzi *et al.*, 2007; Norby and Zak, 2011).This may be the reason for increased total soluble protein content observed under elevated CO_2.

Several contradictory results were reported in soluble protein content under elevated CO_2. Decrease in total soluble protein under CO_2 enrichment was

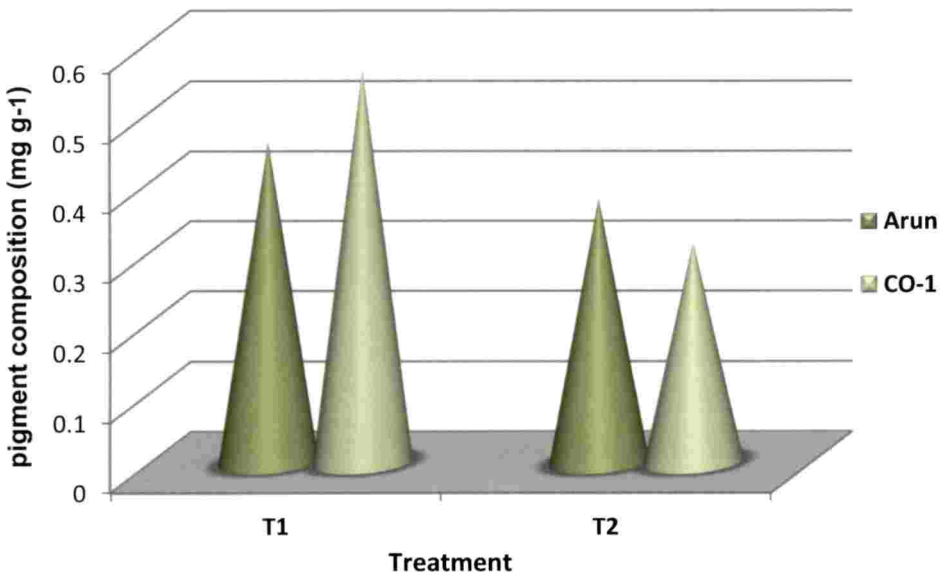

Fig 11: Effect of elevated CO_2 on Chlorophyll a (mg g^{-1}) content in amaranthus.

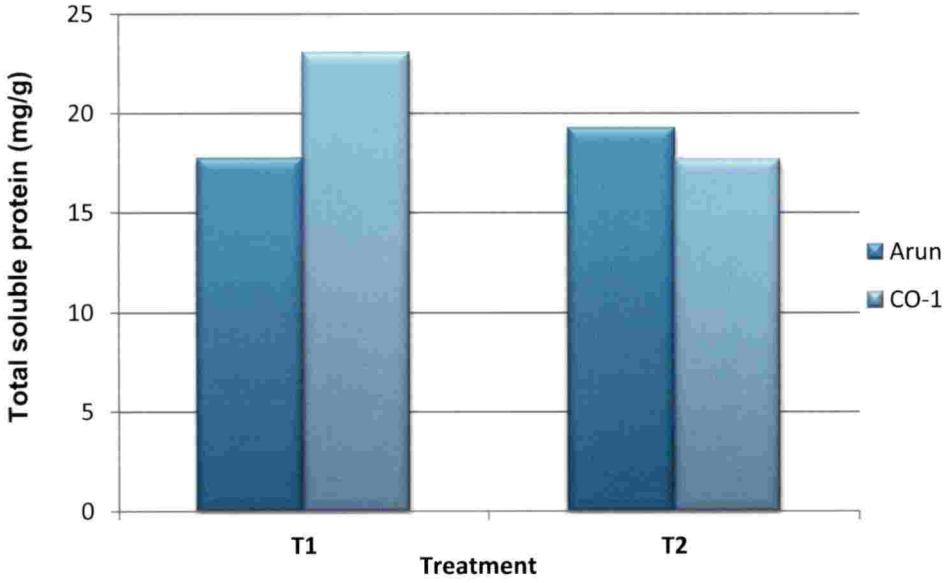

Fig 12: Effect of elevated CO_2 on total soluble protein content (mg/g) in amaranthus.

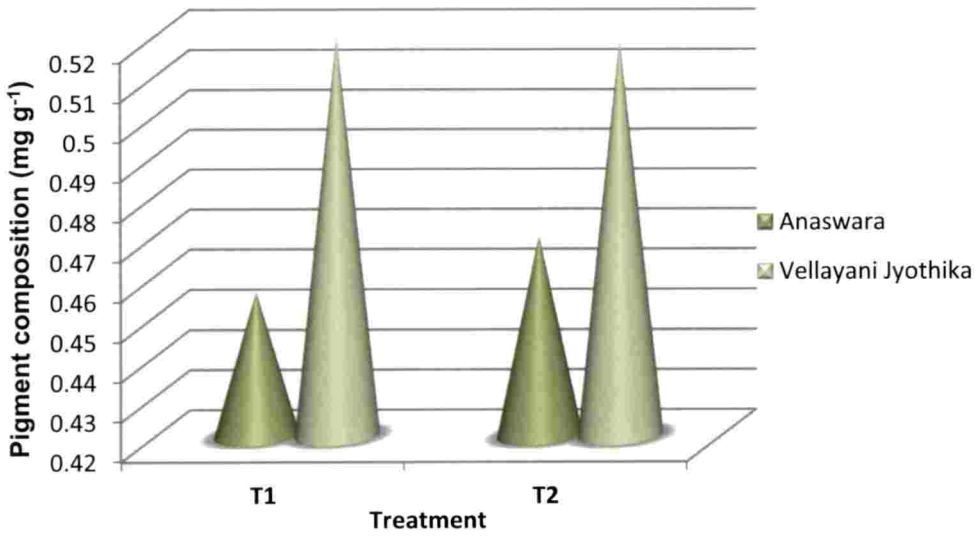

Fig 30: Effect of elevated CO_2 on Chlorophyll a (mg g^{-1}) content in cowpea.

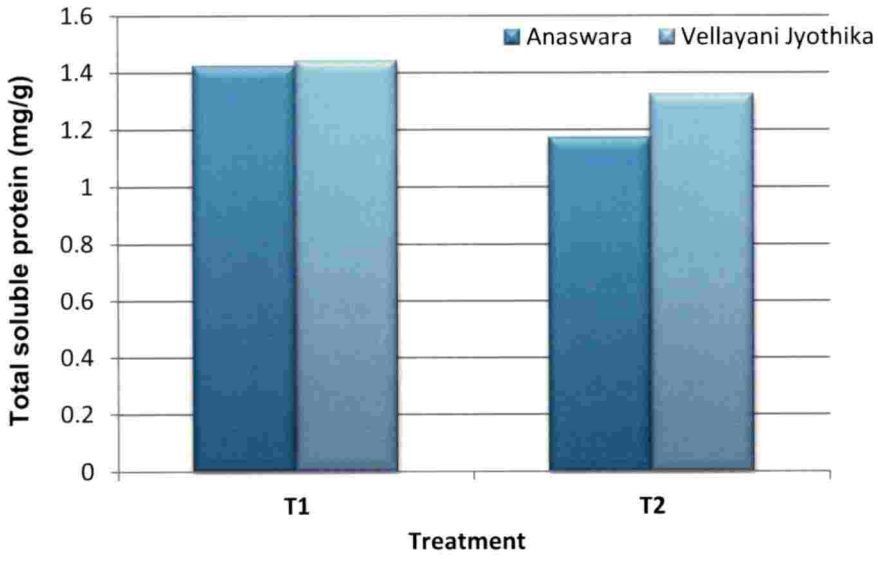

Fig 31: Effect of elevated CO_2 on total soluble protein content (mg/g) in cowpea.

