PHYSIOLOGICAL AND MOLECULAR ANALYSES OF GROWTH RESPONSES IN BLACK PEPPER (*Piper nigrum* L.) UNDER ELEVATEDCARBON DIOXIDE ENVIRONMENTS.

by

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(2013-11-161)

THESIS

Submitted in the partial fulfilment of the requirements for the degree of

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KERALA, INDIA

2015

DECLARATION

I, hereby declare that this thesis entitled **"Physiological and molecular analyses of growth responses in black pepper (*Piper nigrum* L.) under elevated carbon dioxide environments.."** 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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Minu M

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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 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.

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 temperature 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 the burning of fossil fuels, clearing of forest for agriculture or development and agricultural practices is leading to thickening of the insulating blanket and overheating of earth.

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.

The global concentration of carbon dioxide in the atmosphere has reached 400 parts per million (ppm) for the first time in recorded history (NOAA, 2014). Projections suggest that atmospheric CO_2 will reach 700 ppm or more, whereas global temperature will increase by 1.8- 4^0C 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 per 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 a 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 spatial 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 CO2 enrichment studies with respect to specific crops.

Earlier researchers on plant response to high CO2 have been conducted under laboratory greenhouse or controlled field condition. Now a days, number of programmes are being carried out all over the world to study the impact of rising CO2 on agricultural system. Technologies such as FACE (Free Air CO2 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. CO2 enrichment studies in Kerala are being carried out in CPCRI Kasargode and in College of Agriculture Vellayani.

India is widely known as a spice country. Various kinds of spices are grown and developed in the country, especially in southern India. Incredibly popular black pepper (*Piper nigrum* L.) often referred as 'King of spice' has remained the most precious and valuable form of spices in the world since ancient times. It is the most important spice crop of India contributing to lion's share of foreign earnings from the export of spices and hence included under major spices. It is also called as 'Black gold' due its durability and value. The name pepper comes from the Sanskrit word "*Pippali*" meaning berry. Black pepper (*Piper*

nigrum L.) is a flowering vine in the family Piperaceae. It is a perennial, climbing vine indigenous to the Malabar Coast of India. It is a plant of humid tropics, requiring 2000-3000 mm of rainfall, tropical temperature and high relative humidity with little variation in day length throughout the year, but highly sensitive to changing climate.

The hotly pungent spice made from its berries has been used since time immemorial for its flavour and as a medicine. Peppercorns contain an impressive list of plant derived chemical compounds that are known to have disease preventing and health promoting properties. It is being used as traditional medicines in treating flatulence and indigestion. Peppers have been in use since centuries for its anti-inflammatory, carminative, anti-flatulent properties. Peppercorns are composed of health benefiting essential oils such as piperine, an amine alkaloid, which gives strong spicy pungent character to the pepper. Peppercorns are a good source of many anti-oxidant vitamins such as vitamin-C and vitamin-A. They are also rich in flavonoid polyphenolic anti-oxidants like carotenes, cryptoxanthin, zeaxanthin and lycopene. These compounds help the body to remove harmful free radicals and help protect from cancers and diseases.

National Horticultural Board's advance estimate indicated that at present (2013-14) pepper has an area of 1, 17,770 ha with a production of 45, 000 tonnes in India. The distribution pattern of black pepper across various states of India showed the dominance of Kerala accounting for 60 per cent of production followed by Karnataka and Tamil Nadu. As per the National Horticultural Board after 2005, there has been a declining trend in the area and production of black pepper all over India. Similar trend in area, production and yield has also been noticed in Kerala. These changes could be due to lack of developmental efforts, prevailing market price, climate change, statistical estimation, etc.

Kerala's economy, to a great extent, is dependent on production of plantation crops, which is highly influenced by weather factors. Black pepper which is one the most important component in the homesteads of Kerala is highly sensitive to changing climatic conditions.

Considering the role of CO_2 in bringing about the projected changes in climate, the present investigation was undertaken to study the modifications in the physiological, biochemical and growth performance of black pepper under elevated CO_2 conditions. The result of the study will help to understand the performance of pepper under such a predicted situation and also to design improved production technologies for a changing climatic scenario.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Current and predicted patterns of global climate change are a major concern in many areas of socio - economic activities, such as agriculture, forestry, etc. and are a major threat for biodiversity and ecosystem function. The effects have become particularly obvious over the last 30 years in the natural environment and it will affect all levels of life, from the individual, population, species, community and ecosystem to the eco-region level (Lepetz *et al.*, 2009).

Climate change is a result from emission of greenhouse gases (e.g. H_2O, CO_2, CH_4 and N_2O) in the past century that will cause atmospheric warming (IPCC, 2007). Of these greenhouse gases, carbon dioxide is the most important one as its life time in the atmosphere is almost infinite and also due to its relative abundance. Atmospheric concentrations of carbon dioxide have been steadily rising with an average annual increase rate of about 2 ppm (Kiehl, 2011) and will be continued to rise to 500 - 1000 ppm by the year 2100 (Kiehl, 2011; IPCC, 2007) from current atmospheric average of approximately 385 ppm (Keeling *et al.*, 2009).

Since CO_2 is a major plant nutrient, the elevated levels of CO_2 may directly or indirectly affects plant production and development. Plant responses to elevated atmospheric CO_2 vary with species and with environmental conditions. It has been observed that exposure of plants to elevated CO_2 resulted in a higher biomass production, an altered morphology, increased water use efficiency, increased photosynthetic activity and an enhanced carbohydrate content (Kimball, 1983; Smith *et al.*, 1987; Lawlor and Mitchell, 1991; Woodward *et al.*, 1991).

Elevated atmospheric CO_2 in short term exposures has been shown to increase photosynthesis (Noormets *et al.*, 2001), decrease respiration (Volin and Reich, 1996) and stimulate aboveground (Norby *et al.*, 1999) and belowground (King *et al.*, 2001) growth. In long term exposures, the effects of CO_2 are often decreased (Rey and Jarvis, 1998; Tissue *et al.*, 1999) as a result of reduced sink strength (Gesch *et al.*, 1998) or nutrient limited habitats (Oren *et al.*, 2001)

Many controlled environment experiments studied the effects and interactions of these climate change factors on plants (Pickering *et al.*, 1994) which showed alterations in physiology, growth and development in many crops (Allen and Boote, 2000; Reddy *et al.*, 2004). Plants grown at various ambient concentrations of CO_2 show numerous differences, including variation in photosynthesis, respiration, allocation, biochemical composition, morphology, flowering and fruit set (Gates *et al.*, 1983; Pearcy and Bjorkman, 1983; Cure and Acock, 1986; Amthor, 1991).

The most natural way of studying the ultimate outcome of elevated atmospheric CO_2 in plant species is Free Air Carbon dioxide Enrichment (FACE) system. This system allows studying the effects of elevated atmospheric CO_2 on plants grown under natural condition (Ainsworth and Lon g, 2005). In this system CO_2 enriched air is released into the ambient environment without causing appreciable changes in other environmental variables.

In India, impact of elevated CO2 on rice crop grown inside the FACE ring was studied (Uprety, 2003). The most sophisticated FACE system was designed by Brookhaven National Laboratory's FACE Group, which employs computer regulation of CO2 concentration in the FACE rings (Miglietta, 1997). FACE systems had serious constraint in terms of the high installation and operational costs involved in setting up these facilities in agricultural fields.

The least expensive and the most generally applicable technology for estimating the effect of elevated atmospheric CO2 concentration in plants is the Open Top Chamber (OTC) facility (Aronson and McNulty, 2009). Open Top Chamber consist of metal constructions with transparent vertical side walls and a frustum on the top. An opening in the middle of the frustum allows air exchange to reduce temperature and humidity built up in the chamber. Sensors are placed inside the chambers to measure different microclimatic parameters like air temperature, humidity, light intensity etc. on a real time basis. The actual concentration of CO2 within the chamber is measured by CO2 analyzer and controlled by computer supported regulation of inlet valves.

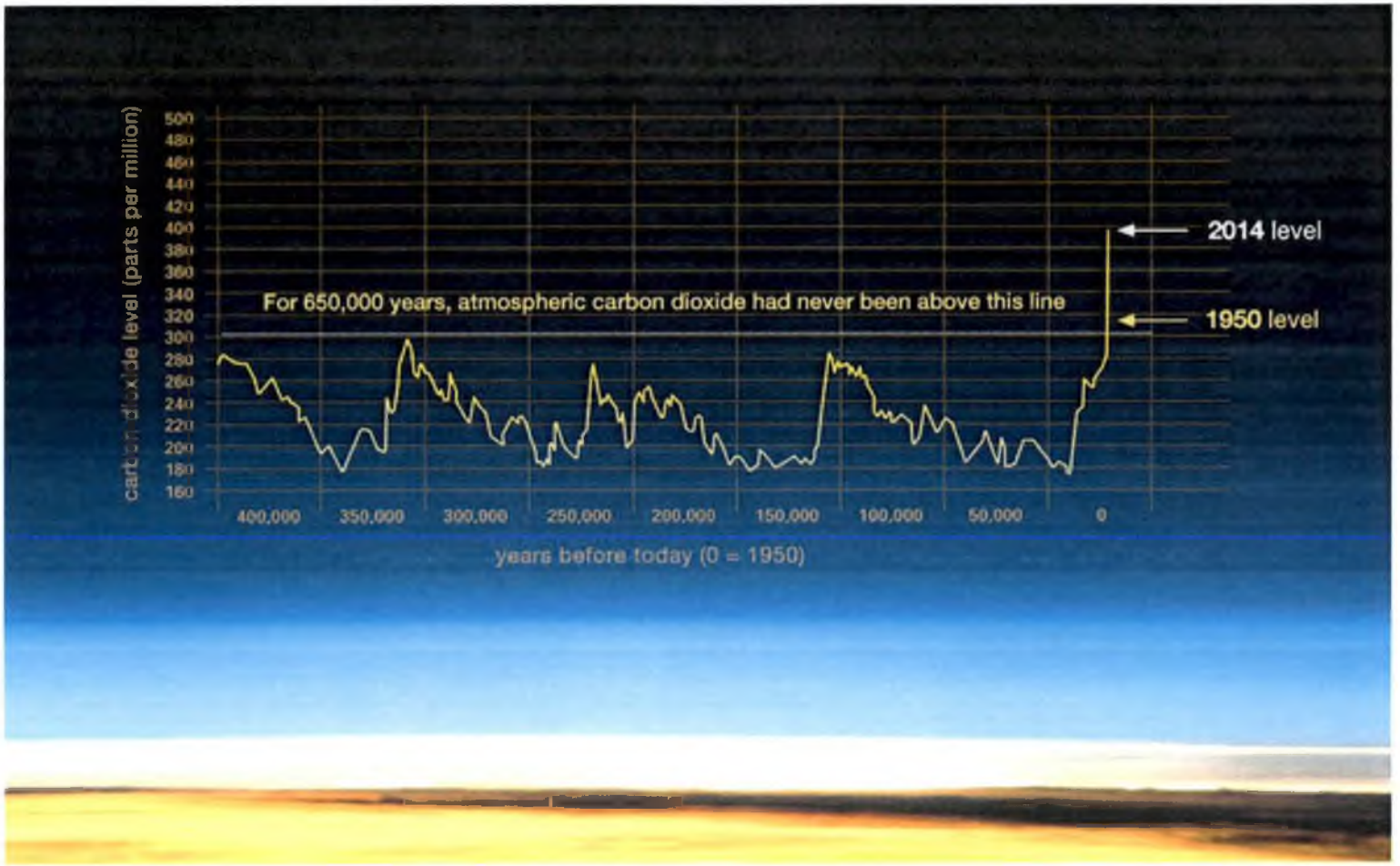


Fig. 1. The relentless rise of carbon dioxide Source: NOAA, 2014

Variation in climate has a great influence in agriculture production scenario of Kerala. There was an increase in maximum temperature over Kerala by 0.64° C during the period of 49 years, commencing from 1956 to 2004 while increase in minimum temperature was 0.23° C (Rao *et al* ., 2008). Therefore, it is high time to project climate change at the site/local/region level and its impact on crops as agriculture is the main sector which suffers to a great extent due to climate change and Indian economy is agrarian based. Such studies in the humid tropics of India are of more relevance where plantation crops are predominant. Unlike in seasonal crops, the impact of weather aberrations will be having long standing ill effects as the crops are perennial in nature which will be reflected upon the state's economy.

Black pepper has played a pivotal role in India's international trade and it is said that the Europeans came to India primarily for this very spice. A wide variety of black pepper is traded at an international level, with India as one of the top five exporters of black pepper, along with Vietnam, Indonesia, Brazil and Malaysia. In India, its production is largely concentrated in South India and other tropical regions. Pepper cultivation is on a never before decline in Kerala, the land of its origin. Production has dropped throughout Kerala because of the terrible infestations in ageing pepper gardens, changes in weather patterns, unfriendly market conditions etc.

Black pepper has been addressed as a master spice and its diversified uses demands more production. Production bases are shrinking. Spatial and temporal variation in weather is a great concern in augmenting the productivity of this rainfed crop. Climate change is evident and it is a great challenge for scientific community to find solutions to mitigate the ill-effect. The work has already been initiated on drought tolerance studies and breeding programme to find better ideotypes and crop management aspects such as water conservation, irrigation, mulching, cropping system etc. to modulate the weather effects. In this context the current programme was formulated to study the physiological and molecular basis of growth responses in black pepper under elevated carbon dioxide environments.

GROWTH PARAMETERS

Plant growth and development depend on a number of endogenous as well as exogenous factors like temperature, relative humidity, light intensity and its duration. Since CO_2 is one of the substrates for the process of photosynthesis, this also influences the growth rates and development of plant species. Most terrestrial plants increase the rate of photosynthesis under elevated CO_2 (Geissler *et al.*, 2009) but growth responses vary from 0 to 50% gain per season depending on the plant age, duration of observations and growth conditions (Beismann *et al.*, 2002).

Under elevated CO_2 and soil water levels, plants modify their leaf morphology and anatomy which enables them to thrive well under environmental stress (McLellan, 2000). The increase in atmospheric CO_2 increases photosynthesis and growth of C_3 plants at least at temperatures above 18°C (Kimball *et al.*, 1993). In general, many plants particularly C_3 plants and trees, will be able to capitalize on elevated CO_2 and translate it into higher photosynthetic rates and better growth and yield (Leakey *et al.*, 2009). Most of the C_3 plants showed a significant positive response to photosynthetic acclimation, while C_4 plants (Sorghum and Panicum) exhibited negative response where as Ananas, Agave and Kalanchoe (CAM plants) showed positive responses to increased CO_2 concentration during growth (Reddy *et al.*, 2010)

Number of Leaves

In most plants, leaves are the major site of food production for the plant. Structures within a leaf convert the energy in sunlight into chemical energy that the plant can use as food. Number of leaves in a plant indicates its physiological age. An increase in biomass due to increase in the number of branches or leaves has been reported in sweet potato and Japanese honey-suckle under CO_2 enrichment (Bhattacharya, 1985; Sasek and Strain, 1991).

An increase in the number of leaves has been also reported in Japanese honey-suckle (Sasek and Strain, 1991), in sweet potato (Bhattacharya, 1985) and

in berseem (Pal, 2004) under elevated CO_2. Elevated CO_2 (800 μmol mol^{-1}) decreases the number of leaves by 23% and 14% in soyabean compared with ambient CO_2 (380 μmol mol^{-1}) at 29 and 44 days after planting (Madhu and Hatfield, 2015).

Leaf Area

A larger leaf area allows for more plant twigs to absorb light energy enabling better photosynthetic performance. Leaf size is determined by cell production and expansion, which are controlled in a coordinated manner during leaf organogenesis (Tsukaya, 2006). Increase in leaf area under CO_2 enrichment has been reported in soybean (Allen, 1990; Cure, 1988) and berseem (Pal, 2004).

Leaf growth is often enhanced following exposure to elevated CO_2 (Volkenburgh and Taylor, 1996; Taylor *et al.*, 2003; Riikonen *et al.*, 2004). Many research groups have highlighted the role of elevated CO_2 concentration on cell division and expansion. Elevated CO_2 may influence both cell production and expansion in poplar tree species (Ferris *et al.*, 2001; Taylor *et al.*, 2003; Tricker *et al.*, 2004).

In Populus spp, under elevated CO_2 the enhanced cell expansion was associated with increased cell-wall extensibility and increased activity of the cell wall loosening enzyme, xyloglucan endotransglycosylase (Ferris, *et al.*, 2001). *Phaseolus vulgaris* when grown under elevated CO_2 concentration showed an increase in leaf area, leaf area index (LAI), leaf area duration and leaf thickness and a decrease in specific leaf area (Bray and Reid, 2002). Leaf area and ratio of leaf fresh weight/ (stem + root) fresh weight, were reported to be higher in *Persea americana grown* under elevated CO_2 (*de la* Vina *et al.*, 1999).

Specific Leaf Area

Specific leaf area (SLA) is the one-sided area of a fresh leaf, divided by its oven-dry mass. Specific leaf area is frequently used in growth analysis because it is often positively related to potential relative growth rate (RGR) across species. It is one of the most widely accepted key leaf characteristics used during the study

of leaf traits (Kraft *et al.*, 2008). Elevated CO_2 enhances leaf size (Heath and Kerstiens, 1997; Kerstiens *et al.*, 1995) and decreases specific leaf area in a number of plant species (Norby and Neill, 1991).

Short term CO_2 exposure does not reflect the changes in leaf anatomy, leaf chemistry, specific leaf area and stomatal conductance in forest tree species (Wullschleger *et al.*, 1997). In a comparative study on five species of field annual plants like *Abutilon theophrasti*(C_3), *Amaranthus retroflexus*(C_4), *Ambrosia artemisiifolia* (C_3), *Chenopodium album*(C_3) and *Seteria faberii* (C_4) a consistent decrease in specific leaf was reported under elevated CO_2 concentration of 700μL/L (Garbut *et al.*, 1990).

Net Assimilation Rate

Net assimilation rate is a useful measure of the photosynthetic efficiency of plants and defined as the rate of increase of dry weight per unit of leaf area. Net photosynthesis per unit leaf area is raised at an increased CO_2 concentration due to a decrease in photorespiration and an increase in substrate supply (Pearcy and Bjorkman, 1983). Zheng *et al.* (2010) reported an increase in net assimilation rate (20-45%) in Artemisia species and *Hedysarum laeve* when grown under an elevated CO_2 level of 800mol mol^{-1}.

Relative Growth Rate

Relative growth rate (RGR) is the growth rate relative to the size of the plant population. It is also called the exponential growth rate, or the continuous growth rate. It is a prominent indicator of plant strategy with respect to productivity as related to environmental stress and disturbance regimes. Relative growth rate is the increase in size relative to the size of the plant present at the start of a given time interval.

In many species the CO_2 induced increase in the relative growth rate during the vegetative growth appeared to be transient; upon prolonged exposure the relative growth rate of plants growing at elevated CO_2 returned to or was

lower than plants growing at ambient CO_2 (Denhertog *et al.*, 1993; Poorter, 1993; Stulen *et al.*, 1994).

Carbon content in leaves of CO_2 enriched plants of Big Sagebrush (*Artemisia tridentate)* was 35 mmol g^{-1} as compared to 36.5 mmol g^{-1} in control plants. If a similar change occurred in stem and roots, this alone could increase the relative growth rate by 4 % (Johnson and Lincoln, 1990). A 6-12% increase in relative growth rate was observed in Artemisia species and *Hedysarum laeve* when grown under an elevated CO_2 level of 800 mol mol^{-1} (Zheng *et al.*, 2010). Carbon dioxide enrichment affects plant structure (Pritchard *et al.*, 1999), transiently enhances the relative growth rate of plants (Lambers *et al.*, 1998) and increases biomass and yield (Kimball, 1983).

Root Weight

Growth of crops in CO_2 enriched atmospheres typically results in significant changes in root growth and development. Increased root carbohydrates stimulate root growth either directly (functioning as substrates) or indirectly (functioning as signal molecules) by enhancing cell division or cell expansion, or both (Pritchard and Rogers, 2000). A shift in biomass allocation from leaves to roots can occur under CO_2 enrichment (Stulen and Den Hertog, 1993). Exposure of crop species to elevated atmospheric CO_2 often results in large shifts in root structure and function in *Senecio vulgaris* (Berntson and Woodward, 1992).

Increasing the atmospheric CO_2 stimulates root biomass more than above ground biomass or leaf area production in many annual plant species (Bernacchi *et al.*, 2000). High carbon gain under CO_2 enrichment increase root length, diameter and number (Lee-Ho *et al.*, 2007) and also stimulates lateral root production in winter wheat (Pritchard and Rogers, 2000). On the basis of FACE experiments Kimball *et al.*, (2002) had reported that in some agricultural crops (wheat, rice, and rye grass) elevated CO_2 stimulated growth of roots more than that of shoots.

Laboratory studies on cotton (*Gossypium hirsutum* L.) plants in a free air carbon dioxide enrichment (FACE) project revealed dry weights, lengths, and volumes of taproots, lateral roots, and fine roots were higher for CO_2 enriched cotton plants with a short-term exposure to increased CO_2 for 6 week (Prior, 1992; Rogers *et al.*, 1993).

Shoot Weight

Shoots consist of stems including their appendages, the leaves and lateral buds, flowering stems and flower buds. Shoot weight is a measure of the productive investment of the plant dealing with the relative expenditure on potentially photosynthesizing organs. Increasing atmospheric CO_2 significantly increased the final plant biomass, aboveground biomass, leaf area and belowground biomass in *Larrea tridentate* (Obrist and Arnone, 2003).

Root Shoot Ratio

Root/shoot ratio is the simple calculation of the ratio of root dry mass to shoot (or stem) dry mass and should serve as a measure of the preferential allocation of C to roots or shoots. It is one measure to assess the overall health of plants. 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).

Results showed root shoot ratio was not significantly affected by higher CO2 concentration in *Larrea tridentate*, a desert herb (Obrist and Arnone, 2003), tall grasses like Indian grass and Switch grass (Mo *et al.*, 1992). The range of response in R/S among crop plants to CO2 doubling ranged from a 8.5% decrease to a 6.4% increase, except in sweet potato (*Ipomoea batatas*) in which a 34.9% increase was observed (Cure, 1985). For *Caragana korshinskii* a desert herb, elevated CO2 significantly increased the ratio of below ground to above ground biomass (Zheng *et al.*, 2010)

Dry matter Production

Dry matter production is the expression of productivity in terms of the dry weight of material produced during a specified time period. Elevated CO_2 stimulates photosynthesis in various intensities during different phenological phases in spring wheat (Mitchell *et al.*, 1999) and its direct consequence is increased dry matter production (Lawlor and Mitchell, 2000; Ziska *et al.*, 2004).

An increase in total dry matter production was reported in soybean (Pan, 1996), dry bean (Prasad, 2002), peanut (Clifford *et al.*, 2000) and cowpea (Ellis, 1995) under CO2 enrichment. A higher biomass accumulation, leaf area and better growth was reported in *Hevea brasilensensis* when exposed to elevated concentration of CO2 (700 +/-25 ppm) compared to ambient air grown plants, for 60 days (Devakumar *et al.*, 1998). Dry matter production of plants was increased significantly for elevated CO2 in soyabean plants (Madhu and Hatfield, 2015).

PHYSIOLOGICAL PARAMETERS

Relative Water Content

Relative water content is the most appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit. Leaf water status is intimately related to several leaf physiological variables, such as leaf turgor, growth, stomatal conductance, transpiration, photosynthesis and respiration (Kramer and Boyer, 1995).

Elevated CO2 can lead to an increase in plant water potential and a delay in the onset of water stress, thus improving growth. It has been reported that elevated CO2 increased biomass production in peach (*Prunus persica*) seedlings by 31% (Centritto *et al.*, 2002) and resulted in higher integrated water use efficiency under water stressed conditions.

Increased water potential and relative water content was reported in *Brassica juncea* plants when exposed to elevated CO2 concentration of about 600±20μmol mol^{-1} (Rabha and Uprety, 1998). Soyabean grown under elevated

CO_2 and subjected to water stress during early reproductive growth avoided the onset of severe water stress by exhibiting decreased water use (Rogers *et al.*, 1984) and increased water use efficiency (Huber *et al.*, 1984) thus enabling them to maintain higher leaf water potentials. No significant difference was reported in relative water content when alfalfa plants were grown under CO_2 enrichment (700 μmol mol^{-1}) and under different levels of temperature (Aranjuelo *et al.*, 2005).

Pigment Composition

The quantity of chlorophyll per unit area is an indication of photosynthetic capacity and productivity of a plant. The amount of chlorophyll in the leaf tissues can be influenced by nutrient availability and environmental factors (Otitoju and Onwurah, 2010). Plants grown in elevated CO_2 environments often exhibit some degree of physiological adaptation. Several studies demonstrated that atmospheric CO_2 enrichment can increase, decrease or can have no effect on leaf chlorophyll concentrations.

In an open-top chamber experiment with alfalfa, plants grown at an atmospheric CO_2 concentration of 600 ppm displayed greater leaf chlorophyll concentrations than those observed in plants grown at 340 ppm (Sgherri *et al.*, 1998). Exposure of an orchid to a super elevated CO_2 concentration of 10,000 ppm resulted in a 64% increase in its leaf chlorophyll concentration relative to that measured in leaves of plants grown at ambient CO_2 (Gouk *et al.*, 1999).

Increases in the air CO_2 content have sometimes been demonstrated to have no significant effect on leaf chlorophyll concentration. Sicher and Bunce (1999) reported that twice-ambient CO_2 concentrations elicited no change in leaf chlorophyll contents of potato plants during a three year study. Even with higher CO_2 enrichment levels (870 ppm above ambient concentrations) Monje and Bugbee (1998) failed to detect any CO_2 induced changes in leaf chlorophyll content of wheat.

A doubling of the atmospheric CO_2 concentration had no significant impact on leaf chlorophyll concentrations within sugar maple (Li *et al.*, 2000)

and oak species (Carter *et al.*, 2000; Stylinski *et al.*, 2000). A decrease in chlorophyll content was reported in pineapple by Zhu *et al.* (1997) when CO_2 enrichment (700 ppm) was imposed for 30 days.

Stomatal Frequency

Stomata are the portals for gas exchange between the leaf mesophyll cells and the environment. They occupy between 0.5% and 5% of the leaf epidermis and are most abundant on the bottom or abaxial surface. They are the integrators of all environmental factors that affect the plant growth (Morison, 1998). Stomatal density is determined by stomatal initiation during ontogenesis and by epidermal cell expansion (Radoglou and Jarvis, 1990). Atmospheric CO_2 enrichment of 700 $\mu mol\ mol^{-1}$ decreases the stomatal densities in the leaves of *Arabidopsis thaliana* (Woodward *et al.*, 2002).

In field studies with broadleaved trees, the CO_2 response had been variable, showing no alteration (Vanhatalo *et al.*, 2001; Herrick *et al.*, 2004; Man kovska *et al.*, 2005; Mc Celrone *et al.*, 2005; Riikonen *et al.*, 2008) or a decrease (Rey and Jarvis, 1997; Bettarini *et al.*, 1998; Paoletti *et al.*, 1998; Ferris *et al.*, 2002) in stomatal densities. In silver birch (*Betula pendula*) and *Fraxinus ornus*, stomatal density decreased under elevated CO_2 as a consequence of an increase in leaf expansion, as stomatal index was not altered (Rey and Jarvis, 1997; Bettarini *et al.*, 1998).

In Populus clones, the reduction in stomatal density was accompanied by decreased stomatal index in young leaves in the upper canopy, suggesting a change in stomatal initiation (Ferris *et al.*, 2002). Carbon dioxide treatment on wild radish showed no significant variation in stomatal characteristics compared to ambient CO_2 treatment (Case *et al.*, 1998). In *Betula pubescens*, stomatal size was increased, whereas stomatal density was not affected by elevated CO_2 after 4 years of open-top chamber exposure (Vanhatalo *et al.*, 2001).

Transpiration Rate

Transpiration is the loss of water in the form of water vapour from the aerial parts of the plant. It is not simply a hazard of plant life. It is the engine that pulls water up from the roots to supply photosynthesis, bring minerals from the roots for biosynthesis within the leaf, cool the leaf and also helps to keep the plant cells turgid. The rate of transpiration is affected by a number of internal (plant factor) and external factors (light, temperature, humidity, wind, atmospheric pressure and water supply).

Increase in the atmospheric CO2 levels enhances water use efficiency because of reduced stomatal conductance that stimulates light saturated photosynthesis particularly in C3 plants (Gruda, 2005). A common response of plants to elevated CO2 is decrease in stomatal conductance (Bazzaz, 1990). This can result in reduced transpiration per unit leaf area and higher soil water content compared with non CO2 enriched plant communities (Field *et al.*, 1995).

Growth in elevated CO2 causes a decrease in stomatal conductance in some tree species (Medlyn *et al.*, 2001). A reduction in transpiration rate was also reported in winter wheat and barley due to partial closure of stomata and decrease in stomatal conductance when grown under elevated CO2 (Morison and Gifford, 1983; Bunce, 2000). Elevated levels of CO_2 in *Podophyllum hexandrum* showed decreased levels of stomatal conductivity and specific leaf area (Chaturvedi *et al.*, 2009).

BIOCHEMICAL PARAMETERS

Total Soluble Protein

Proteins, amino acids make up to 10% of the total dry mass of plant roots and shoots (Rejsek *et al.*, 2010). Growth at elevated CO2 can result in a large decline in Rubisco protein up to 60% (Sage *et al.*, 1989; Besford *et al.*, 1990; Rowland-Bamford *et al.*, 1991).As the levels of CO_2 increased from 400 to 1200 $\mu mol\ mol^{-1}$ a decrease in the soluble protein was reported in *Eleais guineensis* (Oil Palm) seedlings (Ibrahim and Jaafar, 2012).

Soybeans (*Glycine max*) grown under 800 µl CO_2 per liter showed an increase of 31% soluble protein compared to the control plants at 330 µl CO_2 per liter (Joseph *et al.*, 2009). The soluble protein recorded was found to be higher in leaves of *Stylosanthes hamata* grown under 600ppm CO_2 (Baig *et al.*, 2012).Under elevated CO_2 concentration of 700 µmol mol^{-1} a decrease in total soluble protein of barley penultimate leaves and wheat flag leaves were reported with increase in the leaf age (Richard and James, 1997).

Starch and Reducing Sugars

Carbohydrates are chemically bound to other molecules or physically associated or present as isolated molecules (Wang *et al.*, 2003; Ibrahim and Jaafar, 2012). The carbohydrate group includes sugars, starches, and cellulose. CO_2 enrichment enhances the concentration of total carbohydrates in plants (Ibrahim and Jaafer, 2012). Marked increases in foliar carbohydrate content are common at elevated CO_2, even when plants are free from artificial restriction of sink development (Long *et al.*, 2004).

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). Exposure of C3 plants to elevated CO_2 frequently results in an immediate increase in the rate of CO_2 assimilation however a reduction in photosynthetic capacity often occurs after prolonged periods at elevated CO_2 (Stitt, 1991; Griffin and Seemann, 1996). This down-regulation or acclimation of photosynthesis is generally accompanied by a large increase in leaf carbohydrates.

In *Duchesnea indica*, a forest floor herb, elevated CO_2 resulted in increased leaf soluble sugars by 52% and starch content by 160% (Long and Drake, 1992). The influence of elevated CO_2 on plant tissue chemistry has been the subject of many studies and results have been variable, decrease in foliar nitrogen (Mc Guire *et al.*, 1995; Liu *et al.*, 2005; Couture *et al.*, 2012), increase in carbohydrate concentrations (Lincoln *et al.*, 1993; Penuelas and Estiarte, 1998; Lindroth *et al.*, 2001) and altered plant starch and fiber concentrations (Lindroth *et al.*, 2001; Meehan *et al.*, 2010).

CO_2 enrichment generates increased sucrose content in the leaf and intense hydrolytic decomposition into fructose and glucose through acid invertase (Ke, 1993; Ghasemzadeh *et al.*, 2012). 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, 1997) and beech leaf by about 52% (Landolt, 1997). Total reducing sugar and total starch content increased whereas a reduction in total soluble protein was reported under elevated CO_2 (550 ppm and 700 ppm) in black gram (Sathish *et al.*, 2014). Total soluble sugar content was enhanced and total starch content remained unchanged when Alfalfa plants were grown under CO_2enrichment (700 $\mu molmol^{-1}$) under different levels of temperature (Aranjuelo *et al.*, 2005).

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). An increase in starch content was observed in plants of *Desmodium paniculatum* when in air containing 0.1% CO_2 than the plants under normal air of 0.035% CO_2 (Renata *et al.*, 1982).

Phenol Content

Phenolics are aromatic benzene ring compounds with one or more hydroxyl groups produced by plants mainly for protection against stress. These secondary metabolites play important roles in plant development, particularly in lignin and pigment biosynthesis. They also provide structural integrity and scaffolding support to plants.

Several environmental factors such as nutrient supply, temperature, light conditions and atmospheric CO_2 concentrations can influence the levels of total phenolics in plants (Fine *et al.*, 2006). One of these factors is CO_2, and the increases in atmospheric CO_2 due to climate change have a direct impact on plant secondary metabolites. In an experiment conducted under very high atmospheric CO_2 concentrations, Ali *et al.* (2005) found that CO_2 levels of 10000 ppm, 25000

ppm and 50000ppm increased total phenolic concentrations by 58%, 153% and 105% in ginseng roots.

Koricheva *et al.* (1998) reported that the total phenolic concentration increased in temperate species when grown under elevated CO_2 although responses varied among species and environmental conditions (Kinney and Lindroth, 1997).

In a three year open top chamber experiment under elevated CO_2 higher concentration of phenol was reported in silver birch leaves during the last year (Peltonen *et al.*, 2005). In a two year study with open-top chambers using japonica rice variety a decrease in phenolic concentration was reported during seedling stage while an increase in maturity stage under elevated CO_2 concentration of 550 $\mu mol\ mol^{-1}$(Goufo, 2014).

Free Amino Acid

Increase in soluble amino acid content under CO_2 enrichment has been reported in young ginger leaf, in soyabean (Ainsworth *et al.*, 2007), tobacco (Geiger *et al.*, 1998), barley (Manderscheid *et al.*, 1995). Increasing amino acid content can be related to degradation of proteins under elevated CO_2 conditions and hydrolysis to free amino acids (Wrigley *et al.*, 1988). It is widely agreed that plant growth in CO_2 enriched atmospheres enhances the accumulation of both leaf starch and soluble carbohydrates (De Souza *et al.*, 2008; Norby *et al.*, 1986).

Since the metabolism of carbohydrates is essential for the synthesis of amino acids, it is reasonable to assume that the effects of CO_2 enrichment should be similar for these classes of compounds. Ample carbon was available to support amino acid synthesis and the increase in soluble amino acids under CO_2 enrichment (Sicher, 2008). Unlike older leaves, soluble amino acids were increased in young soybean and tobacco leaves exposed to atmospheric CO_2 enrichment (Geiger *et al.*, 1998; Ainsworth *et al.*, 2007).

Wax Content

The production, chemical composition and structure of epicuticular waxes have been found to be sensitive to elevated CO2. Elevated CO2 had increased wax production, modified wax structure, and altered wax chemical composition, leading to stomatal occlusion by wax deposits in several forest tree species (Vanhatalo *et al.*, 2001; Karnosky *et al.*, 2002; Man kovska *et al.*, 2005). Percy *et al.*, (2002) found out that the cuticular wax production of aspen (*Populus tremuloides*) was stimulated by elevated CO2.

Membrane Integrity

A major impact of plant environmental stress is cellular membrane modification, which results in its perturbed function or total dysfunction. Cellular membrane dysfunction due to stress is well expressed in increased permeability and leakage of ions out. High temperature due to elevated CO2 can alter the physical state of the membrane, and lead to fluidization and disintegration (Los and Murata, 2004). Studies have indicated that plant cells can employ synaptotagmin mediated repair to maintain the integrity of the plasma membrane under various conditions of stress; possibly by using exocytosis vesicles to seal the site of injury and or endocytosis to internalize the lesions (Schapire *et al.*, 2008; Yamazaki *et al.*, 2008)

Stable Isotope Discrimination

The isotopic composition of carbon in whole plant and plant organs provides an integrated long term view of carbon assimilation. The isotopes are unevenly distributed among and within different compounds and this isotopic distribution can reveal information about the physical, chemical, and metabolic processes involved in carbon transformations. Several physical factors have been shown to influence the integrated balance of stomatal conductance and carboxylation and thus affect isotopic discrimination in plants (Henderson *et al.*, 1998).

Carbon isotope discrimination can be defined as the molar ratio of 13C/12C (Ra) in atmospheric CO2- the carbon source for plants divided by the same ratio in the plant product (Rp) (Farquhar and Richards, 1984). Atmospheric pCO2 has been shown to influence multiple aspects of plant biology like growth, water use efficiency, chemical profiles in plant cells etc. The basis of the biochemical discrimination against $_{13}C$ in C_3 plants lies with the primary carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) which discriminates against $_{13}C$ because of the intrinsically lower reactivity of $_{13}C$ compared with $_{12}C$ (Farquhar *et al.*, 1982). Records of $\Delta\delta Cp^{13}$ in oak trees have been reported to show a positive correlation with increasing pCO_2 over the last 160 years (Gagen *et al.*, 2007; Loader *et al.*, 2008; Mc Carroll *et al.*, 2009; Treydte *et al.*, 2009).

Studies had showed a positive correlation between $\Delta\delta^{13}Cp$ and pCO2 (Saurer *et al.*, 2003; Hietz *et al.*, 2005; Sharma and Williams, 2009) negative correlation (Beerling and Woodward, 1993; Beerling, 1996) and no correlation (Jahren *et al.*, 2008) was reported in various fossil studies. In C_3 plants, stable carbon isotope discrimination (Δ) has been used to assess genotypic variation in water use efficiency and physiological responses to environmental factors (Hubick *et al.*, 1986; Martin and Thorstenson, 1988; Johnson *et al.*, 1990).

ANTIOXIDANTS

Plant cells involve complex antioxidant defence mechanisms against oxidative stress generated under challenging stress conditions (Matsuura and Fett-Neto, 2013). Factors participating in antioxidative activity are non-enzymatic and enzymatic (Bartels and Sunkar, 2005). Non-enzymatic antioxidants include vitamin C, vitamin E, glutathione, flavonoids, alkaloids, carotenoids etc. Enzymatic antioxidants include catalase, superoxide dismutase peroxidase and metallothionein (Seki *et al.*, 2001).

In some cases, the additional carbon fixed during CO2 enrichment is invested in antioxidative compounds rather than enzymes. Three months exposure to elevated CO2 concentration of 720$\mu L\ L^{-1}$ in open top field chambers reduced

the activities of superoxide dismutase and catalase by an average of 23% and 39% respectively in soyabean (Pritchard *et al.*, 2000). Polle *et al.* (1997) showed that two years of atmospheric CO2 enrichment reduced the activities of several key antioxidative enzymes including catalase and superoxide dismutase in beech seedlings. Lin and Wang (2002) observed that activities of superoxide dismutase and catalase were much higher in CO2 enriched wheat than in ambiently grown wheat.

In a two year field experiment under elevated CO2 (336μmol mol^{-1}) in open top chambers a decreased activity of SOD was observed while the total ascorbic acid concentration increased by 28-72% in lower canopy leaves of soyabean (Booker and Fiscus, 2005). In the early studies of Barbale (1970) and Madsen (1971, 1975), a tripling of the atmospheric CO2 concentration produced a modest (7%) increase in the vitamin C content of tomato plants. Kimball and Mitchell (1981) however could find no effect of a similar CO2 increase on the same species although the extra CO2 of their study stimulated the production of vitamin A. In bean sprouts, on the other hand, a mere one hour per day doubling of atmospheric CO2 concentration actually doubled vitamin C over a 7 day period (Tajiri, 1985).

Molecular Studies

Transcriptional and post-transcriptional processes may regulate protein levels during plant growth in elevated CO2. Growth in elevated CO2 markedly increased non-structural carbohydrates and concomitantly decreased both transcripts and polypeptides corresponding to the large and small subunits of Rubisco in several species of CO2 enriched plants including Arabidopsis (Cheng *et al.*, 1998), wheat (Nie *et al.*, 1995), tomato (Van Oosten and Besford, 1995), and pea (Majeau and Coleman, 1996).