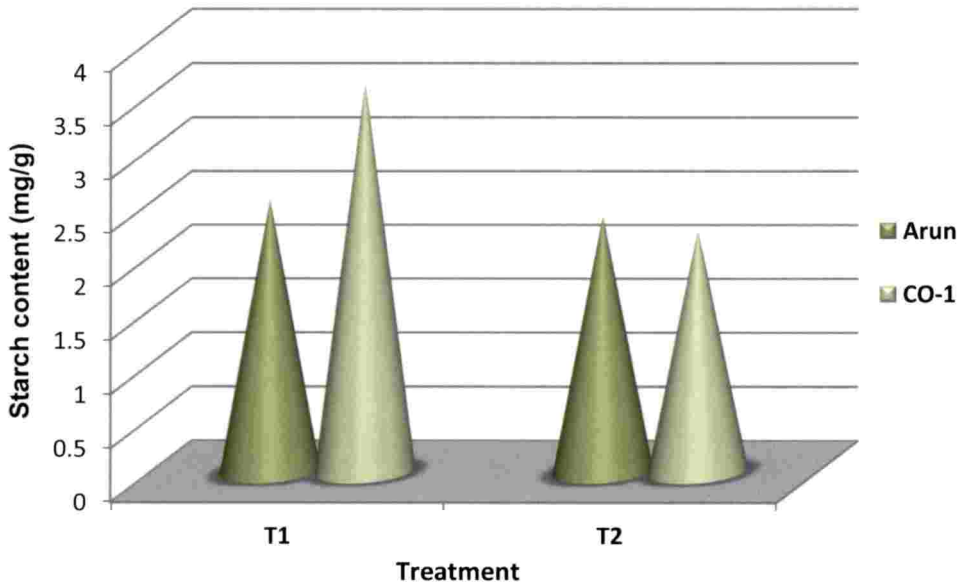

Fig 13: Effect of elevated CO_2 on starch content (mg/g) in amaranthus.

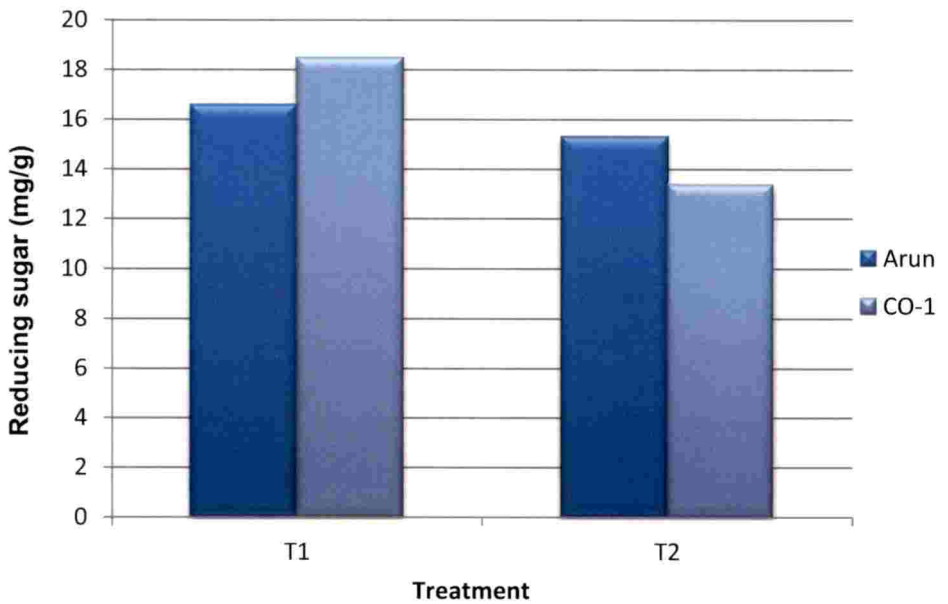

Fig 14: Effect of elevated CO_2 on reducing sugar content (mg/g) in amaranthus.

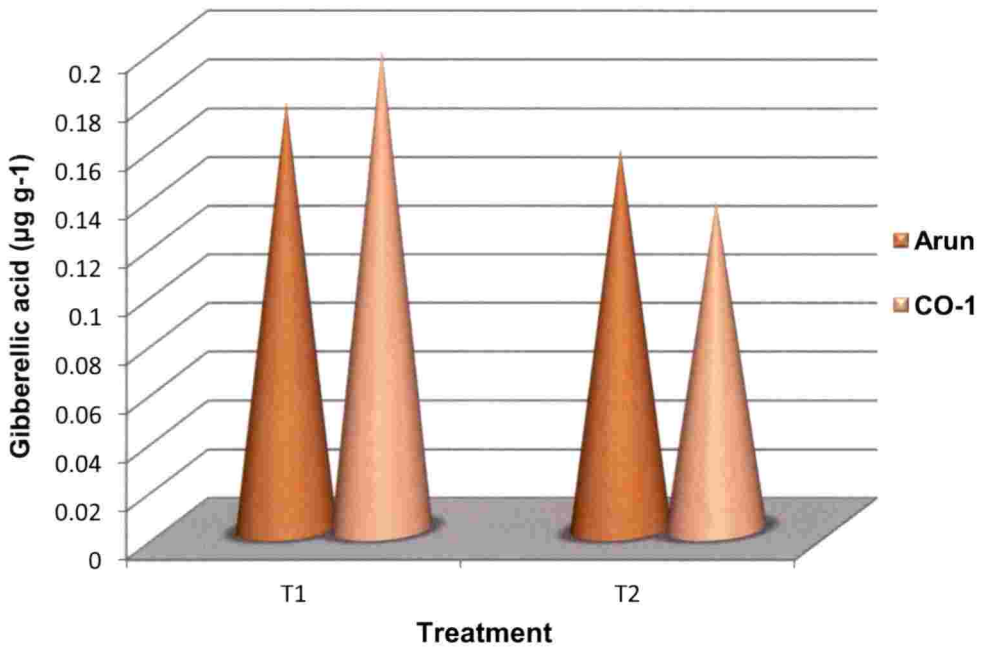

Fig 15: Effect of elevated CO_2 on Gibberellic acid (µg g^{-1}) in amaranthus.