A diurnal relationship was observed between Rubisco small subunit (RBCS) mRNA levels and leaf carbohydrates in Arabidopsis and wheat (Nie *et al.*, 1995; Cheng *et al.*, 1998). Moore *et al.* (1999) using corn, parsley, pea and spinach, reported that Rubisco protein content was unchanged and transcript

levels for the large and small subunits of Rubisco were increased slightly in response to elevated CO2. Idso *et al.* (2001) observed seasonal changes in major 33, 31 and 21 kDa polypeptides in leaves of various citrus species including sour orange trees. Nuclear transcripts or enzyme activities for Rubisco activase in tomato (Van Oosten and Besford, 1995), carbonic anhydrase in cucumber (Peet *et al.*, 1986), in pea (Majeau and Coleman, 1996) and the Chl a/b binding protein (Van Oosten and Besford, 1995; Moore *et al.*, 1998) were decreased in elevated compared to ambient CO2 grown plants.

Enhanced CO2 concentration decreased the intensity of 52 kDa and 51.4 kDa polypeptide at vegetative and flowering stages in black gram (Sathish *et al.*, 2014). Several investigations suggest that most prominent change in leaf photosynthetic apparatus under elevated CO2 is a decrease in the amount of Rubisco protein (Drake *et al.*, 1997).

Pandurangam *et al.* (2006) reported the photosynthetic acclimation to elevated CO2 concentration due to down regulation of Rubisco through limitation imposed on Rubisco small subunit gene expression, as a consequence of sugar accumulation in wheat leaves. However in sunflower and mung bean they reported no down regulation of photosynthetic rate under elevated CO2 condition.

The ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content declined by 60% in spring wheat leaves grown under elevated CO2 concentration of 550μmol mol^{-1} (Nie *et al.*, 1995). Total Ribulose1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity decreased with plant age and was lower in the elevated CO2 (100 Pa) compared to the ambient CO2 treatment (Hanhong and Richard, 2004). Rubisco activity and Rubisco protein in barley penultimate leaves and wheat flag leaves were decreased with leaf age under elevated CO2 concentration of 700 μmol mol^{-1} (Richard and James, 1997).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiment was undertaken with the main objective of assessing the impact of elevated CO_2 on the physiological and molecular basis of growth responses in black pepper (*Piper nigrum* L.). For this, a field experiment was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani. Two technologies were used for subjecting the plants to elevated CO_2 environments - Open Top Chamber and the Trench system. Eight month old rooted cuttings and bush pepper plants of three black pepper varieties were used for both the experimental system. The experiment was conducted for a time period of two months. Biochemical observations were taken at monthly intervals and all other parameters including growth and molecular studies were conducted after the exposure period.

3.1 EXPERIMENT DETAILS

3.1.1 Location

The field experiment was conducted in Open Top Chamber and Trench system located at College of Agriculture Vellayani, situated at 8°5'N latitude and 76°9'E longitude and an altitude of 29 m above mean sea level.

3.1.2 Season

The experiments were conducted from September 2014 to November 2014 in Open Top Chambers and from October 2014 to December 2014 in trench system.

3.1.3 Planting material

Eight months old rooted cuttings and bush pepper plants of Panniyur 1, Panniyur 5 and Karimunda varieties were used for the study. The materials were procured from Regional Agricultural Research Station, Panniyur and Crop Research Station, Balaramapuram.

3.1.4 Layout of the Experiment

The experiment was laid out in CRD with four treatments and three replications.

3.1.5 Techniques for CO_2 enrichment

Two technologies were used for creating CO2 enriched environments.

1. 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 , 45^0slope 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^oC 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 500ppm 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.

2. Trench system

It is a simple and economic technique to subject the plants to elevated CO2. Rectangular trenches of size 2 x 0.75 x 0.75 m were built in the experimental field exposed to open sunlight. All the four sides of the cut ends were provided with a single layer of brick lining to avoid sliding of the edges.

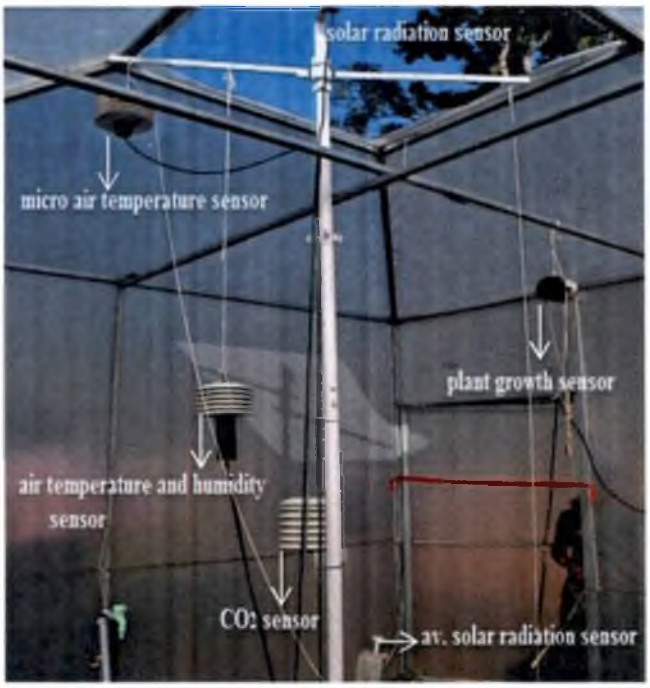


Plate 1. Open Top Chamber system for CO_2 enrichment

Inside the trench a layer of well decomposed organic matter was spread uniformly all along the floor for the release of CO2. CO2 concentration within the trench was monitored using CO2 analyser. Potted plants were kept in the trenches and covered with polythene sheets supported by metallic frame from 4.00 pm to 10.30 am. By covering, CO2 released by dark respiration and soil respiration were trapped during night for making it available to the plants during morning hours up to 10.30am.

For the present study coir pith was selected as the organic manure. A 5cm layer of coir pith was spread uniformly along the floor of two trenches and potted black pepper plants were kept inside the trench. Carbon dioxide concentrations inside the trenches were measured using CO2 analyser. An average concentration of 480 ppm was noted inside the trenches.

3.1.6 Standardisation of Trench

Different types of organic manures viz. vermicompost, farm yard manure, dried leaves, coir pith were kept in glass troughs with control (soil) in another glass trough. The glass troughs were kept undisturbed and sealed tightly. This was maintained for a time period of seven days. CO2 concentration inside all the glass troughs was measured using CO2 analyzer. Based on the concentration of CO2 released from different organic manures coirpith was selected as the best organic material for trench system since a higher CO2 release pattern was observed.

3.1.7 Table 1. CO_2 concentrations inside the Trenches at different intervals

Date of observation	Trench I (ppm)	Trench II (ppm)	Control (ppm)
28/10/14	510	512	360
10/11/14	500	507	361
24/11/14	476	478	360
8/12/14	472	473	358
22/12/14	420	422	360
28/12/14	372	378	361

Plate 2. Trench system for CO_2 enrichment – Standardisation

Plate 3. CO_2 analyser

Plate 4. Trench system

Plate 5. Trench with coirpith spread on the floor

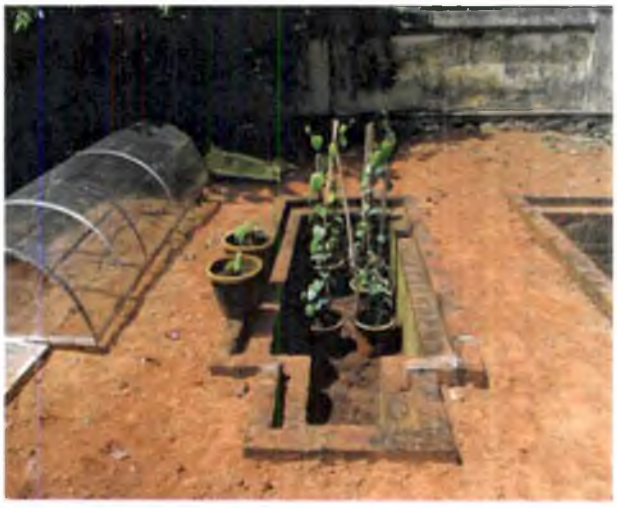


Plate 6. Plants kept in Trenches covered with transparent dome

3.1.8 Treatments

T_1 - OTC with elevated CO_2 concentration (OTC Ec)

T_2 - OTC with ambient CO_2 concentration (OTC Ac)

T_3 - Trench system (T)

T_4 - Absolute control (C)

3.1.9 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. The experiment was laid out in CRD. The potted plants were kept in OTCs and Trench for a period of two 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 after CO_2 exposure period.

3.2.1.2 *Leaf Area*

Leaf area was measured by graphical method.

3.2.1.3 *Specific Leaf Area*

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 80^0C for 2 days and the dry weight was taken. SLA was calculated using the formula.

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

Plate 7. Plants in OTC (Ec)

Plate 8. Plants in OTC (Ac)

Plate 9. Plants kept in Trench system

Plate 10. Plants in Absolute control

3.2.1.4 *Net Assimilation Rate*

The method proposed by Williams (1946) was used for calculating the net assimilation rate (NAR) on leaf dry weight basis and the values were expressed as mg cm^{-2} day^{-1}.

$$NAR = \frac{W_2 - W_1}{t_2 - t_1} \times \frac{\log_e L_2 - \log_e L_1}{L_2 - L_1}$$

W_1 and W_2 = leaf dry weight (mg) at t_1 and t_2 respectively

L_1 and L_2 = leaf area (cm^2) at t_1 and t_2 respectively

$t_2 - t_1$ = time interval in days

3.2.1.5 *Relative Growth Rate*

Relative growth rate (RGR) was determined using the formula of Williams (1946) and expressed in mg $g^{-1}day^{-1}$.

$$RGR = \frac{\log_e W_2 - \log_e W_1}{t_2 - t_1}$$

W_1 and W_2 = plant dry weight (g) at time t_1 and t_2 respectively

$t_2 - t_1$ = time interval in days

3.2.1.6 *Root Weight*

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.

3.2.1.7 *Shoot Weight*

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

3.2.1.8 *Root Shoot Ratio*

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

3.2.1.9 *Dry matter Production*

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

3.2.2. Physiological and Biochemical parameters

3.2.2.1. *Relative Water Content*

Relative water content was estimated as per Barr and Weatherly (1962) by measuring the fresh weight, turgid weight and dry weight of known number of leaf discs from the experimental plants. After measuring the fresh weight of the sample, it was submerged in distilled water for 3 hours and then the turgid weight was taken. The dry weight of the sample was measured after keeping the samples in oven at 80°C for 3 consecutive days. The RWC was calculated using the following formula.

$$RWC = \frac{Fresh\ \ weight - Dry\ \ weight}{Turgid\ \ weight - Dry\ \ weight} \times 100$$

3.2.2.2 *Pigment Composition*

Estimation of Chlorophyll and Carotenoids

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, 480 and 510nm. The chlorophyll content was measured by substituting the absorbance values in the given formulae.

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

$$Chlb = (22.9 \times A_{645} - 4.68 \times A_{663}) \times \frac{V}{1000} \times \frac{1}{fresh\ \ weight}$$

$$TotalChl \quad (a+b) = (8.02 \times A_{663} - 20.2 \times A_{645}) \times \frac{V}{1000} \times \frac{1}{fresh \quad weight}$$

$$Carotenoid \quad = \left(\frac{7.6 \times A_{480} - 1.49 \times A_{510} \times V}{w \times 1000} \right)$$

3.2.2.3 *Stomatal Frequency*

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 microscope and counted using a 40X objective and 10X eyepiece. The field of the microscope was measured using a stage micrometre and stomatal frequency per unit area was calculated.

$$Stomatal \quad frequency \quad = \frac{No \ of \ stomata}{Area \ of \ the \ microscopi \quad c \ field}$$

3.2.2.4 *Transpiration Rate*

Transpiration rate was measured using the SAI-1 Porometer of company Delta T Devices and expressed as mmoles $m^{-2}s^{-1}$.

3.2.2.5 *Stomatal Conductance*

Stomatal conductance was measured using the SAI-1 Porometer of company Delta T Devices and expressed as mmoles $m^{-2}s^{-1}$.

3.2.2.6 *Estimation of Total Soluble Protein*

The total soluble protein of leaf samples were estimated using 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

3.2.2.7 *Estimation of Starch*

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.

3.2.2.8 *Estimation* of *Reducing Sugars*

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 80^0C. 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.

3.2.2.9 *Estimation of Phenols*

Quantification of phenols was done by Folin-Ciocalteau method (Mayr *et al.,* 1995). Phenol was estimated from 0.5g of leaf samples and reflexed in 10 ml 80% methanol for 20 min. The tissue was ground thoroughly in a mortar with pestle and filtered through a double layered cheese cloth. The filtrate was subjected to centrifugation at 1000 rpm for 10 min. The supernatant was collected and made to a known volume using 80% methanol. 0.1 ml aliquot was drawn to a test tube and made up to 3 ml using 80% methanol. To this, 0.5 ml of Folin-Ciocalteau reagent and 2 ml 20% Na_2CO_3 were added.

It was kept in a boiling water bath for 5 min till a white precipitate was formed and was then again centrifuged at 5000 rpm for 5 min. The absorbance of the clear supernatant was read at 650 nm against the blank. Standard curve was prepared using different concentrations of catechol and expressed in catechol equivalents as microgram per gram leaf tissue on fresh weight basis.

3.2.2.10 *Estimation of Total Free Amino Acid*

The total free amino acids were estimated following the Ninhydrin method (Moore and Stein, 1948). The plant sample was weighed (500 mg) and ground in

a mortar and pestle with a small quantity of acid washed sand. To this homogenate 5-10 ml of 80% ethanol was added. The solution was filtered and centrifuged. The filtrate or the supernatant was saved for further use. This extraction was repeated twice with the residue and the supernatants were pooled. The volume was reduced by evaporation and the extract was used for the quantitative estimation of total free amino acids. Ninhydrin solution (1 ml) was added to 1 ml of extract and the volume was made up to 2 ml using distilled water.

The test tube was heated in a boiling water bath for 20 minutes. The contents were mixed after adding 5 ml of the diluents (equal volumes of water and n-propanol). The intensity of the purple colour was read at 570 nm, against a reagent blank, after incubation of 15 minutes. The reagent blank was prepared as above by taking 0.1 ml of 80% ethanol, instead of extract. The standard Leucine (50mg) was dissolved in 50 ml of distilled water in a volumetric flask.

The stock standard of 10 ml was diluted to 100 ml in another volumetric flask to make the working standard solution. A series of volume from 0.1 to 1 ml of this standard solution was prepared to give a concentration range of 10µg-100µg. The procedure was followed as that of sample and the absorbance of purple colour was read at 570 nm. A standard curve was drawn using absorbance versus concentration. The concentration of total free amino acid in the sample was determined from the standard graph and was expressed as % equivalent of leucine.

3.2.2.11 ***Wax Content***

Samples were collected from third fully opened leaves of plants and were cut into $10cm^2$ bits. 10ml of chloroform was taken in beakers after noting down their initial weight. Leaf bits were dipped into chloroform for 30seconds. After removing the leaf bits the beakers were left for evaporation of chloroform. The final weight of beakers was noted after complete evaporation of chloroform. The difference between the final and initial weight of beakers would be noted as the wax content and would be expressed per unit leaf area

3.2.2.12 *Membrane Integrity*

Leaf discs (10 no) from the third fully opened leaves were taken in a 50ml beaker with 10ml distilled water. Initial EC was measured for detecting the small degree of leakage by the discs caused by the punching treatment using conductivity electrode (ECa). After 30 minute incubation the leakage of solutes in this bathing medium was measured (ECb). Then the beakers were boiled at 100°C for 10 minute and the EC was again recorded (ECc).The membrane integrity of leaf tissues was calculated using the following formula.

$$\% \, leakage = \frac{Ecb - Eca}{Ecc} \times 100$$

3.2.2.13 *Stable Isotope Discrimination*

The third fully opened leaves of experimental plants were collected, oven dried at 80^{0}C and were ground to a very fine powder. The samples were sent to the National Facility for stable isotope studies at the Department of Crop Physiology UAS GKVK Bangalore where they were analysed using the isotope ratio mass spectrophotometer (IRMS) coupled with the elemental analyzer for the continuous flow measurement of carbon isotope ratios in plant samples.

3.2.3. Enzymatic Antioxidants

3.2.3.1 *Estimation of Peroxidase (EC.1.11.1.7)*

The peroxidase activity in plants was estimated following the method described by Reddy *et al.* (1995). Leaf sample of 200 mg was homogenised in 1 ml of 0.1 M phosphate buffer (pH 6.5) and centrifuged at 5000 rpm for 15 minute at 4^{0}C. To 3.0ml of pyrogallol solution, 0.1ml of the enzyme extract was added and adjusted to read zero at 430 nm. The enzyme reaction was started by adding 0.5 ml of one percent hydrogen peroxide (H_2O_2) into sample cuvettes and change in absorbance was measured every 30 second up to 3 minute. One unit of peroxidase is defined as the change in absorbance/minute at 430nm.

3.2.3.2 *Estimation of Catalase (EC.1.11.1.6)*

The catalase (CAT) activity in plants was quantified following the method described by Luck (1974). 200mg of leaf sample was prepared in phosphate buffer. The homogenate was centrifuged at 5000 rpm for 15 minute at 4^0C and the supernatant was used for the enzyme assay. The H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette. This was followed by the rapid addition of 40µl of enzyme extract and was mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm. The enzyme solution containing H_2O_2-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

3.2.3.3 *Superoxide dismutase (EC.1.5.1.1)*

Superoxide dismutase (SOD) activity was quantified following the method described by Kakkar *et al.* (1984). Leaf samples (0.5g) from third fully opened leaves were ground with 3.0 ml of potassium phosphate buffer, centrifuged at 2000rpm for 10 minutes and the supernatants were used for the assay. The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2 ml of the enzyme preparation and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2ml of NADH.

The mixture was incubated at 30°C for 90 second and arrested by the addition of 1.0ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minute and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

3.2.3.4 *Estimation of Ascorbic Acid*

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 (V_1 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 (V_2 ml). The amount of ascorbic acid is calculated as follows:

$$Ascorbic \quad acid = \frac{0.5\,mg}{V_1\,ml} \times \frac{V_2}{5\,ml} \times \frac{100}{weight \quad of \quad sample}$$

3.2.4. Molecular studies

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 in1.5 ml of cold denaturing buffer (Appendix III) at 4^0C. 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 4^0C 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 4^0C.

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 4^0C.

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

2-mercapto ethanol - 0.8 ml

Glycerol - 1.6 ml

SDS 20% (w/v) - 1.6 ml

0.5% Bromophenol blue - 0.4 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

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

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

TEMED - 0.1 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).

RESULTS

4. RESULTS

The current programme was undertaken with the main objective of assessing the impact of elevated CO_2 on physiological and molecular basis of growth responses in black pepper (*Piper nigrum* L.). Two technologies were used for subjecting the plants to elevated CO_2 environments - Open Top Chamber and the Trench system. Eight months old rooted cuttings and bush pepper plants of three black pepper varieties (Panniyur 1, Panniyur 5 and Karimunda) were used for both the experimental system and exposed to elevated CO_2 concentration for a period of two months. Biochemical observations were taken at monthly intervals and all other parameters including growth and molecular studies were conducted at the end of exposure. The results based on statistically analysed data pertaining to the experiment conducted during the course of investigation are presented below.

4.1 GROWTH PARAMETERS

4.1.1 Plant Height

The effect of elevated CO_2 environment on plant height is presented in Table 2. Highest mean value (85.20 cm) for plant height was recorded in treatment T_1 (elevated CO_2) at the end of exposure period. The mean values of all other treatments were found to be on par. Irrespective of the variety, plant height of bush pepper was not found to be affected by any of the treatments. The per cent increase in plant height was found to be highest in the cuttings of Panniyur 5 and Karimunda in treatments T_1 (elevated CO_2), T_2 (chamber with ambient CO_2) and T_3 (Trench system). In treatment T_1, a significant reduction in plant height (143.80 cm) was noticed in Panniyur 1 cuttings compared to absolute control (197.0 cm).

Table 2. Effect of elevated CO_2 on plant height, cm

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	143.80	101.45	139.15	197.00	145.35
Panniyur 1 (Bush)	35.90	41.25	29.95	35.00	35.52
Panniyur 5 (Cuttings)	95.00	85.25	94.85	58.00	83.27
Panniyur 5 (Bush)	40.80	31.85	28.25	31.50	33.10
Karimunda (Cuttings)	137.45	111.75	120.80	82.60	113.15
Karimunda (Bush)	58.25	33.70	32.25	29.25	38.36
Mean	85.20	67.54	74.20	72.22	
CD (0.05)	T- 8.939	V- 10.949		T x V- 21.898	

Table 3. Effect of elevated CO_2 on leaf production, no's

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	39	20	20	20	24
Panniyur 1 (Bush)	28	27	11	12	19
Panniyur 5 (Cuttings)	36	21	26	23	26
Panniyur 5 (Bush)	28	16	20	15	20
Karimunda (Cuttings)	35	26	23	19	25
Karimunda (Bush)	14	13	16	9	13
Mean	30	20	19	16	
CD (0.05)	T- 2.519	V- 3.085		T x V- 6.170	

T_1 - OTC with elevated CO_2 concentration (OTC Ec)

T_2 - OTC with ambient CO_2 concentration (OTC Ac)

T_3 - Trench system (T)

T_4 - Absolute control (C)

4.1.2 Number of Leaves

The effect of elevated CO_2 on number of leaves is presented in Table 3. Exposure to 500 ppm CO_2 had a highly significant influence on the number of leaves of plants (30 no's). The number of leaves (20 no's) in the plants of treatment T_2 (chamber with ambient CO_2) and T_3 (Trench system) (19 no's) were found to be on par but significantly higher than control plants. Under elevated CO_2 condition (T_1), per cent increase in leaf production was highest in Panniyur 1 bush compared to absolute control plants.

4.1.3 Leaf Area

All the varieties responded positively and in a highly significant way to CO_2 enrichment (Table 4). A significantly higher leaf area (820.89 cm^2) was recorded in varieties under the treatment T_1 (elevated CO_2) followed by the varieties in treatment T_2 (chamber with ambient CO_2) (427.50 cm^2). Total leaf area (276.18 cm^2) of varieties under treatment T_3 (Trench system) was found to be on par with T_4 (absolute control). Highest per cent increase in leaf area under elevated CO_2 was noticed in Panniyur 5 bushes followed by Panniyur 1 bushes having the values of 904.42 cm^2 and 750.20 cm^2 when compared to absolute control plants.

4.1.4 Specific Leaf Area

The Table 5 shows the effect of elevated CO_2 on specific leaf area. Specific leaf area was found to have a reduction (144.0 $cm^2\ g^{-1}$) in varieties under treatment T_1 (elevated CO_2) followed by the varieties in treatment T_3 i.e. Trench system (132.84 $cm^2\ g^{-1}$) compared to absolute control (166.20 $cm^2\ g^{-1}$). Under elevated CO_2 condition, the cuttings of Panniyur 1 recorded the lowest per cent reduction in specific leaf area having a value of 108.71 $cm^2\ g^{-1}$.

Table 4. Effect of elevated CO_2 on leaf area, cm^2

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	955.10	574.49	420.64	355.96	576.55
Panniyur 1 (Bush)	750.20	671.47	240.39	144.18	451.56
Panniyur 5 (Cuttings)	682.25	432.36	345.74	525.40	496.44
Panniyur 5 (Bush)	904.42	192.77	115.55	137.41	337.54
Karimunda (Cuttings)	1040.47	523.12	348.66	495.78	602.01
Karimunda (Bush)	592.89	170.79	186.09	135.58	271.34
Mean	820.89	427.50	276.18	299.05	
CD (0.05)	T- 60.784	V- 74.444		T x V- 148.890	

Table 5. Effect of elevated CO_2 on specific leaf area, $cm^2\ g^{-1}$

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	108.71	161.12	135.04	162.15	141.75
Panniyur 1 (Bush)	171.45	190.17	187.08	181.50	182.55
Panniyur 5 (Cuttings)	154.30	132.32	103.85	128.40	129.71
Panniyur 5 (Bush)	132.73	168.64	106.05	185.10	148.13
Karimunda (Cuttings)	148.98	153.92	128.59	156.78	147.06
Karimunda (Bush)	147.82	147.28	136.48	183.26	153.71
Mean	144.00	158.90	132.84	166.20	
CD (0.05)	T- 6.648	V- 8.142		T x V- 16.285	

4.1.5 Net Assimilation Rate

Significantly higher net assimilation rate was registered in varieties under treatments T_1 (elevated CO_2) and T_2 (chamber with ambient CO_2) holding the values 0.057 mg cm^{-2} day^{-1} and 0.029 mg $cm^{-2}day^{-1}$ respectively as depicted in Table 6. Net assimilation rate of treatment T_3 (Trench system) was found to be on par with absolute control. The variety Panniyur 1 bush under elevated CO_2 condition (T_1) was observed to have the highest mean value for net assimilation rate (0.066 mg cm^{-2} day^{-1}). Lowest value (0.014 mg^{-1} g^{-1} day^{-1}) was recorded by Panniyur 5 bush in Trench system (T_3).

4.1.6 Relative Growth Rate

The varieties under treatment T_1 (elevated CO_2) condition recorded the highest mean value for relative growth rate (0.0068 mg^{-1} g^{-1} day^{-1}) compared to absolute control (0.0045 mg^{-1} g^{-1} day^{-1}). Table 7 shows the relative growth rate of varieties under elevated CO_2 environments. In treatment T_3 (Trench system) a significant reduction in relative growth rate was observed (0.0037 mg^{-1} g^{-1} day^{-1}). Compared to absolute control condition bushes of Panniyur 1 registered the highest mean value (0.0078 mg^{-1} g^{-1} day^{-1}) for relative growth rate under elevated CO_2 condition.

4.1.7 Root Weight

All the varieties responded positively and in a highly significant way under elevated CO_2 condition (T_1) having a mean value of 2.87 g compared to absolute control condition (1.53 g). Root weights of varieties in Trench system (T_3) were also observed to be superior (1.91 g) to absolute control condition. Under elevated CO_2 (T_1), Karimunda cuttings registered the highest per cent increase in root weight and have a mean value of 2.85 g compared to absolute control (Table 8).

Table 6. Effect of elevated CO_2 on net assimilation rate, mg cm^{-2} day^{-1}

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.056	0.026	0.021	0.027	0.033
Panniyur 1 (Bush)	0.066	0.058	0.017	0.016	0.039
Panniyur 5 (Cuttings)	0.054	0.026	0.031	0.029	0.034
Panniyur 5 (Bush)	0.053	0.016	0.014	0.023	0.027
Karimunda (Cuttings)	0.058	0.026	0.036	0.025	0.036
Karimunda (Bush)	0.058	0.019	0.019	0.022	0.029
Mean	0.057	0.029	0.023	0.023	
CD (0.05)	T- 0.004	V- 0.005		T x V- 0.010	

Table 7. Effect of elevated CO_2 on relative growth rate, mg $g^{-1}day^{-1}$

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.0074	0.0052	0.0049	0.0064	0.0060
Panniyur 1 (Bush)	0.0078	0.0049	0.0056	0.0034	0.0054
Panniyur 5 (Cuttings)	0.0055	0.0028	0.0024	0.0053	0.0040
Panniyur 5 (Bush)	0.0048	0.0035	0.0026	0.0029	0.0033
Karimunda (Cuttings)	0.0057	0.0048	0.0021	0.0043	0.0043
Karimunda (Bush)	0.0094	0.0038	0.0045	0.0056	0.0057
Mean	0.0068	0.0042	0.0037	0.0045	
CD (0.05)	T- 0.00064	V- 0.00078		T x V- 0.0015	

4.1.8 Shoot Weight

The Table 9 shows the shoot weights of different varieties under elevated CO_2 environments. Shoot weights were found to be superior in varieties under elevated CO_2 condition (20.15 g) compared to absolute control (16.07 g). The treatment means of T_2 (ambient CO_2) and T_3 (Trench system) were found to be on par. Compared to control condition highest per cent increase in shoot weight was registered in Panniyur 1 bushes under elevated CO_2 condition (T_1).

4.1.9 Root Shoot Ratio

Highest root shoot ratio was exhibited by the varieties of treatment T_2 (chamber with ambient CO_2) having a value of 0.193 as depicted in Table 10. The varieties under elevated CO_2 (0.162) and Trench system (0.162) also showed a significantly higher root shoot ratio compared to control condition. The per cent increase in root shoot ratio was highest in the cuttings of Karimunda under elevated CO_2 compared to absolute control.

4.1.10 Drymatter Production

Highest mean value (14.15 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 bushes of Panniyur 1 were noticed for its highest per cent increase in drymatter production followed by the bushes of Karimunda (Table 11).

Table 8. Effect of elevated CO_2 on root weight, g

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	3.04	2.20	2.27	2.20	2.43
Panniyur 1 (Bush)	2.17	2.55	2.09	1.17	1.99
Panniyur 5 (Cuttings)	2.62	2.21	2.39	1.56	2.19
Panniyur 5 (Bush)	3.25	2.66	1.48	1.57	2.24
Karimunda (Cuttings)	2.85	1.58	1.15	1.22	1.70
Karimunda (Bush)	3.32	1.90	2.08	1.46	2.19
Mean	2.87	2.18	1.91	1.53	
CD (0.05)	T- 0.220	V- 0.269		T x V- 0.538	

Table 9. Effect of elevated CO_2 on shoot weight, g

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	35.77	26.47	24.95	31.41	29.65
Panniyur 1 (Bush)	13.03	7.77	9.58	7.20	9.39
Panniyur 5 (Cuttings)	17.63	13.04	10.81	17.97	14.86
Panniyur 5 (Bush)	11.34	9.095	9.17	8.77	9.59
Karimunda (Cuttings)	21.32	19.44	13.47	18.59	18.21
Karimunda (Bush)	21.84	9.60	10.57	12.48	13.62
Mean	20.15	14.23	13.09	16.07	
CD (0.05)	T – 1.434	V- 1.756		T x V- 3.512	

Table 10. Effect of elevated CO_2 on root shoot ratio

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.08	0.08	0.09	0.07	0.08
Panniyur 1 (Bush)	0.16	0.33	0.21	0.16	0.22
Panniyur 5 (Cuttings)	0.14	0.17	0.22	0.08	0.15
Panniyur 5 (Bush)	0.28	0.29	0.16	0.17	0.23
Karimunda (Cuttings)	0.13	0.08	0.08	0.06	0.09
Karimunda (Bush)	0.15	0.19	0.19	0.11	0.16
Mean	0.16	0.19	0.16	0.11	
CD (0.05)	T- 0.018	V- 0.022			T x V- 0.045

Table 11. Effect of elevated CO_2 on dry matter production, g

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	25.00	14.86	13.40	19.80	18.26
Panniyur 1 (Bush)	10.07	5.06	6.04	3.24	6.10
Panniyur 5 (Cuttings)	10.90	4.90	3.84	10.18	7.45
Panniyur 5 (Bush)	7.17	4.81	3.23	2.92	4.53
Karimunda (Cuttings)	13.39	10.23	3.84	9.02	9.12
Karimunda (Bush)	18.42	4.76	5.91	7.20	9.07
Mean	14.15	7.44	6.04	8.72	
CD (0.05)	T- 1.640	V- 2.009			T x V- 4.018

4.2 PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS

4.2.1 Relative Water Content

During the first month of exposure, highest mean value (93.56%) for relative water content was registered in treatment T_1 (elevated CO_2). Relative water content of varieties under treatments T_2 (ambient CO_2) and T_3 (Trench system) was found to be on par with absolute control. There was no significant variation observed for relative water content between the varieties and interactions (Table 12).

During the second month, the relative water content of treatments T_1 (84.65%) and T_3 (85.00%) was found to be on par with absolute control (85.82%) as presented in Table 13. The varieties under treatment T_2 (ambient CO_2) registered a significant reduction in RWC (81.11%). Under elevated CO_2 condition (T_1), the bush types of Panniyur 1 were observed to have significantly higher mean value for RWC (89.92%).

4.2.2 Pigment Composition

4.2.2.1 Chlorophyll a

During the first month of exposure, no significant variation in chlorophyll a content was observed between the treatments T_1 (elevated CO_2) and T_2 (ambient CO_2) and the treatment means of T_1 (0.663 mg g^{-1} of fresh weight) and T_2 (0.663 mg g^{-1} of fresh weight) were found to be on par with absolute control as depicted in Table 14. A significant reduction in chlorophyll a content was noticed in treatment T_3 (Trench system) holding a value of 0.286 mg g^{-1} of fresh weight. Compared to absolute control per cent increase in chlorophyll a content was found to be highest in the cuttings of Karimunda under elevated CO_2 (0.685 mg g^{-1} of fresh weight).

During the second month (Table 15), elevated CO_2 (T_1) was found to enhance the chlorophyll a content significantly (1.20 mg g^{-1} of fresh weight). The treatment means of T_2 (0.93 mg g^{-1} of fresh weight) and T_3 (0.99 mg g^{-1} of fresh

weight) were found to be on par with absolute control. There was no significant variation observed between varieties and interactions.

Table 12. Effect of elevated CO_2 on relative water content, (%) after one month of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	96.58	83.63	88.96	91.56	90.18
Panniyur 1 (Bush)	91.11	87.30	81.93	86	86.58
Panniyur 5 (Cuttings)	93.43	84.66	86.41	83.09	86.90
Panniyur 5 (Bush)	95.04	84.11	90.89	86.65	88.92
Karimunda (Cuttings)	93.08	84.11	84.36	83.80	86.34
Karimunda (Bush)	92.13	80.60	88.90	87.86	87.37
Mean	93.56	86.91	86.32	84.07	
CD (0.05)	T- 3.140	V- NS		T x V- NS	

Table 13. Effect of elevated CO_2 on relative water content, (%) at the end of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	85.67	84.64	89.10	82.47	85.47
Panniyur 1 (Bush)	89.92	81.91	85.37	85.42	85.65
Panniyur 5 (Cuttings)	81.69	80.04	85.10	86.91	83.43
Panniyur 5 (Bush)	78.23	79.81	88.20	86.68	83.23
Karimunda (Cuttings)	84.63	78.02	78.63	89.69	82.74
Karimunda (Bush)	87.81	82.25	83.59	83.79	84.36
Mean	84.65	81.11	85.00	85.82	
CD (0.05)	T- 2.894	V- NS		T x V- 7.089	

Table 14. Effect of elevated CO_2 on chlorophyll a, (mg g^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.685	0.675	0.300	0.585	0.561
Panniyur 1 (Bush)	0.600	0.685	0.345	0.870	0.625
Panniyur 5 (Cuttings)	0.655	0.675	0.235	0.605	0.542
Panniyur 5 (Bush)	0.685	0.610	0.245	0.695	0.558
Karimunda (Cuttings)	0.685	0.685	0.315	0.530	0.553
Karimunda (Bush)	0.670	0.650	0.280	0.665	0.566
Mean	0.663	0.663	0.286	0.658	
CD (0.05)	T- 0.042		V-NS	T x V- 0.105	

Table 15. Effect of elevated CO_2 on chlorophyll a, (mg g^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	1.12	0.88	0.95	0.71	0.91
Panniyur 1 (Bush)	1.24	1.03	0.94	0.87	1.02
Panniyur 5 (Cuttings)	1.42	1.04	1.02	0.84	1.08
Panniyur 5 (Bush)	1.28	1.02	1.07	0.89	1.06
Karimunda (Cuttings)	1.25	0.89	1.06	0.95	1.04
Karimunda (Bush)	0.91	0.75	0.89	0.82	0.84
Mean	1.20	0.93	0.99	0.84	
CD (0.05)	T- 0.151		V- NS	T x V- NS	

4.2.2.2 Chlorophyll b

During first month, the varieties under treatment T_1 (elevated CO_2) registered the highest mean value for chlorophyll b content (0.40 mg g^{-1} of fresh weight). Chlorophyll b content (0.16 mg g^{-1} of fresh weight) of treatment T_3 (Trench system) was found to be on par with absolute control (0.18 mg g^{-1} of fresh weight). Under elevated CO_2 condition (T_1) the cuttings of Panniyur 1 recorded the highest chlorophyll b content and lowest chlorophyll b content was noticed in the cuttings of Karimunda in Trench system compared to absolute control (Table 16).

During the second month also, significantly higher mean value of 0.52 mg g^{-1} of fresh weight was recorded in treatment T_1 (elevated CO_2). The treatment means of T_2 (ambient CO_2) and T_3 (Trench system) were found to be on par but was superior to absolute control as presented in Table 17. When compared to absolute control, highest per cent increase in chlorophyll b content was noticed in Panniyur 5 bush under elevated CO_2 condition and in trench system. When compared to absolute control chlorophyll b content was found to be highest in the bush types of Panniyur 5 under elevated CO_2 and in Trench system.

4.2.2.3 Total Chlorophyll

During first month, total chlorophyll content (1.07 mg g^{-1} of fresh weight) was found to be superior in treatment T_1 (elevated CO_2). A significant reduction (0.40 mg g^{-1} of fresh weight) in total chlorophyll was noticed in treatment T_3 (Trench system). Under elevated CO_2 and in trench system total chlorophyll was found to be highest in Panniyur 5 bush when compared to absolute control (Table 18).

During second month, no significant variation was observed between the treatments T_1 (1.45 mg g^{-1} of fresh weight), T_2 (1.36 mg g^{-1} of fresh weight) and T_3 (1.43 mg g^{-1} of fresh weight) but was found superior to absolute control (1.18 mg/g of fresh weight). Under the treatment T_1, total chlorophyll was highest in

Panniyur 5 bush (1.79 mg g^{-1} of fresh weight) and in trench system it was found to be highest in the cuttings of Panniyur 5 (Table 19).

Table 16. Effect of elevated CO_2 on chlorophyll b, (mg g^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.37	0.25	0.19	0.12	0.23
Panniyur 1 (Bush)	0.34	0.38	0.13	0.18	0.26
Panniyur 5 (Cuttings)	0.63	0.46	0.18	0.24	0.38
Panniyur 5 (Bush)	0.35	0.16	0.18	0.15	0.21
Karimunda (Cuttings)	0.37	0.27	0.12	0.25	0.25
Karimunda (Bush)	0.35	0.29	0.17	0.13	0.23
Mean	0.40	0.30	0.16	0.18	
CD(0.05)	T- 0.035		V- 0.043	T x V- 0.087	

Table 17. Effect of elevated CO_2 on chlorophyll b, (mg g^{-1}) at the end of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.465	0.465	0.365	0.270	0.391
Panniyur 1 (Bush)	0.495	0.705	0.640	0.590	0.607
Panniyur 5 (Cuttings)	0.665	0.595	0.355	0.385	0.500
Panniyur 5 (Bush)	0.500	0.360	0.435	0.245	0.385
Karimunda (Cuttings)	0.635	0.300	0.540	0.360	0.458
Karimunda (Bush)	0.365	0.365	0.345	0.270	0.336
Mean	0.520	0.465	0.446	0.353	
CD (0.05)	T- 0.053		V- 0.066	T x V- 0.132	

Table 18. Effect of elevated CO_2 on total chlorophyll, (mg g^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	1.05	1.01	0.56	0.70	0.83
Panniyur 1 (Bush)	1.04	1.11	0.47	1.05	0.92
Panniyur 5 (Cuttings)	1.31	1.14	0.32	0.84	0.90
Panniyur 5 (Bush)	0.92	1.07	0.37	0.34	0.67
Karimunda (Cuttings)	1.06	0.94	0.44	0.59	0.76
Karimunda (Bush)	1.06	0.88	0.24	1.09	0.82
Mean	1.07	1.02	0.40	0.77	
CD (0.05)	T-0.044		V- 0.054	T x V- 0.108	

Table 19. Effect of elevated CO_2 on total chlorophyll, (mg g^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	1.59	1.61	1.55	1.03	1.44
Panniyur 1 (Bush)	1.24	1.84	1.39	1.82	1.57
Panniyur 5 (Cuttings)	1.50	1.16	1.62	1.07	1.33
Panniyur 5 (Bush)	1.79	1.07	1.47	1.06	1.34
Karimunda (Cuttings)	1.29	1.20	1.18	1.08	1.19
Karimunda (Bush)	1.28	1.32	1.36	1.05	1.25
Mean	1.45	1.36	1.43	1.18	
CD(0.05)	T- 0.154		V- 0.189	T x V- 0.378	

4.2.2.4 Carotenoids

During the first month, - no significant variation was observed in carotenoids between the treatments T_1 (0.43 mg g^{-1} of fresh weight) and T_2 (0.44 mg g^{-1} of fresh weight) but was superior to absolute control. In trench system (T_3) a significant reduction was noticed. In treatment T_1, carotenoid content was found to have the highest per cent increase in Panniyur 5 bush having a value of 0.44 mg g^{-1} of fresh weight compared to absolute control as depicted in Table 20

During the second month, carotenoid content was found to be highest in treatment T_3 (0.60 mg g^{-1} of fresh weight) followed by the treatment T_1 (0.55 mg g^{-1} of fresh weight). Under elevated CO_2 condition (Table 21), highest per cent increase in carotenoid content was noticed in the cuttings of Panniyur 5 and in trench system per cent increase was highest in bush types of Panniyur 5 compared to absolute control.