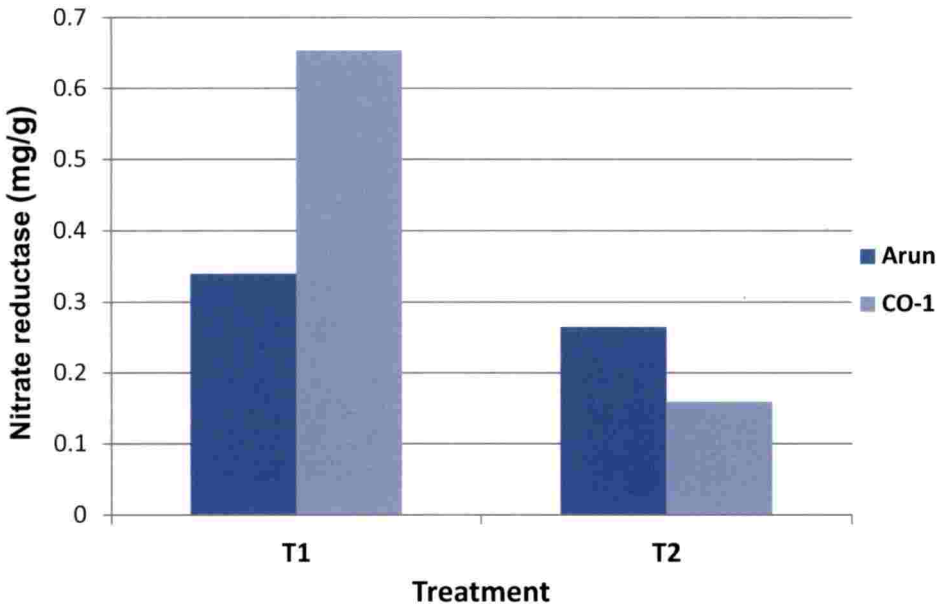

Fig 16: Effect of elevated CO_2 on Nitrate reductase (µg g^{-1}) in amaranthus.

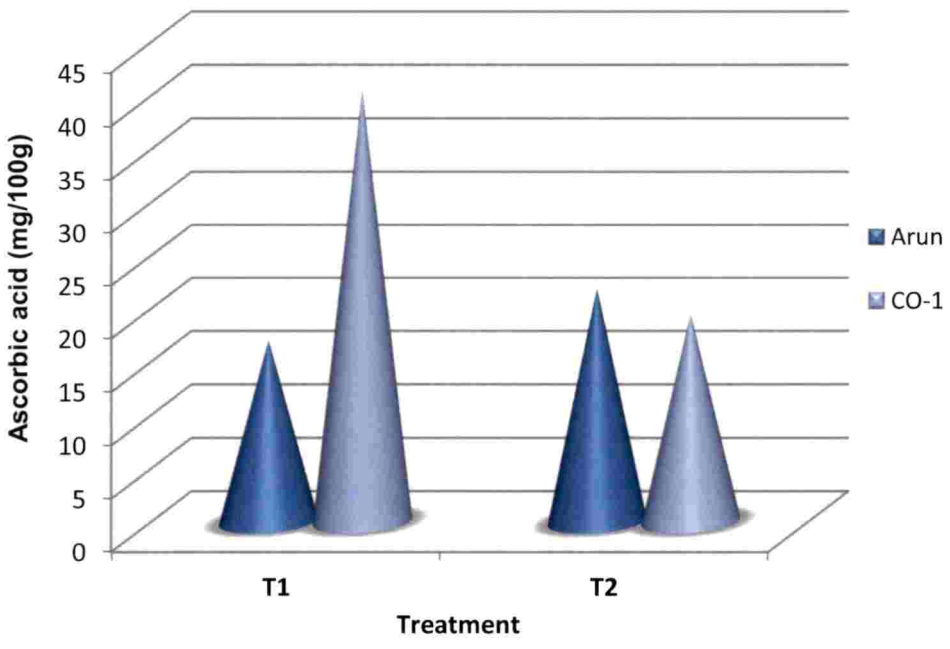

Fig 17: Effect of elevated CO_2 on ascorbic acid (mg/100g) in amaranthus.

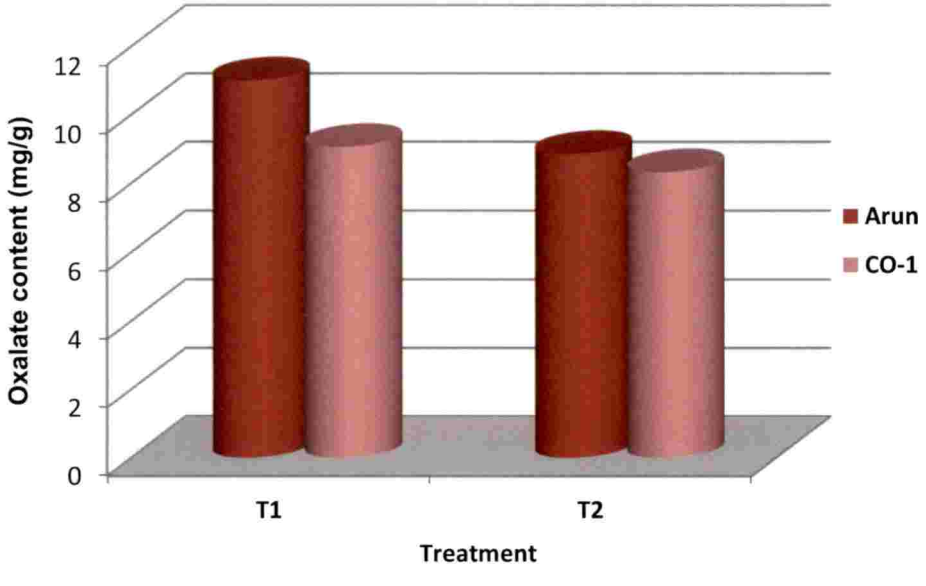

Fig 18: Effect of elevated CO_2 on oxalate content (mg/g) in amaranthus.

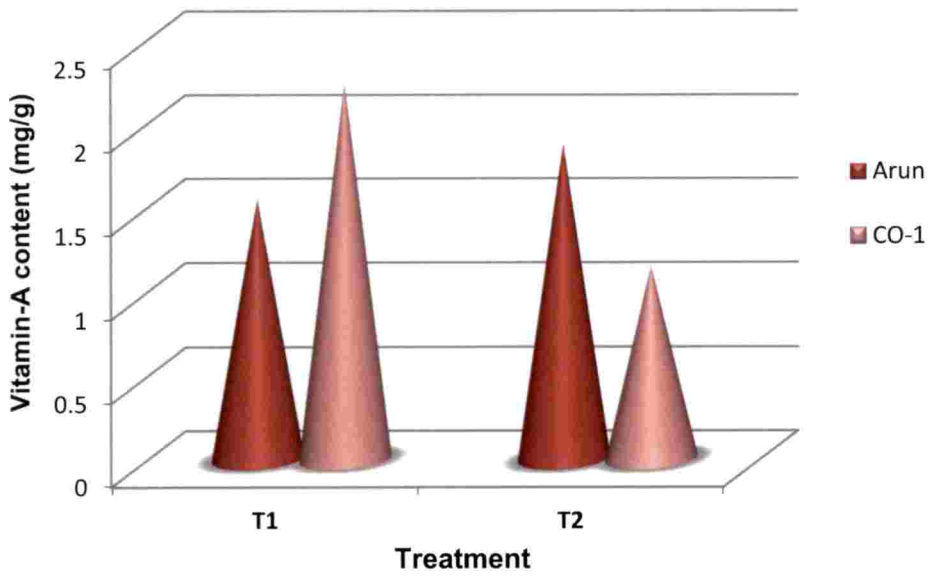

Fig 19: Effect of elevated CO_2 on Vitamin-A content (mg/g) in amaranthus.

reported in *Eleais guineensis* by Ibrahim and Jaafar, (2012). Richard and James, (1997) found out that elevated CO_2 concentration of 700 μmol mol^{-1} leads to decreased total soluble protein of barley penultimate leaves and wheat flag leaves. The protein accumulation was found to be lowest in barley leaves (Robredo *et al.*, 2011) enriched with high CO_2 concentration.

Accumulation of carbohydrates in leaves is one of the most pronounced and universally observed responses of C_3 plants to elevated atmospheric CO_2 (Long *et al.*, 2004). However, there is considerable variation in the response of different species, with increases ranging from almost zero to over 100% (Wong, 1990; Korner and Miglietta, 1994). Under elevated CO_2 condition, carbohydrates accumulate in plant tissues since their usage intensity is lower than their production under these conditions (Moore *et al.*, 1998). Studies have revealed that elevated CO_2 conditions enhances the soluble sugar content of *Labisia pumila* (Ibrahim, 2011), *Urtica diocia* and *Plantago major* (Den-Hertog, 1996), *Poa alpinia* (Baxter, 1994) and beech leaf.

In the present study, from the initial period onwards high accumulation of starch was noticed in varieties under elevated CO_2. In varieties under high CO_2 level, an average of 37.96 % increase in starch production was noticed. Several reports on increased carbohydrate fractions in plants under elevated CO_2 were reported by several workers. Wang *et al.*, in 2003 reported a 52% increase of total soluble carbohydrate content in beech leaves. High carbohydrate accumulation was reported in strawberry under elevated CO_2 condition (Wang *et al.*, 2003). Elevated CO_2 condition increases the accumulation of starch, total soluble sugars and reducing sugars in black gram during the flowering stage (Sathish *et al.*, 2014). Growth under elevated CO_2 levels increases the leaf starch content on an average of 160% and soluble carbohydrate by 52% in Alpine tundra (Moore *et al.*, 1999). Starch and sucrose levels were increased by 132% and 43% respectively in the leaves of 6 week old plants of *Arabidopsis thaliana* in response to CO_2 enrichment level of 100 Pa (Hanhong Bae and Richard Sicher, 2004). Lilley *et al.* (2001) reported that elevated CO_2 conditions produced an average increase in total

non-structural carbohydrate contents of 28% for clover and 16% for phalaris. Rising levels of atmospheric CO_2 can alter plant growth and partitioning to secondary metabolites (Mattson and Julkunen-Tiitto, 2005).

The effects of CO_2 enrichment on leaf ultra structure, mineral nutrition and plant hormone concentrations have not been extensively studied in any of the model plants; nevertheless, these aspects, in particular leaf ultra structure and plant hormone concentrations, are very important for an integrative understanding of plant responses to increased atmospheric CO_2. In present study, an increment by 31.62 % in the gibberellic acid content was observed in CO-1 variety of amaranthus and 10.25 % average increment in the Anaswara variety of cowpea under elevated CO_2 condition.

CO_2 enrichment also affected leaf nitrate reductase (NR) activity in both the genotypes but no particular trend was observed. In Pusa 1103, NR activity increased significantly during vegetative and flowering stage by 68% and 48%, respectively but declined by 15% at podding stage. NR activity was higher during vegetative stage followed by flowering and podding stage. In Pusa 1105, NR activity declined by 12% and 10% during flowering and podding stage, respectively. In present study, increase in the nitrate reductase content was observed in all the varieties of amaranthus (Arun and CO-1), and cowpea (Anaswara and Vellayani jyothika) under elevated CO_2 condition.

In present study, the amount of ascorbic acid in the variety CO-1 (amaranthus) was found to be more under elevated CO_2 treatment. This increment can be attributed to the fact that the additional carbon fixed by plants during CO_2-enrichment is invested in antioxidative compounds; and one of the most prominent of these products is *ascorbate* or vitamin C. Chemical analysis revealed that plantlets grown with elevated CO_2 had somewhat higher levels of oxalates in the leaves and shoots. The extra CO_2 of their study stimulated the production of vitamin A. (Kimball and Mitchell, 1981).

Ploidy level can often produce significant changes in basic biology and result in strong ecological consequences. Higher ploidy level of CO-1 which is a

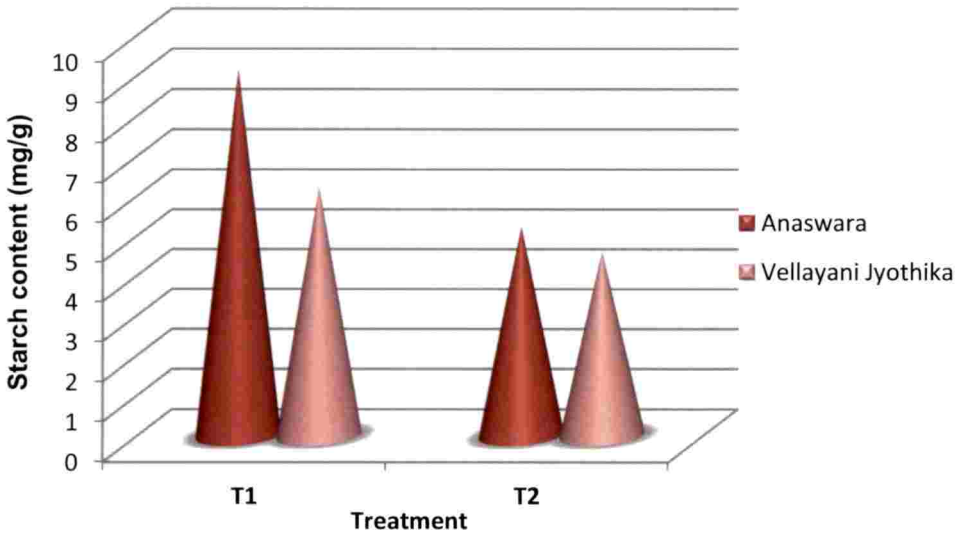

Fig 32: Effect of elevated CO_2 on starch content (mg/g) in cowpea.