4.2.3 Stomatal Frequency

Effect of elevated CO_2 on stomatal frequency at the end of exposure is presented in Table 22. Lower values for stomatal frequencies were noticed in varieties under all the treatments compared to absolute control. Stomatal frequencies of treatment T_1 (1311 no cm^{-2}), T_2 (1367 no cm^{-2}) and T_3 (1442 no cm^{-2}) were found to be on par. Lowest per cent reduction in stomatal frequencies was noticed in Panniyur 1 bush followed by Karimunda cuttings under elevated CO_2 condition when compared with absolute control plants.

4.2.4 Stomatal Conductance

Stomatal conductance in the varieties exposed to elevated CO_2 were noticed for lowest mean value (28.70 mmoles m^{-2} s^{-1}) compared to control plants (40.98 mmoles m^{-2} s^{-1}). The mean values for stomatal conductance in treatment T_2 was found to be on par with absolute control as presented in Table 23. Compared to absolute control stomatal conductance was found to be lower in the cuttings of Karimunda under elevated CO_2.

Table 20. Effect of elevated CO_2 on carotenoid, (mg g^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.42	0.42	0.14	0.36	0.33
Panniyur 1 (Bush)	0.43	0.43	0.16	0.48	0.37
Panniyur 5 (Cuttings)	0.42	0.46	0.11	0.39	0.34
Panniyur 5 (Bush)	0.44	0.46	0.11	0.22	0.31
Karimunda (Cuttings)	0.43	0.43	0.15	0.31	0.33
Karimunda (Bush)	0.45	0.42	0.08	0.39	0.33
Mean	0.43	0.44	0.12	0.36	
CD (0.05)	T- 0.021		V- 0.026	T x V- 0.053	

Table 21. Effect of elevated CO_2 on carotenoid, (mg g^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.63	0.78	0.55	0.40	0.59
Panniyur 1 (Bush)	0.40	0.85	0.79	0.75	0.70
Panniyur 5 (Cuttings)	0.83	0.61	0.57	0.35	0.59
Panniyur 5 (Bush)	0.54	0.74	0.57	0.32	0.54
Karimunda (Cuttings)	0.48	0.66	0.74	0.61	0.62
Karimunda (Bush)	0.44	0.62	0.40	0.36	0.45
Mean	0.55	0.711	0.60	0.46	
CD (0.05)	T- 0.108		V- 0.132	T x V- 0.265	

Table 22. Effect of elevated CO_2 on stomatal frequency, (no cm^{-2}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	1674.10	1729.91	1339.28	2287.94	1757.80
Panniyur 1 (Bush)	1116.07	1283.48	1729.91	1953.12	1520.60
Panniyur 5 (Cuttings)	1227.67	1283.48	1618.30	1060.26	1297.40
Panniyur 5 (Bush)	1227.67	1171.87	1562.50	1674.10	1409.00
Karimunda (Cuttings)	1227.67	1116.07	1283.48	1841.51	1367.20
Karimunda (Bush)	1395.08	1618.30	1116.07	1450.89	1395.10
Mean	1311.38	1367.19	1441.59	1711.31	
CD (0.05)	T- 182.610		V- 223.650	T x V- 447.300	

Table 23. Effect of elevated CO_2 on stomatal conductance, mmoles m^{-2} s^{-1}

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	27.95	22.30	26.65	44.30	30.30
Panniyur 1 (Bush)	33.75	91.95	97.00	41.95	66.16
Panniyur 5 (Cuttings)	20.55	52.20	64.70	39.80	44.31
Panniyur 5 (Bush)	50.20	74.15	62.85	18.95	51.53
Karimunda (Cuttings)	13.35	18.00	47.30	39.05	29.42
Karimunda (Bush)	26.40	19.95	62.85	61.85	42.76
Mean	28.70	46.42	60.22	40.98	
CD (0.05)	T-7.148		V- 8.754	T x V- 17.509	

4.2.5 Transpiration Rate

All the treatments induced a reduction in transpiration rate compared to absolute control as depicted in Table 24. During the first month of treatment, the lowest rate of transpiration was registered in varieties under elevated CO_2 (0.5 mmoles m^{-2} s^{-1}). When compared to absolute control, the cuttings of Panniyur 5 registered the lowest rate of transpiration (0.45 mmoles m^{-2} s^{-1}) under elevated CO_2 while the rate of transpiration was found to be lowest in Panniyur 1 bush of trench system.

During the second month, except for the treatment T_1 (elevated CO_2) which holds the lowest value for transpiration rate (0.72 mmoles m^{-2} s^{-1}) all other treatments had no significant influence on the rate of transpiration. Under the treatments T_1 (elevated CO_2) and T_3 (Trench system) lowest transpiration rate was noticed in Panniyur 5 cuttings having the values of 0.65 mmoles m^{-2} s^{-1} and 0.68 mmoles m^{-2} s^{-1} respectively when compared to absolute control (Table 25).

4.2.6 Total Soluble Protein

Exposure to 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) and T_3 (Trench system) during the first month of exposure. As presented in Table 26, under elevated CO_2 condition highest per cent increase in total soluble protein was registered in Panniyur 1 bush (5.20 mg g^{-1}) and in trench system it was found to be highest in the cuttings of Panniyur 1 (5.95 mg g^{-1}) compared to absolute control.

During second month, total soluble protein was found to be superior in treatments T_1 (elevated CO_2) and T_3 (Trench system) having mean values of 6.40 mg g^{-1} and 6.22 mg g^{-1} respectively. Among the varieties of treatment T_3 (Trench system) and T_1 (elevated CO_2), Panniyur 5 cuttings registered the highest mean

value for total soluble protein content (7.40 mg g^{-1}) when compared to absolute control as depicted in Table 27.

Table 24. Effect of elevated CO_2 on transpiration rate, (mmoles $m^{-2}s^{-1}$) after one month of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.69	0.47	1.39	1.17	0.93
Panniyur 1 (Bush)	0.37	1.19	0.81	1.35	0.93
Panniyur 5 (Cuttings)	0.45	1.44	1.06	1.75	1.17
Panniyur 5 (Bush)	0.79	1.15	1.51	1.54	1.25
Karimunda (Cuttings)	0.57	0.82	1.16	1.17	0.93
Karimunda (Bush)	0.60	0.82	1.47	1.63	1.13
Mean	0.58	0.98	1.23	1.43	
CD (0.05)	T- 0.076		V- 0.093		T x V- 0.187

Table 25. . Effect of elevated CO_2 on transpiration rate, (mmoles $m^{-2}s^{-1}$) at the end of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.50	0.81	0.53	0.87	0.68
Panniyur 1 (Bush)	0.48	0.55	1.06	0.88	0.74
Panniyur 5 (Cuttings)	0.65	1.55	0.68	1.40	1.07
Panniyur 5 (Bush)	1.78	0.99	1.05	0.51	1.08
Karimunda (Cuttings)	0.44	0.61	0.98	0.59	0.65
Karimunda (Bush)	0.51	0.78	0.73	0.81	0.71
Mean	0.72	0.88	0.84	0.84	
CD (0.05)	T- 0.073		V- 0.090		T x V- 0.180

Table 26. Effect of elevated CO_2 on total soluble protein, (mg g^{-1}) after one month of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	4.20	3.15	5.95	3.20	4.12
Panniyur 1 (Bush)	5.20	3.95	5.15	3.20	4.37
Panniyur 5 (Cuttings)	4.50	4.30	5.05	3.95	4.45
Panniyur 5 (Bush)	4.85	3.95	3.00	5.05	4.21
Karimunda (Cuttings)	3.30	3.25	2.00	3.35	2.97
Karimunda (Bush)	3.30	3.40	4.30	3.20	3.55
Mean	4.22	3.66	4.24	3.65	
CD (0.05)	T- 0.305		V- 0.374	T x V- 0.748	

Table 27. Effect of elevated CO_2 on total soluble protein, (mg g^{-1}) at the end of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	6.65	4.35	8.35	4.15	5.87
Panniyur 1 (Bush)	4.50	4.15	7.40	4.30	5.08
Panniyur 5 (Cuttings)	7.40	4.60	7.40	3.50	5.72
Panniyur 5 (Bush)	7.85	3.40	4.35	4.10	4.92
Karimunda (Cuttings)	6.00	6.05	5.05	3.60	5.17
Karimunda (Bush)	6.00	5.15	4.80	4.75	5.17
Mean	6.40	4.61	6.22	4.06	
CD (0.05)	T- 0.482		V- 0.591	T x V- 1.182	

4.2.7 Starch

During the first month of exposure, starch content was found to be highest in treatment T_1 (under elevated CO_2) (31.91 mg g^{-1}) followed by the treatment T_3 (Trench system) (10.61 mg g^{-1}). All the varieties under elevated CO_2 hold its significantly higher mean values for starch content which was followed by the varieties in Trench system (Table 28). In treatment T_1, cuttings of Karimunda recorded the highest mean value for starch content (40.27 mg g^{-1}) whereas in T_3 bush types of Panniyur 1 holds the highest value for starch content compared to absolute control.

The same trend was noticed during the second month also with significantly higher mean values of 29.92 mg g^{-1} under the treatment T_1 (under elevated CO_2) and 14.43 mg g^{-1} in treatment T_3 (Trench system) as presented in Table 29. In both the treatments T_1 and T_3 starch content was found to be highest in the cuttings of Karimunda when compared to absolute control.

4.2.8 Reducing Sugar

During first month, the treatment T_3 (trench system) holds the highest mean value for reducing sugar content (9.25 mg g^{-1}) whereas no significant variation in reducing sugar content was noticed between the other treatments (Table 30). In treatment T_3 reducing sugar content was found to be highest in Karimunda bush compared to absolute control and have a value of 12.50 mg g^{-1}.

During the second month of exposure (Table 31), among different treatments reducing sugar content was found to be highest in treatment T_1 (under elevated CO_2) followed by the treatment T_3 (Trench system) which holds a mean values 15.06 mg g^{-1} of 11.91 mg g^{-1} respectively. Under elevated CO_2 condition (T_1) reducing sugar content was found to be highest in the bush types of Panniyur 5 (18.50 mg g^{-1}) and in trench system Karimunda bush registered the highest value for reducing sugar (15.90 mg g^{-1}) compared to absolute control.

Table 28. Effect of elevated CO_2 on starch content, (mg g^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	29.47	7.42	10.57	7.87	13.83
Panniyur 1 (Bush)	32.40	7.42	10.57	7.42	14.45
Panniyur 5 (Cuttings)	28.12	7.87	10.35	8.55	13.72
Panniyur 5 (Bush)	24.07	8.77	10.57	8.55	12.99
Karimunda (Cuttings)	40.27	7.65	11.02	7.87	16.70
Karimunda (Bush)	37.12	7.60	10.57	7.87	15.79
Mean	31.91	7.79	10.61	8.02	
CD (0.05)	T- 0.989		V- 1.211	T x V- 2.423	

Table 29. Effect of elevated CO_2 on starch content, (mg g^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	28.80	8.10	10.35	5.62	13.21
Panniyur 1 (Bush)	28.12	6.52	12.15	6.07	13.21
Panniyur 5 (Cuttings)	29.47	7.65	15.07	5.62	14.45
Panniyur 5 (Bush)	33.30	7.65	16.20	5.40	15.63
Karimunda (Cuttings)	33.07	7.87	15.97	4.27	15.30
Karimunda (Bush)	26.77	8.00	16.87	5.62	14.31
Mean	29.92	7.63	14.43	5.43	
CD (0.05)	T- 1.024		V- 1.254	T x V- 2.508	

Table 30. Effect of elevated CO_2 on reducing sugar, (mg g^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	6.50	4.20	8.90	8.20	6.95
Panniyur 1 (Bush)	6.80	5.50	8.70	6.50	6.87
Panniyur 5 (Cuttings)	6.00	7.50	8.90	6.90	7.32
Panniyur 5 (Bush)	5.60	6.60	9.60	5.60	6.85
Karimunda (Cuttings)	5.60	7.10	6.90	5.10	6.17
Karimunda (Bush)	7.00	6.95	12.50	7.00	8.36
Mean	6.25	6.30	9.25	6.55	
CD (0.05)	T-0.786		V-0.963	T x V- 1.927	

Table 31. Effect of elevated CO_2 on reducing sugar, (mg g^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	11.90	10.00	11.40	10.0	10.82
Panniyur 1 (Bush)	14.00	9.50	10.80	9.10	10.85
Panniyur 5 (Cuttings)	15.70	9.50	11.70	8.90	11.45
Panniyur 5 (Bush)	18.50	10.50	12.10	9.00	12.52
Karimunda (Cuttings)	16.30	9.10	9.60	9.00	11.00
Karimunda (Bush)	14.00	9.30	15.90	8.30	11.87
Mean	15.06	9.65	11.91	9.05	
CD (0.05)	T- 0.883		V- 1.081	T x V- 2.163	

4.2.9 Phenol Content

Elevated CO_2 negatively influence the phenol content of varieties during the first month of exposure (Table 32). A reduction in phenol content was registered in treatments T_3 (Trench system) and T_1 (elevated CO_2) which holds the values of 0.357 mg g^{-1} and 0.664 mg g^{-1} respectively. Phenol content of treatment T_1 (elevated CO_2) was found to be on par with treatment T_2 (ambient CO_2). Phenol content was found to be lowest in Panniyur 1 cuttings under elevated CO_2 and in the bushes of same variety in Trench system compared to absolute control.

During the second month of exposure, the treatments T_1 (0.70 mg g^{-1}) and T_2 (0.70 mg g^{-1}) were noticed to have the highest mean values for phenol content which were found to be on par also. The treatment T_3 (trench system) exhibited a reduction in phenol content (0.42 mg g^{-1}) compared to absolute control. Under elevated CO_2 the highest phenol content was noticed in the cuttings of Karimunda as presented in Table 33.

4.2.10 Free Aminoacid

During the first month of exposure, a significant reduction in free aminoacid content was registered in varieties under the treatments T_3 (Trench system) (1.03 mg g^{-1}) and T_1 (elevated CO_2) (1.03 mg g^{-1}). A significant reduction in free aminoacid content was noticed in treatment T_2 (1.61 mg g^{-1}) when compared to absolute control. Among the varieties under treatment T_1, free aminoacid content was observed to be highest in bush types of Panniyur 1(Table 34).

During the second month, there occurs a significant increase in free aminoacid content in varieties under treatment T_1 (elevated CO_2) having a mean value of 2.59 mg g^{-1}as depicted in Table 35. All other treatment means were found to be on par. Both in treatments T_1 and T_3 free aminoacid content was found to be highest in the bushes of Panniyur 1 when compared with absolute control.

Table 32. Effect of elevated CO_2 on phenol content, (mg g^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.58	0.80	0.31	0.81	0.62
Panniyur 1 (Bush)	0.73	0.70	0.26	0.82	0.63
Panniyur 5 (Cuttings)	0.62	0.71	0.41	0.67	0.60
Panniyur 5 (Bush)	0.64	0.51	0.37	0.87	0.60
Karimunda (Cuttings)	0.69	0.68	0.41	0.79	0.64
Karimunda (Bush)	0.70	0.69	0.36	0.64	0.60
Mean	0.66	0.68	0.35	0.77	
CD (0.05)	T- 0.032		V- NS		T x V- 0.079

Table 33. Effect of elevated CO_2 on phenol content, (mg g^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.63	0.70	0.44	0.66	0.61
Panniyur 1 (Bush)	0.71	0.71	0.38	0.67	0.62
Panniyur 5 (Cuttings)	0.63	0.70	0.42	0.64	0.59
Panniyur 5 (Bush)	0.70	0.72	0.44	0.55	0.60
Karimunda (Cuttings)	0.86	0.68	0.42	0.58	0.64
Karimunda (Bush)	0.65	0.67	0.42	0.58	0.58
Mean	0.70	0.70	0.42	0.61	
CD (0.05)	T- 0.017		V- 0.021		T x V- 0.043

Table 34. Effect of elevated CO_2 on free aminoacid content, (mg g^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	1.02	1.48	0.92	1.82	1.31
Panniyur 1 (Bush)	2.42	1.80	1.38	1.40	1.75
Panniyur 5 (Cuttings)	0.88	1.20	1.06	1.66	1.20
Panniyur 5 (Bush)	0.88	1.62	0.66	1.70	1.21
Karimunda (Cuttings)	1.64	1.94	1.32	2.16	1.76
Karimunda (Bush)	1.96	1.62	0.86	2.51	1.73
Mean	1.46	1.61	1.03	1.87	
CD (0.05)	T- 0.127		V- 0.155		T x V-0.311

Table 35. Effect of elevated CO_2 on free aminoacid content, (mg g^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	3.26	1.72	1.88	1.86	2.18
Panniyur 1 (Bush)	2.94	2.30	2.28	1.40	2.23
Panniyur 5 (Cuttings)	2.46	1.58	1.80	2.14	1.99
Panniyur 5 (Bush)	2.42	1.74	0.84	1.54	1.63
Karimunda (Cuttings)	2.00	1.80	2.12	2.14	2.01
Karimunda (Bush)	2.48	1.90	2.40	2.22	2.25
Mean	2.59	1.84	1.88	1.88	
CD (0.05)	T- 0.149		V- 0.183		T x V- 0.366

4.2.11 Wax Content

During first month, significantly higher amount of wax production was noticed in treatment T_1 (under elevated CO_2) having a value of 2.95 mg cm^{-2}. The wax content in varieties under treatment T_3 was on par with absolute control as presented in Table 36. Compared to absolute control highest per cent increase in wax production was registered in Karimunda bush under elevated CO_2 and in Panniyur 1 cuttings of Trench system.

The effect of elevated CO_2 on wax production at the end of exposure is presented in Table 37. Highest wax production was noticed under the treatment T_1 (elevated CO_2) (2.65 mg cm^{-2}). In varieties under the treatment T_3 a significant increase in wax production was noticed (2.31 mg cm^{-2}). The mean value for wax content in varieties under treatment T_2 (ambient CO_2) was found to be on par with absolute control. Both in treatments T_1 and T_3 highest per cent increase in wax content was noticed in Panniyur 5 cuttings compared to absolute control.

4.2.12 Membrane Integrity

Membrane integrity is expressed in terms of % leakage. During first month, % leakage was found to be highest (32.77%) in varieties of treatment T_1 (elevated CO_2) and lowest (13.44%) for the varieties under the treatment T_3 (Trench system) compared to absolute control condition. In treatments T_1 and T_3 % leakage was found to be lowest in the bush types of Panniyur 1 compared to absolute control (Table 38).

During second month, there occurs a reduction in % leakage of varieties under treatment T_1 (elevated CO_2) and was found to be on par with varieties under the treatment T_3 (Trench system) and T_4 (absolute control). When compared to absolute control Panniyur 5 bush under elevated CO_2 registered the lowest % leakage where as in trench system lowest % leakage was noticed in the cuttings of Panniyur 5 as depicted in Table 39.