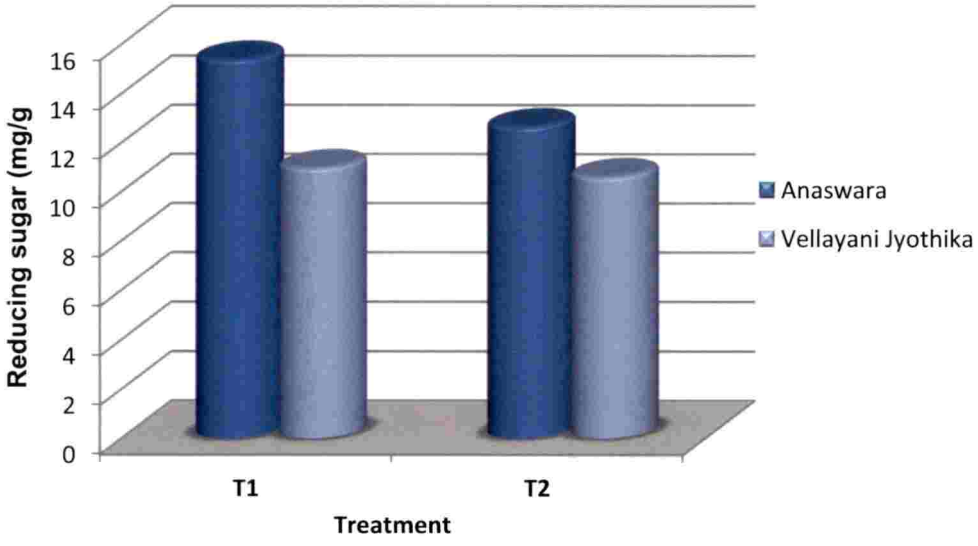

Fig 33: Effect of elevated CO_2 on reducing sugar content (mg/g) in cowpea.

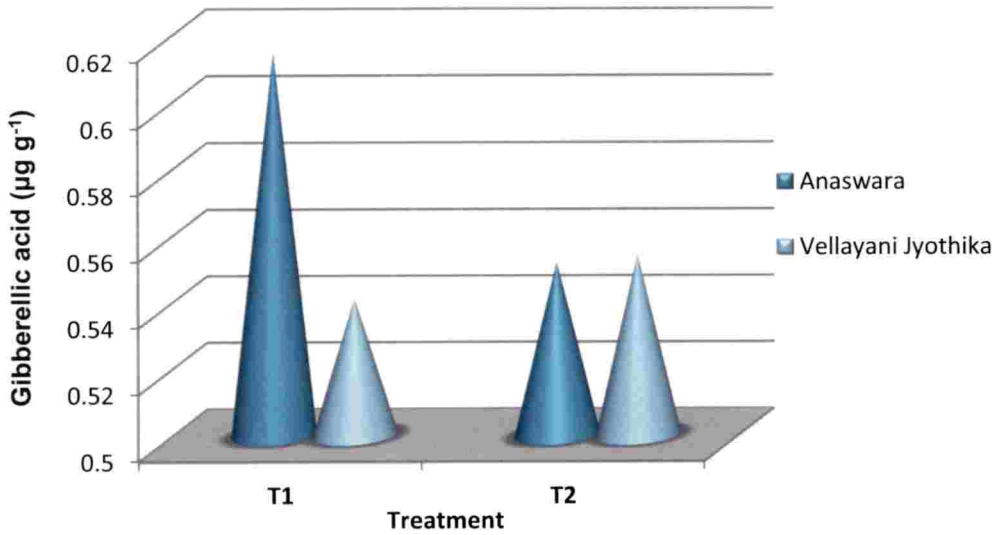

Fig 34: Effect of elevated CO_2 on Gibberellic acid (µg g^{-1}) in cowpea.

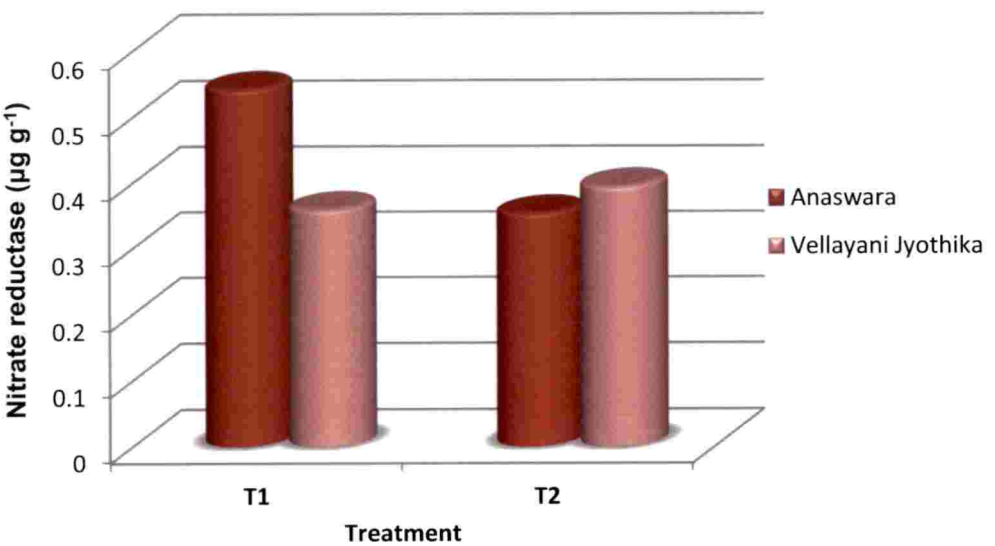

Fig 35: Effect of elevated CO_2 on Nitrate reductase (µg g^{-1}) in cowpea.

selection from local type can make it perform better under CO_2 condition.

Molecular studies:

Flowering time gene expression using RT-PCR

Effect of CO_2 enrichment on the expression of the floral integrator gene-Flowering locus T (FT) was studied in both in amaranthus and cowpea. There was distinct variation in the expression levels of FT between experimental plants grown under open condition and elevated CO_2 environment as evidenced by RT-PCR. Plants exposed to CO_2 enrichment showed the highest FT expression levels in both the crops. Plate No 8 depicts the FT expression pattern under different environments. The interaction between CO_2 and FT can represent the up-stream effects on photoreceptors and time keeping mechanism.

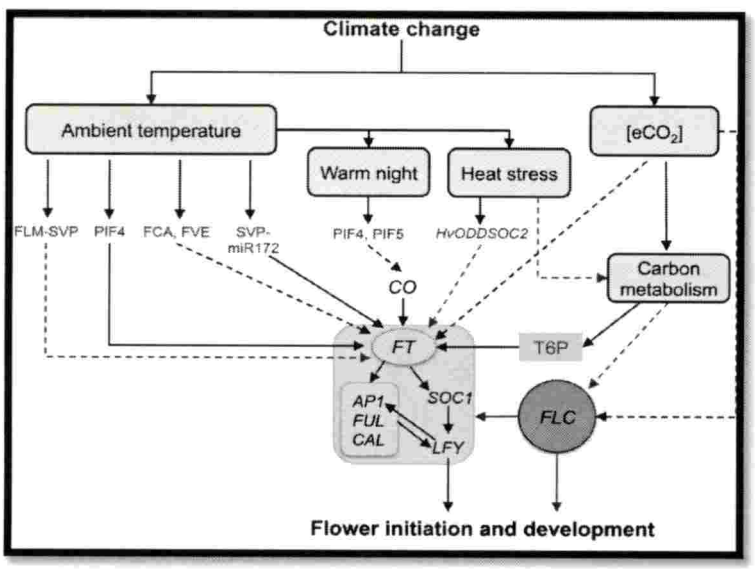

Plate 08: Flowering regulation by ambient temperature and elevated CO_2.

Increasing CO_2 concentration in the atmosphere will have an influence on carbon metabolism. Involvement of sugars in the regulation of flowering has been reported in Arabidopsis where production of Trehalose-6-phosphate (T-6-P) by shoot apical meristem act as a signal for floral transition and initiation under inductive environmental condition (Wahl *et al,* 2013). It was also reported that elevated CO_2 can induce floral transition with enhanced substrate supply through increased photosynthesis, though excess foliar sugar accumulation under elevated CO_2 may delay flowering (Springer and Ward, 2007). The increase in temperature associated with CO_2 enrichment can affect flowering time by directly affecting the rate of development (Craufurd and Wheeler 2009). This can be due to the compound effect of temperature on the processes of photosynthesis and respiration.

In addition to this integral signals like plant hormones can be involved in the cross-talk between reproduction and other developmental process. The enhanced levels of Gibberellins found in amaranthus and cowpea under CO_2 enrichment decisive because of its involvement in modification of the floral integrators.

In the present programme, which was undertaken to study the CO_2 enrichment induces modifications in flowering time in amaranthus and cowpea, time to first flowering and time to 50% flowering were found to be influenced by CO_2 enrichment. And there was varietal variation in the extent of variation. CO-1 in the case of amaranthus and Anaswara in the case of cowpea responded more to the modified atmosphere. Based on the physiological, biochemical and molecular analyses carried out during the study, it was shown that the important factors which can directly be correlated with flowering time are enhanced photosynthetic activity reflected in terms of higher starch and sugar accumulation and dry matter accumulation and GA. There was differential expression of FT both in both the crops under elevated CO_2 condition which can be mediated through carbohydrate accumulation and GA signals. The increase in the rate of development reflected in terms of growth parameters also give an indication of CO_2 enrichment associated increase in temperature mediated interaction between developmental and reproductive processes.

The results of the study highlights the influence of environmental factors like CO_2 concentration and temperature on plant development and flowering time. Infield conditions, factors like temperature can be influenced by crop management practices such as sowing dates. Even in crop breeding programmes, which are targeted towards earlier or late flowering to increase cultivar adaptability to environment faced with abiotic stress factors, efforts should be undertaken with causion taking into consideration all these different factors and their impacts. In the present, changing climatic scenario, elevated CO_2 and increasing temperature are the key factors that can offset plant fitness and flowering related events. Addressing the effects of these environmental factors on flowering events is critical and is of utmost importance to understand the adaptation of crops to changing climate.

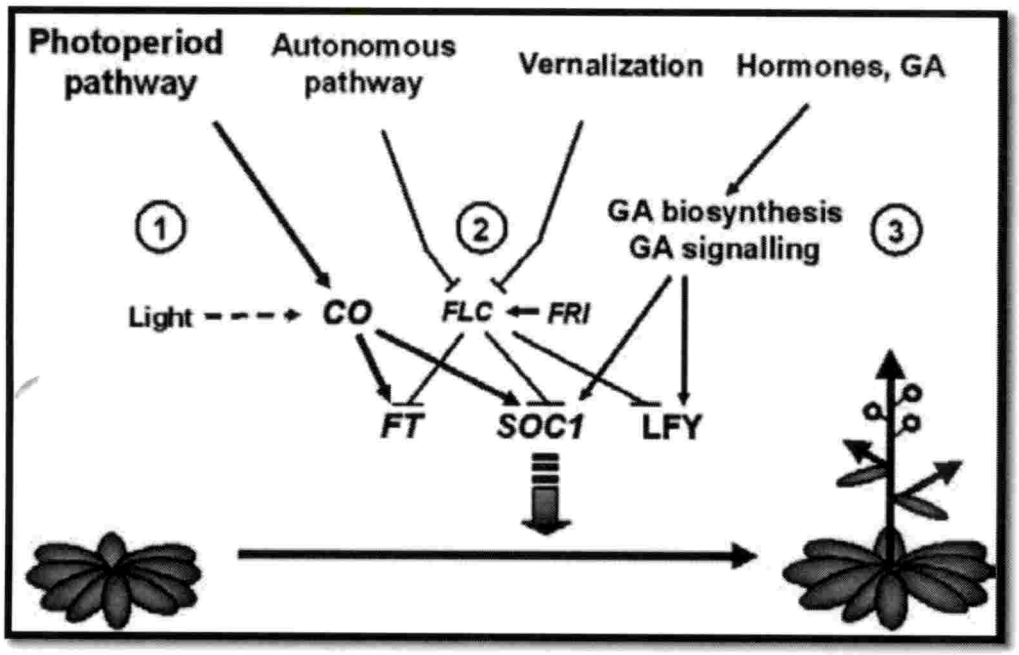

Plate 09: Floral pathways and signals that trigger flowering.

SUMMARY

6. SUMMARY

The level of CO_2 in the atmosphere is rising at an unprecedented rate. According to NOAA (National Oceanographic and Atmospheric Administration) 2016, global concentration of CO_2 has reached 400 ppm for the first time in recorded history. This rise, along with other trace gases in the atmosphere is widely thought to be a primary factor driving global climate change. It has emerged as an important environmental challenge due to its potential impact on biological systems on earth.