Table 36. Effect of elevated CO_2 on wax content, (mg cm^{-2}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	2.70	1.99	2.51	1.92	2.28
Panniyur 1 (Bush)	2.68	1.58	1.27	2.18	1.93
Panniyur 5 (Cuttings)	2.74	1.95	2.33	2.25	2.31
Panniyur 5 (Bush)	2.95	1.98	2.20	1.91	2.26
Karimunda (Cuttings)	3.32	2.30	2.03	2.06	2.42
Karimunda (Bush)	3.36	2.15	1.75	1.79	2.26
Mean	2.95	1.99	2.01	2.02	
CD (0.05)	T- 0.147		V- 0.180		T x V- 0.360

Table 37. Effect of elevated CO_2 on wax content, (mg cm^{-2}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	2.99	2.02	2.81	1.99	2.45
Panniyur 1 (Bush)	3.01	2.31	1.24	2.09	2.16
Panniyur 5 (Cuttings)	2.98	1.75	2.60	1.11	2.11
Panniyur 5 (Bush)	1.81	2.11	2.44	2.94	2.32
Karimunda (Cuttings)	2.76	2.48	2.19	2.22	2.41
Karimunda (Bush)	2.35	2.29	2.62	2.11	2.34
Mean	2.65	2.16	2.31	2.07	
CD (0.05)	T-0.110		V-0.135		T x V-0.270

Table 38. Effect of elevated CO_2 on membrane integrity, (in terms of % leakage) one month after exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	27.62	27.98	14.27	16.91	21.69
Panniyur 1 (Bush)	34.59	37.28	10.93	25.16	26.99
Panniyur 5 (Cuttings)	46.22	25.65	12.44	16.60	25.23
Panniyur 5 (Bush)	28.84	22.11	14.61	15.95	20.38
Karimunda (Cuttings)	24.39	25.59	17.64	15.75	20.84
Karimunda (Bush)	34.95	26.53	10.76	8.42	20.16
Mean	32.77	27.52	13.44	16.46	
CD (0.05)	T- 2.445		V- 2.995	T x V- 5.990	

Table 39. Effect of elevated CO_2 on membrane integrity, (in terms of % leakage) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	14.67	20.64	12.73	11.65	14.92
Panniyur 1 (Bush)	12.97	17.02	13.90	11.55	13.86
Panniyur 5 (Cuttings)	15.48	24.79	17.36	23.95	20.39
Panniyur 5 (Bush)	13.98	15.25	15.54	12.96	14.43
Karimunda (Cuttings)	14.13	23.50	15.83	16.32	17.44
Karimunda (Bush)	16.53	19.75	25.47	11.68	18.36
Mean	14.62	20.16	16.80	14.68	
CD (0.05)	T-2.413		V- 2.956	T x V- 5.912	

4.2.13 Stable Isotope Discrimination

The effect of elevated CO_2 on isotope discrimination values is presented in Fig.2. Highest mean value for isotope discrimination was observed in treatment T_1 (elevated CO_2) followed by the treatment T_2 (chamber with ambient CO_2). Among the varieties under elevated CO_2 condition isotope discrimination values were found to be highest in the bushes and cuttings of Karimunda.

4.3 ANTIOXIDANTS

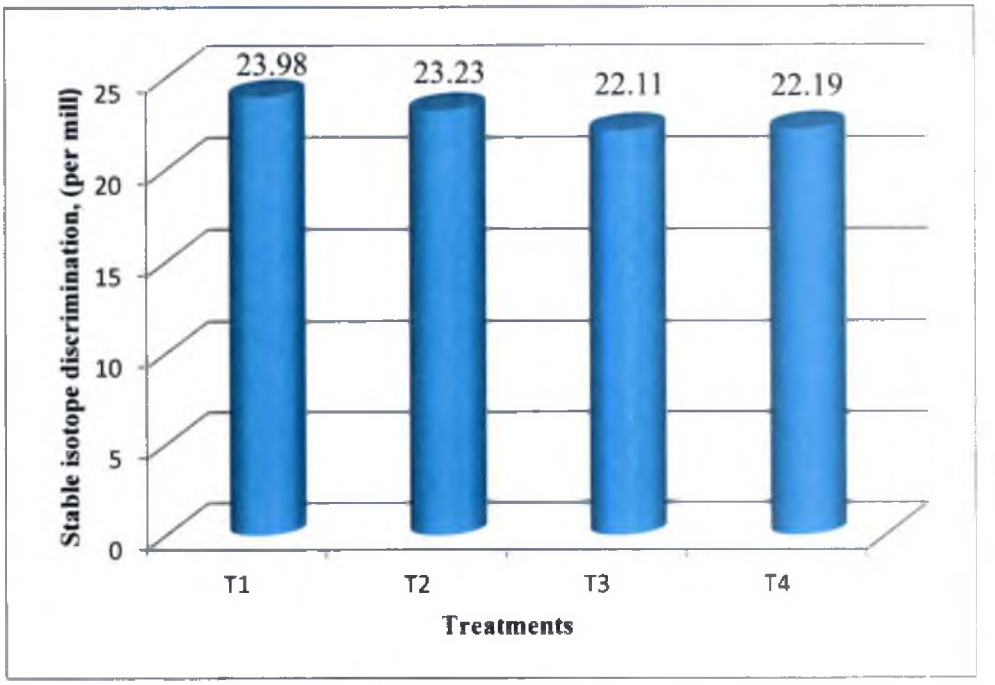
4.3.1 Peroxidase

During the first month of exposure, peroxidase activity was found to be highest (2.91 activity g^{-1} min^{-1}) in varieties under treatment T_3 (Trench system) compared to control plants (1.66 activity g^{-1} min^{-1}). The treatment means of T_1 (2.33 activity g^{-1} min^{-1}) and T_2 (2.18 activity g^{-1} min^{-1}) were found to be on par but superior than absolute control. In both the treatments T_1 and T_3 peroxidase activity was found to be highest in the cuttings of Karimunda compared to absolute control (Table 40).

During second month also highest peroxidase activity (2.67 activity g^{-1} min^{-1}) was registered in varieties under treatment T_3 (Trench system) followed by the treatment T_1 (2.41 activity g^{-1} min^{-1}). A significant reduction (1.58 activity g^{-1} min^{-1}) in peroxidase activity was noticed under treatment T_2 (chamber with ambient CO_2). As presented in Table 41, when compared to absolute control peroxidase activity was found to be highest in Karimunda bush of Trench system and in Panniyur 1 cuttings under elevated CO_2.

4.3.2 Catalase

During the first month of exposure, the treatment T_3 (Trench system) was noticed for its highest mean value for catalase activity (0.368 activity g^{-1} min^{-1}) while the mean values of all other treatments were found to be on par. Under elevated CO_2 and in Trench system the cuttings of Panniyur 5 were observed to have the highest catalase activity when compared to absolute control, depicted in Table 42.



T1: OTC (Ec) T2: OTC (Ac) T3: Trench system T4: Absolute control

Fig.2. Effect of elevated CO_2 on stable isotope discrimination, per mill

Table 40. Effect of elevated CO_2 on peroxidase activity, (activity g^{-1} min^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	2.77	1.95	3.60	1.07	2.35
Panniyur 1 (Bush)	2.37	3.38	3.50	2.35	2.90
Panniyur 5 (Cuttings)	3.42	2.35	1.98	2.48	2.55
Panniyur 5 (Bush)	2.20	1.83	2.82	1.56	2.10
Karimunda (Cuttings)	1.82	1.78	2.77	0.70	1.77
Karimunda (Bush)	1.40	1.79	2.80	1.82	1.95
Mean	2.33	2.18	2.91	1.66	
CD (0.05)	T- 0.205		V- 0.251		T x V- 0.505

Table 41. Effect of elevated CO_2 on peroxidase activity, (activity g^{-1} min^{-1}) at the end of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	3.02	1.65	2.22	1.90	2.20
Panniyur 1 (Bush)	2.37	1.63	2.22	1.68	1.97
Panniyur 5 (Cuttings)	2.40	1.95	2.67	1.80	2.20
Panniyur 5 (Bush)	2.00	1.50	2.50	2.23	2.05
Karimunda (Cuttings)	2.67	1.38	2.42	2.05	2.13
Karimunda (Bush)	2.02	1.38	4.02	1.88	2.32
Mean	2.41	1.58	2.67	1.92	
CD (0.05)	T- 0.220		V-0.267		T x V-0.539

Table 42. Effect of elevated CO_2 on catalase activity, (activity g^{-1} min^{-1}) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.187	0.162	0.312	0.218	0.220
Panniyur 1 (Bush)	0.125	0.231	0.468	0.337	0.290
Panniyur 5 (Cuttings)	0.243	0.312	0.268	0.093	0.229
Panniyur 5 (Bush)	0.156	0.125	0.343	0.250	0.218
Karimunda (Cuttings)	0.256	0.162	0.506	0.231	0.289
Karimunda (Bush)	0.231	0.188	0.312	0.200	0.233
Mean	0.200	0.197	0.368	0.221	
CD (0.05)	T- 0.045		V-0.055		T x V- 0.111

The effect of elevated CO_2 on catalase activity at the end of exposure is presented in Table 43. The catalase activity (0.436 activity g^{-1} min^{-1}) under elevated CO_2 condition (T_1) was found to be on par with absolute control (0.438 activity g^{-1} min^{-1}). Highest mean value for catalase activity was registered in treatment T_2 (0.974 activity g^{-1} min^{-1}) followed by the treatment T_3 (Trench system). Compared to absolute control both in treatments T_1 and T_3 Panniyur 5 cuttings was noticed to have the highest catalase activity.

4.3.3 SOD

The effect of elevated CO_2 on SOD activity at the end of exposure period is presented in Table 44. The highest mean value (0.91 activity g^{-1} min^{-1}) for SOD activity was recorded in varieties under treatment T_1 (elevated CO_2) followed by the treatment T_3 (Trench system) (0.77 activity g^{-1} min^{-1}). Compared to absolute control SOD activity was significantly higher under the treatment T_2 (0.63 activity g^{-1} min^{-1}). The per cent increase in SOD activity was found to be highest in the bushes of Panniyur 5 under elevated CO_2 and in the cuttings of Panniyur 5 of trench system when compared to absolute control.

4.3.4 Ascorbic Acid

During first month, highest mean value (11.87 mg $100g^{-1}$) for ascorbic acid content was noticed in varieties of treatment T_1 (elevated CO_2). A significant reduction in ascorbic acid content was noticed in treatments T_3 (8.87 mg $100g^{-1}$) and T_2 (7.65 mg $100g^{-1}$). In treatment T_1 (elevated CO_2) per cent increase in ascorbic acid content was highest in the cuttings of Karimunda whereas in T_3 (Trench system) it was found to be highest in the cuttings of Panniyur 5 compared to absolute control (Table 45).

During second month, the varieties under treatment T_3 (Trench system) recorded the highest mean value (9.02 mg $100g^{-1}$) while the mean values of all other treatments were found to be on par. When compared to absolute control, the per cent increase in ascorbic acid was highest in the bush types of Panniyur 1 under elevated CO_2 as presented in Table 46. In trench system the percent

increase was highest in the cuttings of Panniyur 1 when compared to absolute control.

Table 43. Effect of elevated CO_2 on catalase activity, (activity g^{-1} min^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	0.131	0.531	0.625	0.156	0.360
Panniyur 1 (Bush)	0.313	0.498	0.937	0.331	0.520
Panniyur 5 (Cuttings)	0.625	0.968	0.687	0.168	0.612
Panniyur 5 (Bush)	0.575	1.500	0.781	0.625	0.870
Karimunda (Cuttings)	0.812	1.375	0.281	0.669	0.784
Karimunda (Bush)	0.162	0.973	0.156	0.681	0.493
Mean	0.436	0.974	0.578	0.438	
CD (0.05)	T- 0.103		V-0.127	T x V-0.254	

Table 44. Effect of elevated CO_2 on SOD activity, (activity g^{-1} min^{-1}) at the end of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	1.09	0.43	0.63	0.43	0.64
Panniyur 1 (Bush)	0.35	0.27	0.85	0.40	0.47
Panniyur 5 (Cuttings)	0.77	0.67	0.98	0.43	0.71
Panniyur 5 (Bush)	0.85	0.93	0.97	0.13	0.72
Karimunda (Cuttings)	1.04	0.80	0.68	0.67	0.79
Karimunda (Bush)	1.38	0.70	0.52	0.53	0.78
Mean	0.91	0.63	0.77	0.43	
CD (0.05)	T- 0.090		V-0.111	T x V- 0.222	

Table 45. Effect of elevated CO_2 on ascorbic acid content, (mg $100g^{-1}$) after one month of exposure.

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	9.59	8.77	7.30	10.27	8.98
Panniyur 1 (Bush)	11.64	8.53	9.46	9.46	9.77
Panniyur 5 (Cuttings)	10.96	6.85	8.65	7.90	8.59
Panniyur 5 (Bush)	13.70	6.16	8.38	11.64	9.97
Karimunda (Cuttings)	13.70	8.22	10.00	9.59	10.37
Karimunda (Bush)	11.64	7.37	9.46	11.64	10.03
Mean	11.87	7.65	8.87	10.08	
CD (0.05)	T- 1.015		V- NS		T x V- 2.486

Table 46. Effect of elevated CO_2 on ascorbic acid content, (mg $100g^{-1}$) at the end of exposure

Varieties (V)	T_1	T_2	T_3	T_4	Mean
Panniyur 1 (Cuttings)	7.03	5.13	11.64	6.22	7.50
Panniyur 1 (Bush)	7.30	6.22	6.85	5.13	6.37
Panniyur 5 (Cuttings)	5.67	6.49	8.96	7.57	7.17
Panniyur 5 (Bush)	9.73	7.84	8.90	9.46	8.98
Karimunda (Cuttings)	7.30	7.84	10.27	6.49	7.97
Karimunda (Bush)	5.95	7.74	7.53	10.00	7.80
Mean	7.16	6.87	9.02	7.47	
CD (0.05)	T- 0.900		V- 1.102		T x V- 2.205

4.3 MOLECULAR STUDIES

The electrophoretic analysis of proteins was carried out using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein profile revealed that elevated CO_2 induced the production of a few new proteins but suppressed certain others. When compared to absolute control, in the cuttings of Panniyur 1 protein bands of approximately 66kDa, 58kDa, 31kDa, 26kDa and 19kDa were found to be absent in treatments T_1, T_2 and T_3. Under elevated CO_2 condition and in trench system new band of about 20.1kDa was observed. New protein band of approximately 43kDa was observed in trench system. In the bush types of Panniyur 1, in treatments T_1 and T_2 protein bands of about 18kDa 17kDa and 15kDa were absent and new bands of 22kDa and 14.7 kDa was observed in treatment T_3.

Under elevated CO_2 condition (T_1), in the cuttings of Panniyur 5 a new polypeptide band of about 37kDa was observed when compared to absolute control. Both in treatments T_1 and T_2 a new band of about 20.1kDa was observed whereas a band of approximately 23kDa was absent when compared to absolute control. In bush types of Panniyur 5, a new band of about 92kDa was observed under elevated CO_2. Polypeptide bands of approximately 43kDa was absent in treatments T1 and T_2 compared to absolute control. Under elevated CO_2 and in treatment T_2 a new protein band of about 27kDa was noticed in the cuttings of Karimunda when compared to absolute control whereas a band of approximately 40kDa was absent in treatments T_1 and T_2. In the bushes of Karimunda approximately 82kDa protein band was absent under elevated CO_2 when compared to absolute control condition. In trench system new bands of about 40kDa and 25kDa was noticed and under elevated CO_2 and trench system a new band of approximately 20.1kDa was observed when compared to absolute control.

In all the varieties, the expression levels of both the large and small subunits of Rubisco were slightly brought down by CO_2 enrichment.

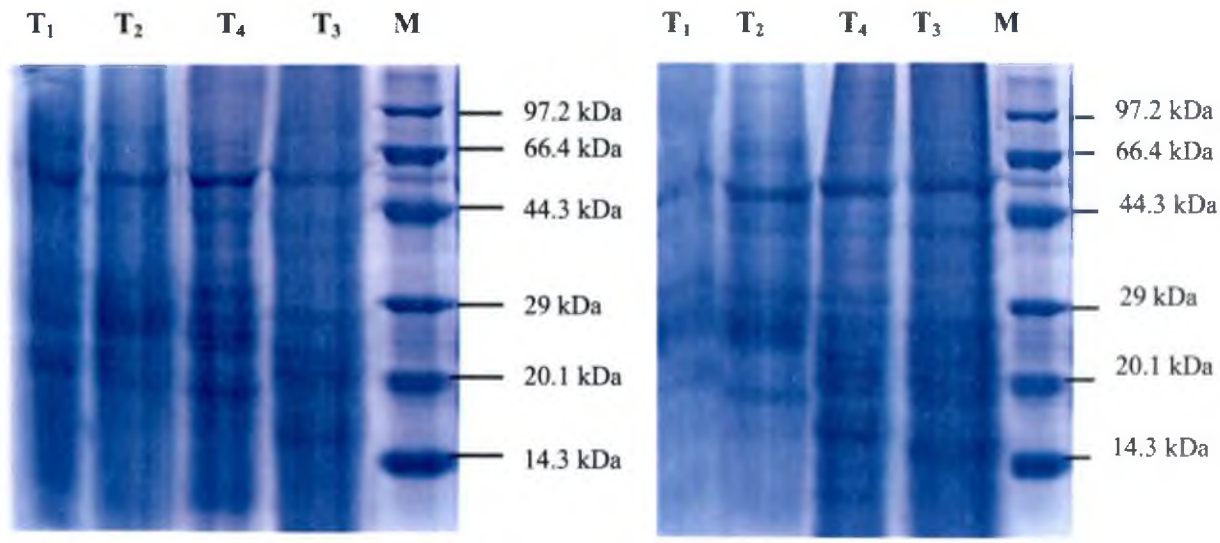


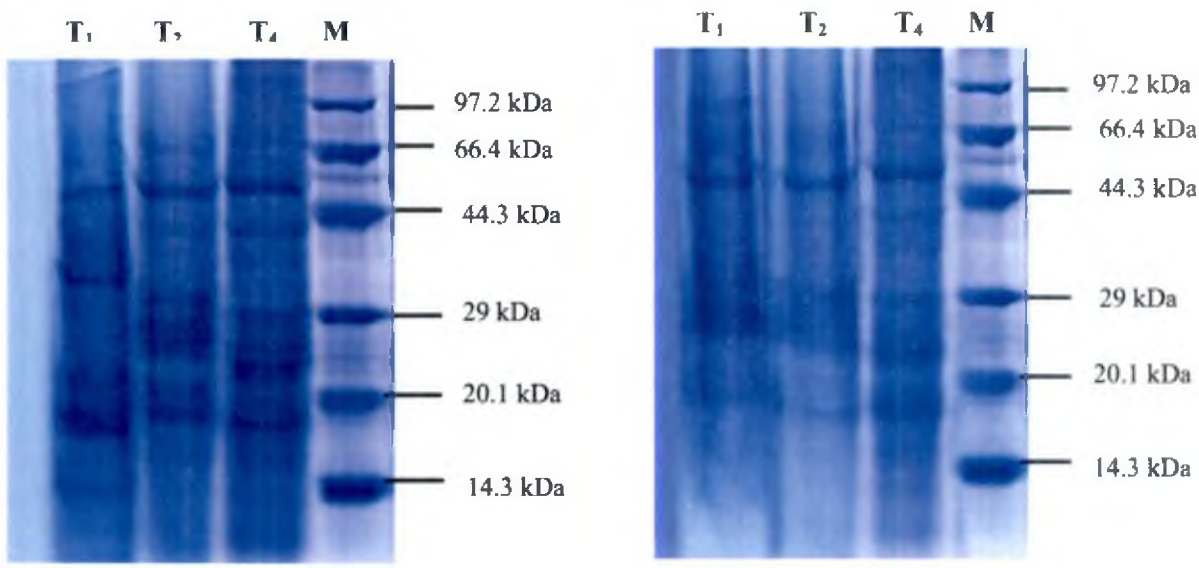
Plate 11. Protein profile of Panniyur 1 variety

T_1 - OTC with elevated CO_2 concentration (OTC Ec)

T_2 - OTC with ambient CO_2 concentration (OTC Ac)

T_3 - Trench system (T)

T_4 - Absolute control (C)



Panniyur 5 (cuttings)

Panniyur 5 (bush)

Plate 12. Protein profile of Panniyur 5 variety

T_1 - OTC with elevated CO_2 concentration (OTC Ec)

T_2 - OTC with ambient CO_2 concentration (OTC Ac)

T_3 - Trench system (T)

T_4 - Absolute control (C)

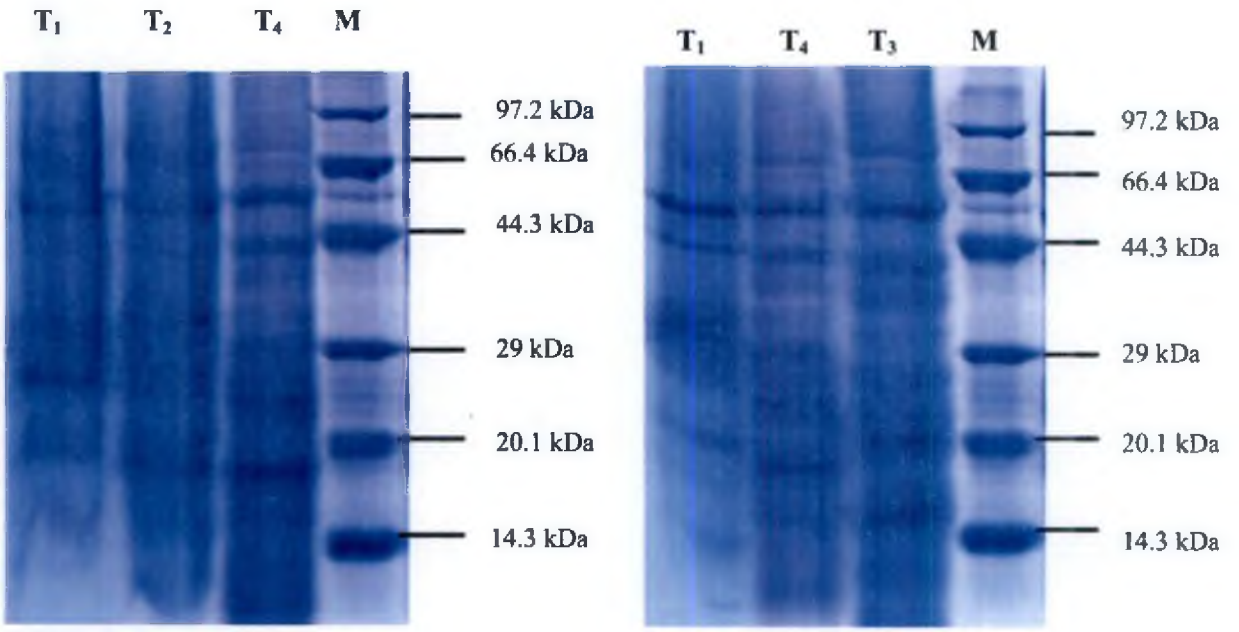


Plate 13. Protein profile of Karimunda variety

T_1 - OTC with elevated CO_2 concentration (OTC Ec)

T_2 - OTC with ambient CO_2 concentration (OTC Ac)

T_3 - Trench system (T)

T_4 - Absolute control (C)

DISCUSSION

5. DISCUSSION

The level of CO_2 in the atmosphere is rising at an unprecedented rate. According to NOAA, 2014 global concentration of CO_2 has reached 400ppm 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. Moreover the report of IPCC, 2007 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.