The geographic distribution of plant species, vegetation types and agricultural cropping patterns demonstrate the very strong control that climate has on plant growth. The most significant factor that contributes to climate change is the accumulation of heat trapping greenhouse gases in the atmosphere. Environmental Protection Agency considers many molecules like water vapour (H_2O), Carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) as greenhouse gases. Among these CO_2 has contributed the most to climate change (IPCC, 2007) mainly due to its radiative forcing character, longer residence time in the atmosphere and also due to its relative abundance in the atmosphere.

Moreover the report of IPCC, 2012 has reconfirmed the increasingly strong evidence of global climate change and projected that the globally averaged temperature of the air would rise by 1.8–6.4°C by the end of the century.

Kerala is blessed with an amazing range of green leafy vegetables. Each green leafy vegetable has got a wide range of health benefits. Amaranth belongs to the family Amaranthaceae. It is the most popular leafy vegetable of Kerala. It can be grown throughout the year. The leaves and succulent stems are good sources of iron (305 mg/100 g), calcium (397 mg/100 g), vitamin A (8340 microgram /100 g) and vitamin C (99 mg /100 g). Therefore, it is popularly called as poor man's Spinach. The crop is adapted to a wide range of soil conditions.

Pulses are the main sources of protein and is commonly called poor man's meat. They are also used as fodder and concentrate for cattle. Pulses are responsible for improving soil fertility by increasing the amount of N_2 in the soil. Cowpea belongs to the family Leguminoseae. It is a twining annual herbaceous plant. The stem is slightly ridged and glabrous. The leaves are trifoliate and alternate. Pods are long and cylindrical. Cowpea can be grown throughout the year under Kerala conditions.

Studies emphasizing on the consequences of changing climate scenario on the growth, physiological and phenological aspects of amaranthus and cowpea are of utmost importance. The knowledge generated can strengthen modeled projections on future plant evolution. Moreover, Future studies on physiological & molecular realms can provide insight into how environmental and endogenous cues interface with the floral development pathways.

Agro-ecosystems may be strongly influenced by the projected increase in atmospheric CO_2 concentration and associated climate change. In general, under elevated CO_2, plants will have altered growing cycles (phenology), increased photosynthesis, altered phenology, reproductive output, potential fitness, decreased nutritional content (Jablonski *et al.*, 2002).

In this context, the current programme "Physiological and molecular analyses of flowering responses in amaranthus (*Amaranthus* spp.) and cowpea (*Vigna* spp.) under elevated CO_2 environment" was designed to study the potential implications of elevated CO_2 condition on the flowering phenology of Amaranthus and cowpea. The knowledge generated through this investigation will help to improve incorporation of such mechanisms into crop breeding programs which will be beneficial for maximizing crop productivity in the face of future increases in atmospheric CO_2 concentration.

The experiment was laid out in CRD with two treatments and four replications for each treatment. Treatment one was chamber A with elevated CO_2 facility and treatment second was open control with ambient CO_2 condition.

The pot culture experiments were conducted and plants were exposed to the elevated CO_2 condition. The control plants were maintained in the open field with ambient CO_2 concentration. The control sets were kept under open field condition. Growth analysis and analyses of physiological and biochemical parameters were done at the time of harvest. The varieties which showed modification in flowering time to a greater extent under exposure to elevated CO_2 were chosen for molecular analyses. The technology used for subjecting the plants to elevated CO_2 environments is the Open Top Chambers (OTC) system. In both set of experiment entire crop period was completed in OTCs.

In the case of amaranthus, CO-1 variety recorded highest values of growth, physiological and biochemical parameters and was performing better when exposed to elevated CO_2 condition. CO-1 recorded highest values for number of leaves (42.44), specific leaf area (219.13), root weight (1.45g), shoot weight (3.17g), total dry matter (4.93g), stomatal frequency (595.78cm-2), pigment composition (0.56mg g-1), total soluble protein (23.02mg g-1), starch (3.61mg g-1), reducing sugar (18.46mg g-1), GA (0.198µg g-1) and nitrate reductase (0.65µg g-1).

Flowering time was modified in CO-1 in terms of days to first flowering and days to 50% flowering (2 days); but Arun did not show any significant response in flowering time and hence CO-1 was selected for molecular analyses. Regarding quality parameters, Arun showed a reduction in ascorbic acid and vitamin A content under CO_2 enrichment with an increase in oxalate content. In the case of CO-1, though ascorbic acid and vitamin A contents were less under open condition, upon exposure to higher concentrations of CO_2, there was tremendous increase in these quality parameters along with oxalate content.

Both the varieties of cowpea recorded significant variations in growth, physiological and biochemical parameters when exposed to higher concentrations of CO_2. But Anaswara recorded higher values for number of leaves (74.25), specific leaf area (454.53), root weight (15.04g), shoot weight (63.15g), total dry matter (78.76g), starch content (9.16mg g-1), reducing sugar (15.36mg g-1,), GA (0.615µg g-1) and nitrate reductase (0.54µg g-1). Vellayani Jyothika recorded higher values for stomatal distribution (2893.8 cm-1) and physiological and biochemical parameters like pigment composition (0.52mg g-1) and total soluble protein (1.44 mg g-1).Flowering time was modified to a greater extent in Anaswara - 2 days to first flowering and days to 50% flowering and so Anaswara was selected for molecular analyses.

CO_2 enrichment was found to influence the quality parameters in amaranthus. CO-1 showed a tremendous increase in ascorbic acid and Vitamin A, but there was an increase in oxalate content also.

During the period of study, environmental factors like temperature, humidity and sun shine hours were measured. There was an increase of 7°C on an average during the period and also an increase in leaf temperature. For gene expression studies FLOWERING LOCUS T (FT) was selected. The DNA of FLOWERING LOCUS T was amplified from Anaswara and CO-1. Differential expression was observed in both the crops under elevated CO_2 condition.

In the present study, both cowpea and amaranthus were found to be responding to elevated CO_2 in terms of flowering time. This can be correlated with the higher photosynthate accumulation with a net positive effect on growth parameters. The increased gibberellic acid level displayed by both the crops upon CO_2 enrichment can also play a role in signaling the crosstalk between reproduction and other developmental processes.

The results of the study highlights the influence of environmental factors like CO_2 concentration and temperature on plant development and flowering time. Infield conditions, factors like temperature can be influenced by crop management

practices such as sowing dates. Even in crop breeding programmes, which are targeted towards earlier or late flowering to increase cultivar adaptability to environment faced with abiotic stress factors , efforts should be undertaken with causion taking into consideration all these different factors and their impacts.

Understanding the mechanisms involved in the regulatory network modulating floral initiation in response to elevated CO_2 and elevated temperature will facilitate understanding and identifying options to develop plants better adapted to changing climate.