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.

Black pepper is a fragile plant, especially when it is young, and requires great care, being quite vulnerable to variations in the weather. Black pepper cultivation is showing a never-before decline in Kerala. Production has dropped throughout Kerala because of many reasons and one among is the changing climatic scenario. Because of the economical relevance of black pepper and its medicinal value, it is important to understand how this crop will respond to the foreseen increase in atmospheric CO_2. There is no research report available about the response of black pepper under elevated CO_2. Hence an experiment was proposed to analyse the physiological, molecular and biochemical basis of growth responses in black pepper under elevated carbon dioxide conditions.

Black pepper varieties viz. Panniyur 1, Panniyur 5 and Karimunda were used for the study. The rooted cuttings and bush types of the same varieties were kept in Open Top Chambers and Trench system for exposing them to elevated CO_2 concentrations for a period of two months. Observations on growth

parameters and molecular studies were taken at the end of the exposure period and biochemical parameters were taken at monthly intervals. The influence of elevated CO_2 on different growth parameters, physiological and biochemical parameters and antioxidant levels of black pepper are discussed in this section.

5.1 EFFECT OF ELEVATED CO_2 ON GROWTH PARAMETERS

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. Elevated CO_2 stimulates photosynthesis leading to increased carbon uptake and assimilation, thereby increasing plant growth. 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 plant height, number of leaves, leaf area, specific leaf area, net assimilation rate, relative growth rate, root weight, shoot weight, root shoot ratio and dry matter production.

According to Pritchard *et al.* (1999) alternations in developmental processes at the shoot apex and within the vascular cambium can contribute to increased plant height, altered branching characteristics and increased stem diameters under elevated CO_2 conditions.

In the present study, 18% increase in plant height was observed under elevated CO_2 condition. The increase in plant height could be related to the increased node number and stimulation of internodes elongation under CO_2 enrichment. The result was in total agreement with the findings of Roggers *et al.* (1984) in soybean. Downton *et al.* (1990) reported a 33% increase in plant height of *Garcinia mangostana* at elevated CO_2. In another study by Wu, 2004 and Prasad, 2005 the lateral growth was enhanced more than the vertical growth when wheat plants are exposed to elevated CO_2 concentration.

Of all plant organs, leaves are most morphologically diverse (Poethig, 1997) 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 84.5% under elevated CO_2 and 17.5% in trench system. 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 specialised state in which they either become components of organ primordia or contribute to internodes between organs (Clark, 1997).

Leaf growth is frequently altered by differences in plant water potential (Taylor *et al.*, 1994). Growth under elevated CO_2 enhances the efficiency of water use which resulted in increased leaf expansion rates and greater leaf expansivity. Improved water status of plants due to partial closure of stomata causes a higher turgor pressure which stimulates leaf expansion (Lenssen and Rozema, 1990). The present experimental result revealed an increase in leaf area about 174.4% under elevated CO_2. Enhancement in leaf area under elevated CO_2 was also reported in soyabean (Madhu and Hatfield, 2015), populus (Gardner *et al.*, 1995), berseem (Pal, 2004) *Phaseolus vulgaris* (Bray and Reid, 2002) etc. Increase in total leaf area under elevated CO_2 was also reported in eucalyptus (Roden and Ball, 1996), pea and soybean (Xu *et al.*, 1994) and in cotton (Wong, 1990).

Specific leaf area was found to be 13.3% less in varieties exposed to elevated CO_2 and 20.07% less in Trench system. The result was in accordance with a recent study in soyabean where 22.2% reduction in specific leaf area was

reported at 29DAP (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).

The beneficial effect of elevated CO_2 on plant production may result from various reasons, including direct photosynthetic enhancements due to CO_2 enrichment (Geissler *et al.*, 2009; Niklaus and Korner, 2004) and an increase in the amount of photosynthate available for the development of resource acquisition structures due to enhanced water use efficiency (Bazzaz, 1990). Mbikayi *et al.* (1983) reported that the increased production of biomass with CO_2 enrichment was associated with an increased net assimilation rate and relative growth rate.

The results showed 147.8% enhancement in net assimilation rate (Fig.4) and an increase in relative growth rate of 51.1% under elevated CO_2. Similar results were obtained by Zheng *et al.* (2010) in Artemisia species when grown under elevated CO_2. An initial increase in RGR was reported in rice plants grown under 100Pa CO_2 (Makino *et al.*, 1997).

Increasing atmospheric CO_2 significantly increased the final plant biomass, aboveground biomass, and belowground biomass (Obrist and Arnone, 2003). 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).

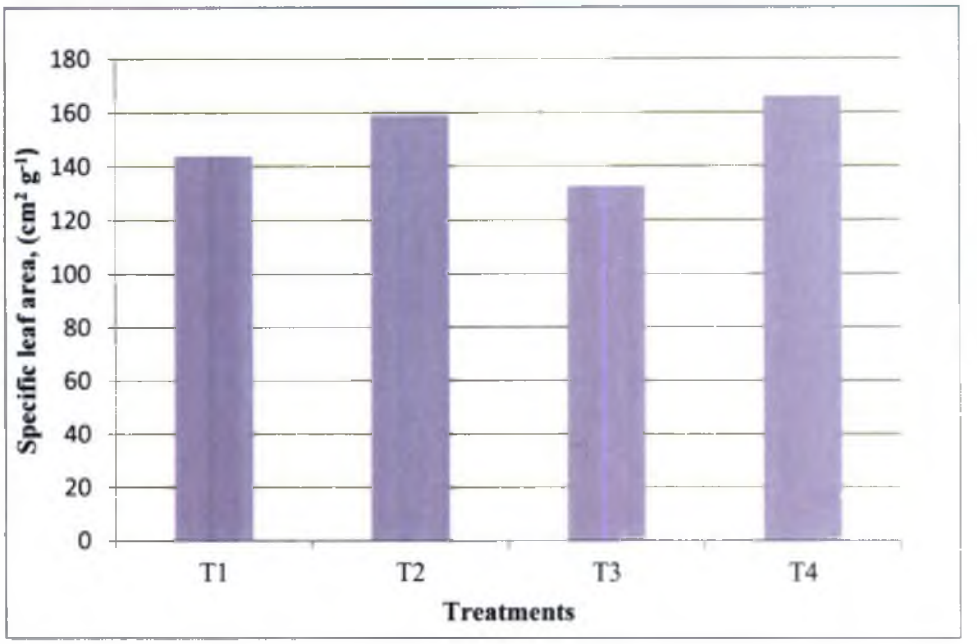
In the present study 87.5% increase in root biomass was observed under elevated CO_2 whereas 24.8% increase was noticed in trench system (Fig.5). This agrees with many results of previous studies in black gram (Vanaja *et al.*, 2007), soybean (Rogers *et al.*, 1992,) wheat (Chaudhuri *et al.*, 1990) 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

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 25.3% increase in shoot dry weight whereas in trench system 18.5% reduction was recorded. The reduction noticed in trenches was due to severe infestation of pathogens leading to a considerable loss in shoot biomass.

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.

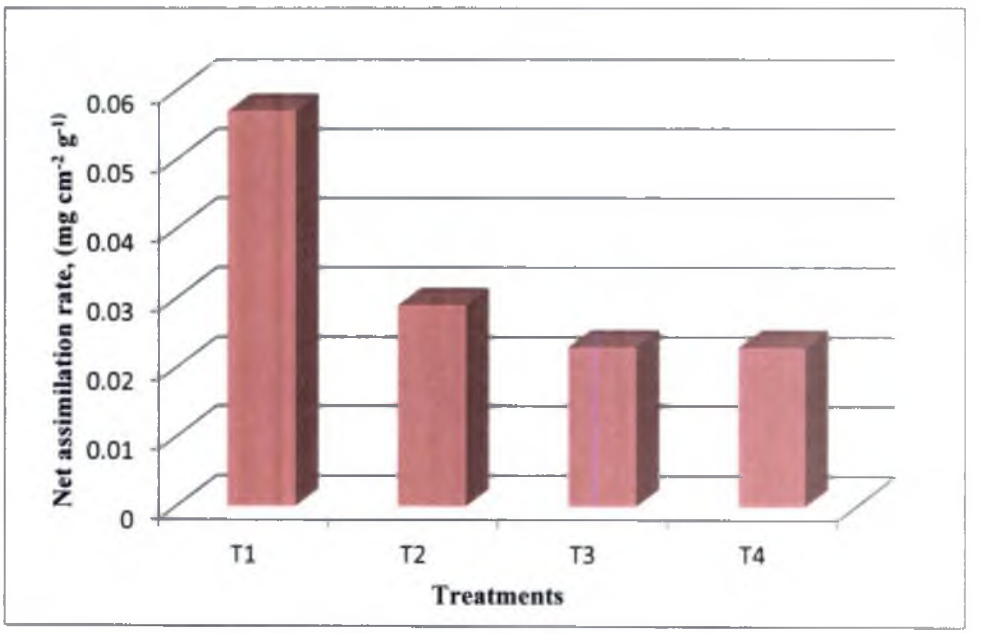
In this experiment, an increase in root shoot ratio of 42.1% was reported under elevated CO_2 and Trench system. The result was in agreement with former reports by Cure, (1985) in sweet potato, Baxter *et al.* (1994) in montane grass species, Norby *et al.*, (1992) in yellow poplar trees. Zheng *et al.* (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 (Mitchell *et al.*, 1999) and its direct consequence is increased dry matter production (Lawlor and Mitchell, 2000; Ziska *et al.*, 2004). An increase in dry matter production was seen in the present study (Fig.6). Under elevated CO_2 conditions 62.27% 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 increase



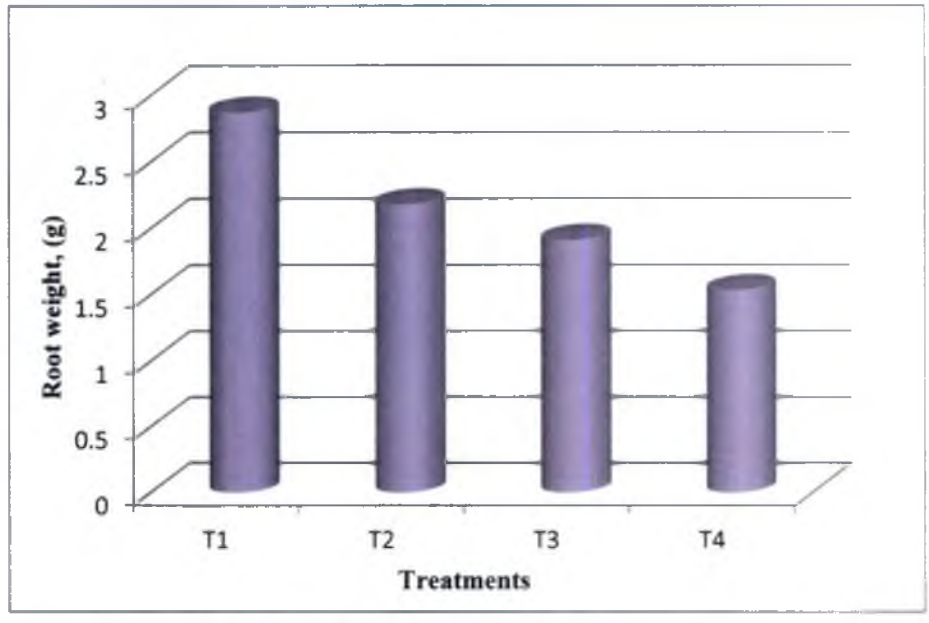
T1: OTC (Ec), T2: OTC (Ac), T3: Trench system, T4: Absolute control

Fig.3. Effect of elevated CO_2 on specific leaf area, $cm^2\ g^{-1}$



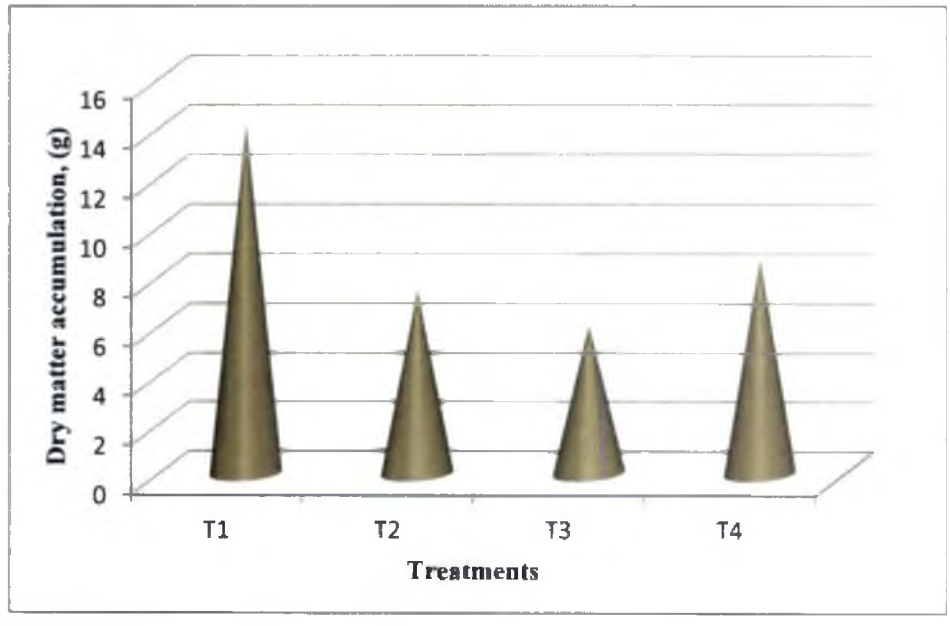
T1: OTC (Ec), T2: OTC (Ac), T3: Trench system, T4: Absolute control

Fig.4. Effect of elevated CO_2 on net assimilation rate, mg $cm^{-2}\ g^{-1}$



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system, T4: Absolute control

Fig. 5. Effect of elevated CO_2 on root weight, g



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system, T4: Absolute control

Fig. 6. Effect of elevated CO_2 on drymatter accumulation, g

in 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.

5.2 EFFECT OF ELEVATED CO_2 ON PHYSIOLOGICAL PARAMETERS

The effect of high CO_2 level on various physiological parameters like RWC, stomatal frequency, stomatal conductance, transpiration rate, pigment composition, wax content, membrane integrity and stable isotope discrimination were analysed. The results of these parameters are discussed below.

Elevated CO_2 concentration can have significant impact on the water status of plants through its direct influence on the stomatal characteristics. Leaf water status influences several physiological variables, such as leaf turgor, stomatal conductance, transpiration, photosynthesis, respiration etc. and ultimately the growth and development. Relative water content is a useful indicator of the state of water balance of a plant (Yamasaki and Dilenberg, 1999).

In addition to the effects of CO_2 on photosynthesis and C allocation elevated CO_2 can impact growth through improved plant water relations (Rogers and Dahlman, 1993). From a physiological standpoint, increased WUE can represent as one of the most significant plant responses to elevated CO_2 (Rogers *et al.*, 1994). Plant water use efficiency is strongly affected by stomatal density (Woodward and Kelly, 1995). Reduced stomatal opening leads to improved water use efficiency (Guy and Reid 1986; Clifford *et al.*, 2000) resulting in lower water stress of plants (Kimball, 1983).

In this experiment, during the initial phase of exposure the water status of the plants under elevated CO_2 was found superior to control condition. At the end of exposure, water status under elevated CO_2 and trench system was found to be on par with control. No significant difference in water status of alfalfa plants under elevated CO_2 level was reported by Aranjuelo *et al.* (2005). Increase in water status of plants under elevated CO_2 was reported in several studies. Rabha and Uprety, (1998) reported that in *Brassica juncea* exposure of elevated CO_2

concentration about 600±20μmol mol^{-1} resulted in increased relative water content. Similar result was obtained in soyabean by Rogers *et al.* (1984).

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 23% reduction in stomatal density (Fig.7) was observed under elevated CO_2 and in Trench system. Similar results were obtained in *Betula pendula* and *Fraxinus ornus* (Rey and Jarvis, 1997; Bettarini *et al.*, 1998) 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.

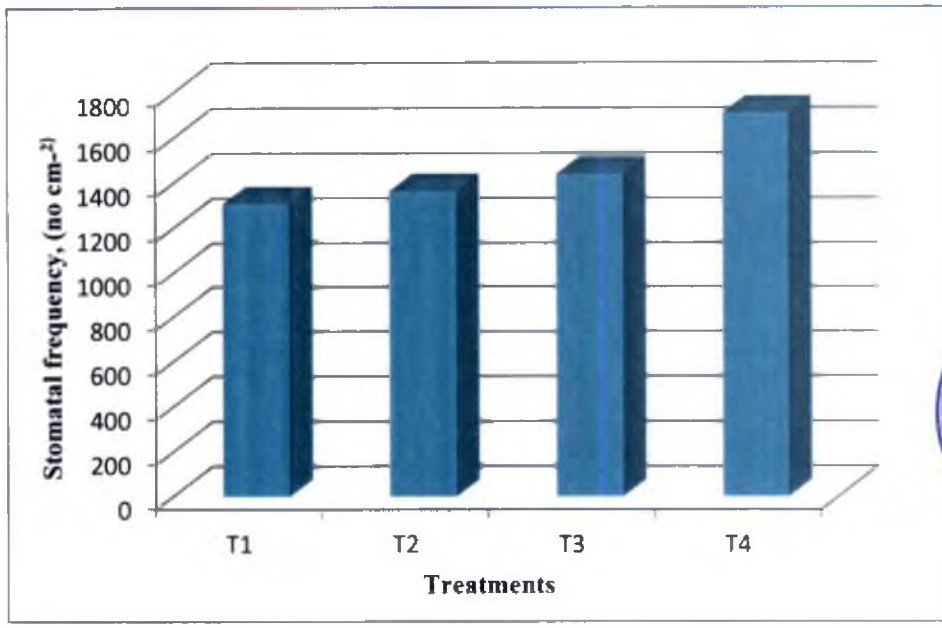
CO_2 sensing is an intrinsic property of guard cells, which are thought to respond to the intercellular CO_2 (ci) rather than CO_2 at the leaf surface (Mott, 1988). Stomatal closure requires the guard cell membrane potential to be depolarized, i.e. made less negative (Assmann, 1999). Electrophysiological studies showed that exposure to elevated CO_2 can lead to increase in the activity of outward rectifying K^+ channels, a decrease in the activity of inward rectifying K^+ channels, stimulation of Cl^- release from guard cells and increase in guard cell Ca^{2+}concentration (Webb *et al.*, 1996; Brearley *et al.*, 1997; Hanstein and Felle, 2002; Raschke *et al.*, 2003). These changes collectively depolarize the membrane potential of guard cells and cause stomatal closure (Assmann, 1993).

Around 21.26% reduction in stomatal conductance was observed under increased CO_2 concentration followed by 29.82% reduction in trench system during the initial period. At the end of exposure period 29.96% reduction in stomatal conductance was noticed. Similar results were obtained by Garcia *et al.*

(1998) in wheat leaves, in sorghum by Wall *et al.* (2001), wheat and barley (Bunce, 2000), maize and soybean (Wilson *et al.*, 1999).