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PHYSIOLOGICAL AND MOLECULAR ANALYSES OF FLOWERING RESPONSES IN AMARANTHUS (*Amaranthus* spp.) AND COWPEA (*Vigna* spp.) UNDER ELEVATED CO_2 ENVIRONMENT.

by

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ABSTRACT

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ABSTRACT

The study entitled "Physiological and molecular analyses of flowering responses in amaranthus (*Amaranthus* spp.) and cowpea (*Vigna* spp.) under elevated CO_2 environment" was undertaken with the objective to study the physiological ,molecular and biochemical basis of elevated CO_2 mediated modifications in the flowering responses of amaranthus and cowpea. The experiments were conducted at the Department of Plant Physiology, College of Agriculture, and Vellayani during 2015-2017. Two pot culture experiments were conducted with two varieties of amaranthus – Arun and CO-1 and two varieties of cowpea- Anaswara and Vellayani Jyothika. The technology used for CO_2 enrichment was Open Top Chamber system (OTC). CO_2 was released from cylinders to OTC bringing the CO_2 level to 600ppm. Amaranthus and cowpea plants were raised and maintained in pots as per POP (KAU) recommendations under elevated CO_2. The control sets were kept under open field condition. Growth analysis and analyses of physiological and biochemical parameters were done at the time of harvest. The varieties which showed modification in flowering time to a greater extent under exposure to elevated CO_2 were chosen for molecular analyses.

In the case of amaranthus, CO-1 variety recorded highest values of growth, physiological and biochemical parameters and was performing better when exposed to elevated CO_2 condition. CO-1 recorded highest values for number of leaves (42.44), specific leaf area (219.13), root weight (1.45g), shoot weight (3.17g), total dry matter (4.93g), stomatal frequency (595.78cm^{-2}), pigment composition (0.56mg g^{-1}), total soluble protein (23.02mg g^{-1}), starch (3.61mg g^{-1}), reducing sugar (18.46mg g^{-1}), GA (0.198µg g^{-1}) and nitrate reductase (0.65µg g-1).

Flowering time was modified in CO-1 in terms of days to first flowering and days to 50% flowering (2 days); but Arun did not show any significant response in flowering time and hence CO-1 was selected for molecular analyses. Regarding

quality parameters, Arun showed a reduction in ascorbic acid and vitamin A content under CO_2 enrichment with an increase in oxalate content. In the case of CO-1, though ascorbic acid and vitamin A contents were less under open condition, upon exposure to higher concentrations of CO_2, there was tremendous increase in these quality parameters along with oxalate content.

Both the varieties of cowpea recorded significant variations in growth, physiological and biochemical parameters when exposed to higher concentrations of CO_2. But Anaswara recorded higher values for number of leaves (74.25), specific leaf area (454.53), root weight (15.04g), shoot weight (63.15g), total dry matter (78.76g), starch content (9.16mg g^{-1}), reducing sugar (15.36mg g^{-1}), GA (0.615µg g^{-1}) and nitrate reductase (0.54µg g^{-1}). Vellayani Jyothika recorded higher values for stomatal distribution (2893.8 cm^{-1}) and physiological and biochemical parameters like pigment composition (0.52mg g^{-1}) and total soluble protein (1.44 mg g^{-1}). Flowering time was modified to a greater extent in Anaswara - 2 days to first flowering and days to 50% flowering and so Anaswara was selected for molecular analyses.

CO_2 enrichment was found to influence the quality parameters in amaranthus. CO-1 showed a tremendous increase in ascorbic acid and Vitamin A, but there was an increase in oxalate content also. During the period of study, environmental factors like temperature, humidity and sun shine hours were measured. There was an increase of 7°C on an average during the period and also an increase in leaf temperature.

For gene expression studies FLOWERING LOCUS T (FT) was selected. The DNA of FLOWERING LOCUS T was amplified from Anaswara and CO-1. Differential expression was observed in both the crops under elevated CO_2 condition.

In the present study, both cowpea and amaranthus were found to be responding to elevated CO_2 in terms of flowering time. This can be correlated with the higher photosynthate accumulation with a net positive effect on growth

parameters. The increased gibberellic acid level displayed by both the crops upon CO_2 enrichment can also play a role in signaling the crosstalk between reproduction and other developmental processes. Understanding the mechanisms involved in the regulatory network modulating floral initiation in response to elevated CO_2 and elevated temperature will facilitate understanding and identifying options to develop plants better adapted to changing climate.

APPENDICES

Table 36. Weather data during the crop period of cowpea in open condition (August 2016 to October 2016)

Period	Air Temperature (^{0}C)	Soil temperature (^{0}C)	Relative Humidity (%)	Sunshine Duration (min.)
August	39.72	38.26	56.51	902.34
September	34.66	34.68	69.86	2640.07
October	36.99	35.38	63.36	2031.72
Average	37.12	36.10	63.24	1858.02

Table 37. Weather data during cropping period of cowpea in OTC (August 2016 to October 2016).

Period	Air temperature (^{0}C)	Soil temperature (^{0}C)	Relative Humidity (%)	Sunshine Duration (min.)
August	42.46	45.91	81.37	898.72
September	43.22	40.53	78.46	2132.46
October	40.64	37.42	76.51	2646.32
Average	42.10	41.28	78.78	1892.51

Table 38. Weather data during the crop period in open condition (January 2017 to March 2017)

Period	Air Temperature (^{0}C)	Soil temperature (^{0}C)	Relative Humidity (%)	Sunshine Duration (min.)
January	32.51	31.26	80.36	982.34
February	32.62	32.47	85.31	1772.26
March	33.36	30.72	83.87	2031.72
Average	32.83	31.48	83.18	1595.44

Table 39. Weather data during the crop period in OTC open top chamber (January 2017 to March 2017)

Period	Air Temperature (^{0}C)	Soil temperature (^{0}C)	Relative Humidity (%)	Sunshine Duration (min.)
January	39.43	37.46	87.34	672.89
February	40.62	38.34	82.76	1689.23
March	41.86	42.29	79.43	1938.47
Average	40.63	39.36	83.17	1433.53

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Table 40. Weather data during the crop period in open condition (December 2017 to February 2018)

Period	Air Temperature (^{0}C)	Soil temperature (^{0}C)	Relative Humidity (%)	Sunshine Duration (min.)
December	33.72	29.84	90.81	728.36
January	32.43	30.01	92.62	1628.72
February	34.51	29.42	93.72	2028.36
Average	33.55	29.75	92.37	1461.81

Table 41. Weather data during the crop period in OTC open top chamber (December 2017 to February 2018)

Period	Air Temperature (^{0}C)	Soil temperature (^{0}C)	Relative Humidity (%)	Sunshine Duration (min.)
December	41.13	37.35	93.32	893.49
January	40.82	34.26	94.47	1432.81
February	42.56	36.17	94.82	1963.42
Average	41.50	35.92	94.20	1429.90