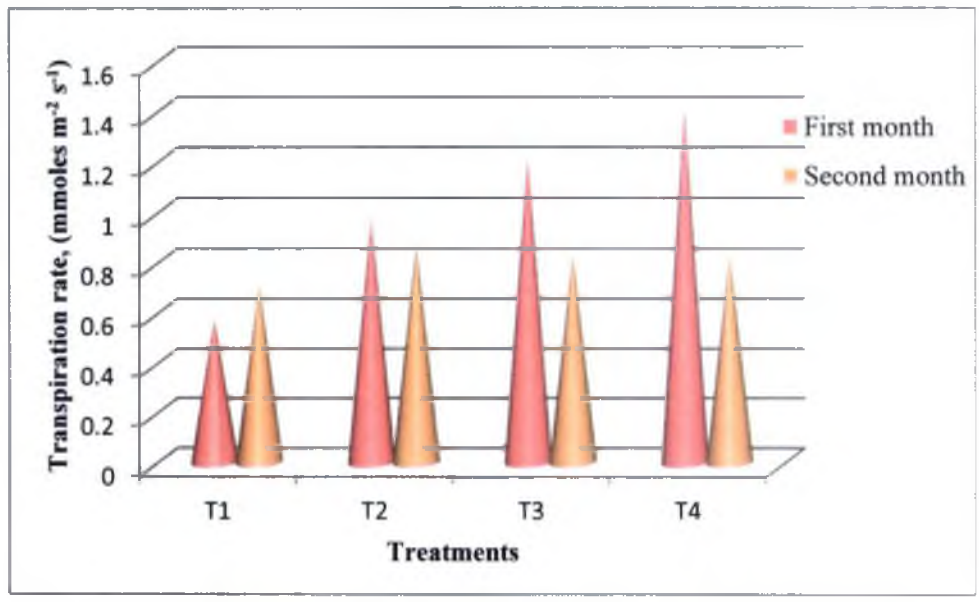
Plants respond to increases in the air's CO_2 content by displaying reduced stomatal conductance, which typically leads to reduced rates of transpirational water loss (Apple *et al.*, 2000). In crops like corn and soybean growth under elevated CO_2 caused both reversible and irreversible decreases in hydraulic conductance, which could have been related to decreased transpiration (Bunce and Ziska 1998). Robredo *et al.* (2007) have also shown that hydraulic conductance decreased markedly in barley plants grown under elevated CO_2 than those grown under the ambient CO_2 level. High levels of CO_2 also cause partial closure of leaf stomata and reduce transpiration (Bazzaz, 1991). Elevated CO_2 reduces transpiration by partially closing the stomata and decreasing stomatal conductance (Morison and Gifford 1983; Bunce, 2000).

In this experiment 59.4% reduction in transpiration rate was noticed under elevated CO_2 whereas 13.98% reduction occurred in varieties of trench system during the initial period of exposure. During the second month 14.28% reduction was noticed under elevated CO_2 (Fig.8). Reduction in transpiration rate under elevated CO_2 was reported in many studies. Wullschleger *et al.* (2002) reported reduced rates of transpiration in sweetgum trees growing in FACE plots at 540ppm of CO_2. Douglas fir seedlings grown for three years in environmental chambers under CO_2 concentration of 530ppm + 3.5°C resulted in 12% reduction of transpiration (Apple *et al.*, 2000). Kellomaki and Wang (1998) found that mature Scots pines growing at twice ambient atmospheric CO_2 concentrations displayed a 14% reduction in cumulative sap flow and also suggests significant CO_2 induced reductions in transpirational water loss. A reduction in transpiration rate was also reported in winter wheat and barley due to partial closure of stomata and decrease in stomatal conductance under elevated CO_2 (Morison and Gifford, 1983; Bunce, 2000).



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system T4: Absolute control

Fig.7. Effect of elevated CO_2 on stomatal frequency, no cm^{-2}



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system T4: Absolute control

Fig.8. Effect of elevated CO_2 on transpiration rate, mmoles m^{-2} s^{-1}

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; Karacan, 2006; Onwurah *et al.*, 2007). 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 (Karacan, 2006). 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 42.85% increase in chlorophyll a content. In the case of trench system, significant increase was found during the initial period of exposure. A significant increase in chlorophyll b content of 122.2% and 47.30% was noticed under elevated CO_2 conditions during the first and second months. In trench system 26.34% increase in chlorophyll b content was noticed at the end of exposure. During first and second month, 38.96% and 22.88% increase in total chlorophyll was noticed under elevated CO_2. In trench system at the end of exposure 21.18% increase in total chlorophyll was observed. Compared to absolute control condition carotenoid content under elevated CO_2 showed 19.5% increase during the exposure period. In trench system also 30.43% increase in carotenoid content was noticed.

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

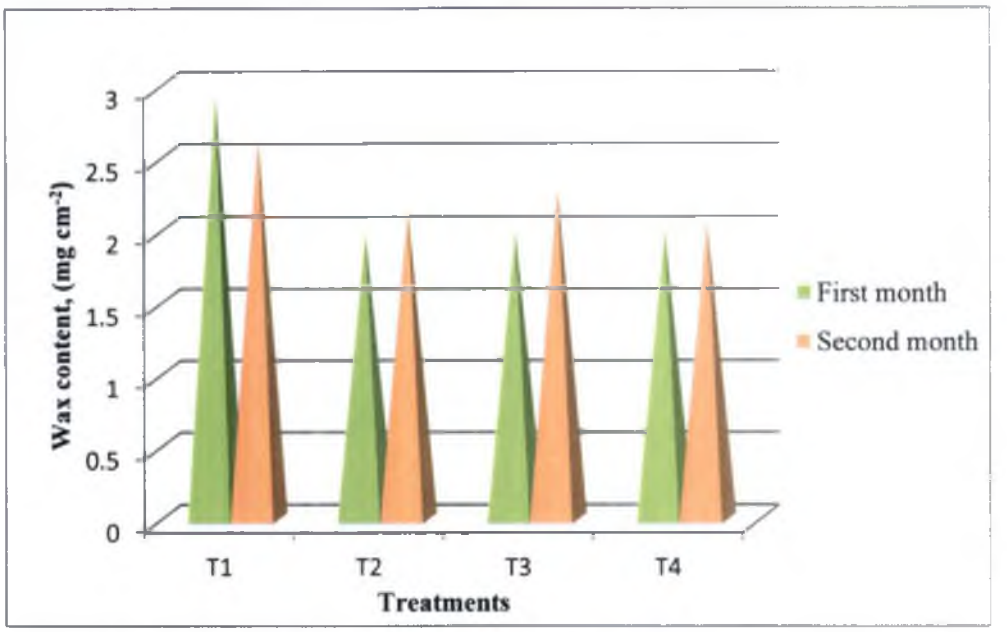
alfalfa plants grown under 600ppm of CO_2. Orchid plants subjected to elevated CO_2 showed a 64% increase in chlorophyll content (Gouk *et al.*, 1999).

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.

The aerial surfaces of all higher plants carry a partial or continuous coverage of amorphous epicuticular wax. These thin films are often surmounted by embedded crystalline wax structures, the shape of which is predetermined by the chemical composition of wax precursors (Jeffree *et al.*, 1975). The alterations in epicuticular wax structure and chemical composition are likely to affect the plant responses to abiotic and biotic stresses (Riikonen *et al.*, 2010). Elevated CO_2 had significant influence in wax production but only limited researches were carried out in this aspect.

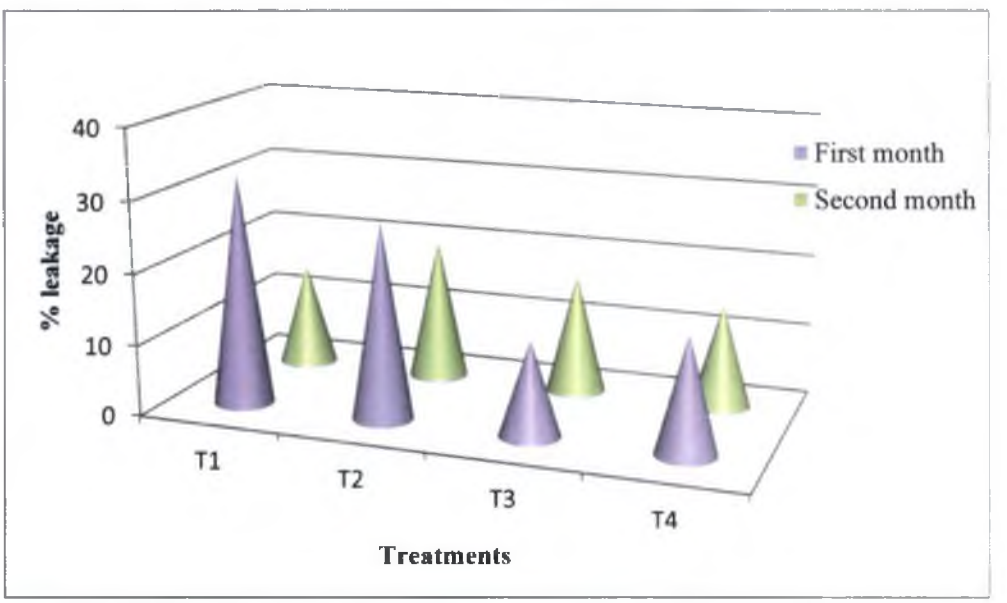
In the present study, an increase in wax content of 46.03% and 28% was noticed under elevated CO_2 during first and second month of exposure. In trench system also, 11.59% increase in wax production occurred (Fig.9). Elevated CO_2 influences wax production and composition in several tree species (Vanhatalo *et al.*, 2001; Karnosky *et al.*, 2002; Man Kovska *et al.*, 2005).

The plasma membrane is the selectively permeable lipid bilayer that surrounds the living cells. As one of the first points of contact for environmental signals upon the cell, the plasma membrane plays an important role in stress responses. This is of particular relevance in plants, which cannot move or take shelter from potentially damaging environmental conditions. So the maintenance of membrane integrity is very important to thrive under stress conditions (Eckardt, 2008).



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system T4: Absolute control

Fig.9. Effect of elevated CO_2 on wax content, mg cm^{-2}



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system T4: Absolute control

Fig.10. Effect of elevated CO_2 on membrane integrity expressed as % leakage

Significant variation was observed in the loss of membrane integrity in plants exposed to elevated CO_2. During the first month of exposure a reduction in membrane integrity was noticed in plants exposed to high CO_2 level. Due to the acclimatization of plants under elevated CO_2 there occurred an increase in membrane integrity at the end of exposure period (Fig.10).

Several physical factors have been shown to influence the integrated balance of stomatal conductance and carboxylation and thus affect isotopic discrimination in plants (Henderson *et al.*, 1998). In this study, carbon isotope discrimination values were found to be varying across the treatments and varieties. Higher discrimination values indicate higher internal CO_2 concentrations. The plants in OTC with elevated CO_2 concentration showed the highest discrimination values and this may be due the drastic reduction noticed in stomatal frequency and stomatal conductance under CO_2 enrichment that leads to a steeper gradient in CO_2 concentrations between the atmosphere and sub stomatal cavity. Among the other tested parameters drymatter accumulation was found to be enhanced about 62.27% under elevated CO_2 condition. CO_2 enrichment helps to generate better mesophyll efficiency in pepper plants which will be an added advantage leading to better photosynthetic efficiency. Across the varieties, Karimunda (cuttings and bush) exhibited the highest discrimination values indicating the better mesophyll efficiency possessed by this particular variety upon exposure to CO_2 enrichment.

5.3 EFFECT OF ELEVATED CO_2 ON BIOCHEMICAL PARAMETERS

The major biochemical compounds studied in the current experiment are total soluble proteins, starch, reducing sugar, phenols and free amino acids. Elevated CO_2 affects growth through changes in chemical composition of plants (Poorter *et al.*, 1997). Plant cells produce two types of metabolites, primary and secondary. Primary metabolites are involved directly in the growth and metabolic processes, viz. carbohydrates, lipids and proteins. Additionally, they are produced as a result of photosynthesis and are also involved in cell component synthesis. Most natural products consist of compounds derived from primary metabolites

such as amino acids, carbohydrates and fatty acids, and consequently, they are generally categorized as secondary metabolites. Exposure of plants to elevated CO_2 conditions in general enhances photosynthesis rates by inducing Rubisco enzyme activity (Moore *et al.*, 1999) and following that production of primary and secondary metabolites were enhanced (Ibrahim and Jaafar, 2012)

Elemental (e.g. Zinc, iodine) and macro molecular (Protein) composition in plant tissues are expected to change under high CO_2 (Taub and Wang, 2008). The content and type of protein of plant can also get altered due to CO_2 enrichment, as reported in wheat (Hogy and Fangmeier, 2009) and rice (Terao *et al.*, 2005).

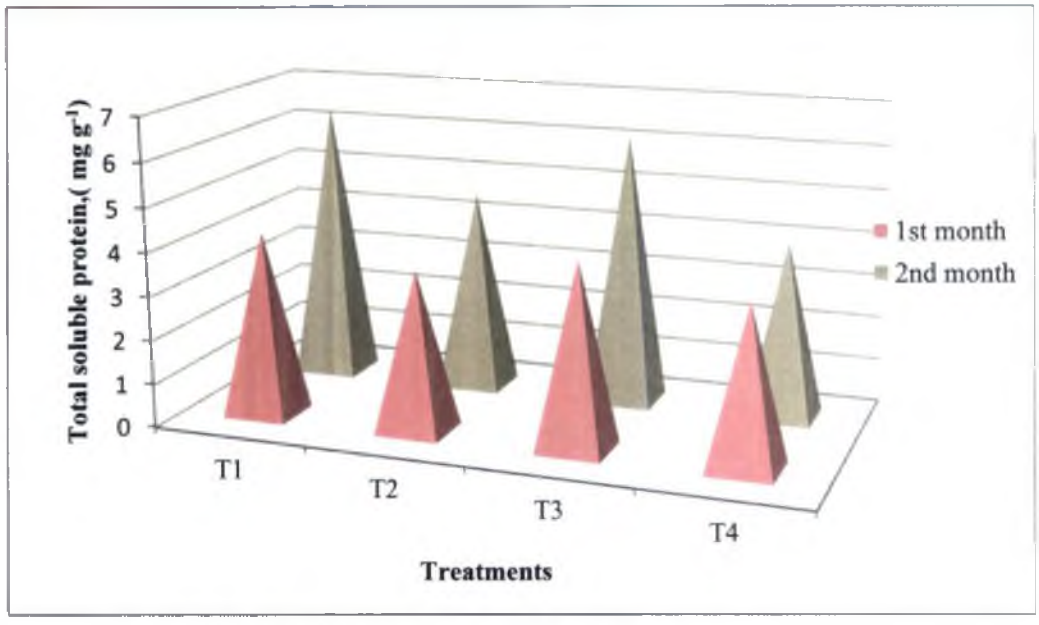
In this experiment 15.6% increase in total soluble protein was observed under elevated CO_2 whereas 16.1% increment occurred in trenches. The same trend was noticed during the second month also. Under elevated CO_2, 57.6% increase was noticed and in trenches total soluble protein increases up to 53.29% (Fig.11). 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; Zak *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 reported in *Eleais guineensis* by Ibrahim and Jaafar, (2012). Richard and James, (1997) found out that elevated CO_2 concentration of 700 $\mu mol\ mol^{-1}$ 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

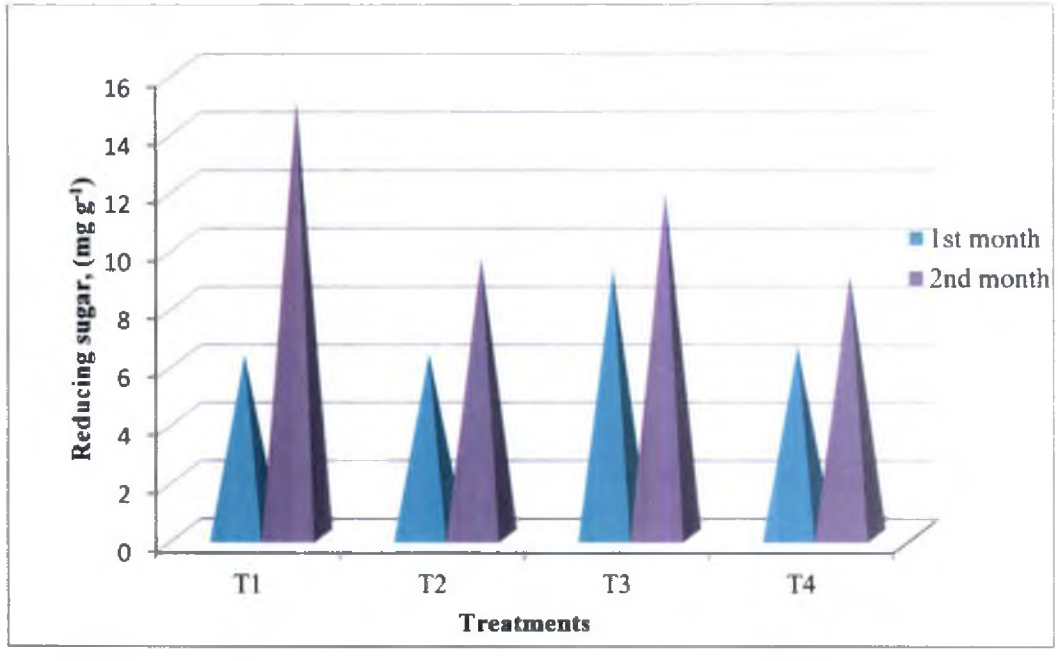
(Long *et al.*, 2004). However, there is considerable variation in the response of different species, with increases ranging from almost zero to over 100% (Wong, 1990; Korner and Miglietta, 1994). Under elevated CO_2 condition, carbohydrates accumulate in plant tissues since their usage intensity is lower than their production under these conditions (Moore *et al.*, 1998; Wolfie *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, 1997) and beech leaf (Landolt, 1997).

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 297% increase in starch production was noticed during first month and 451% in second month. In trench system also 32.29% and 165.7% increase in starch accumulation was noticed during the first and second month of exposure. In the case of reducing sugars, at the end of exposure 66.4% and 31.60% increase was reported under elevated CO_2 condition and trench system respectively (Fig.12). Several reports on increased carbohydrate fractions in plants under elevated CO_2 were reported by several workers. Landolt, (1997) 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). Elevated CO_2 can lead to the



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system T4: Absolute control

Fig.11. Effect of elevated CO_2 on total soluble protein, mg g^{-1}



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system T4: Absolute control

Fig.12. Effect of elevated CO_2 on reducing sugar, mg g^{-1}

accumulation of non-structural carbohydrates and increased concentration of soluble phenolic compounds in leaves (Poorter *et al.*, 1997). Carbohydrates are the basic compounds required to produce phenolics compound through shikimic acid pathway where extra carbohydrates derived from glycolysis and the penthose phosphate pathway are converted into the aromatic amino acid (Reddy *et al.*, 1996). Greater the source-sink ratio is, the greater the production of secondary metabolites (Makino *et al.*, 1994).

In this study phenol content was found to be reduced during the initial phase of exposure but continous exposure led to 14.75% increase in phenol content under elevated CO_2. In trench system, during the first and second month 53.7% and 31.14% reduction in phenol content was noticed. Goncalves *et al.* (2009) reported that elevated CO_2 increases the total phenolic content in wheat leaves. Similar reports were obtained by Saravanan and Karthi, (2014), Lindroth *et al.* (1993) and Ghasemzadeh *et al.* (2010).

Amino acids are the basic structures of proteins and each type of protein depends on the arrangement of the amino acids. Plant growth in CO_2 enriched atmospheres enhances the accumulation of both leaf starch and soluble carbohydrates (De Souza *et al.*, 2008; Norby *et al.*, 1986). Since the metabolism of carbohydrates is essential for the synthesis of amino acids, it is reasonable to assume that the effects of CO_2 enrichment should be similar for these classes of compounds (Sicher, 2008). Ample carbon was available to support amino acid synthesis and the increase in soluble amino acids under CO_2 enrichment (Sicher. 2008). Rising of CO_2 concentration from ambient (400 µmol/mol) to elevated levels (800 µmol/mol) resulted in enhanced levels of most amino acids in the leaf and rhizome of ginger varieties (Ghasemzadeh *et al.*, 2014).

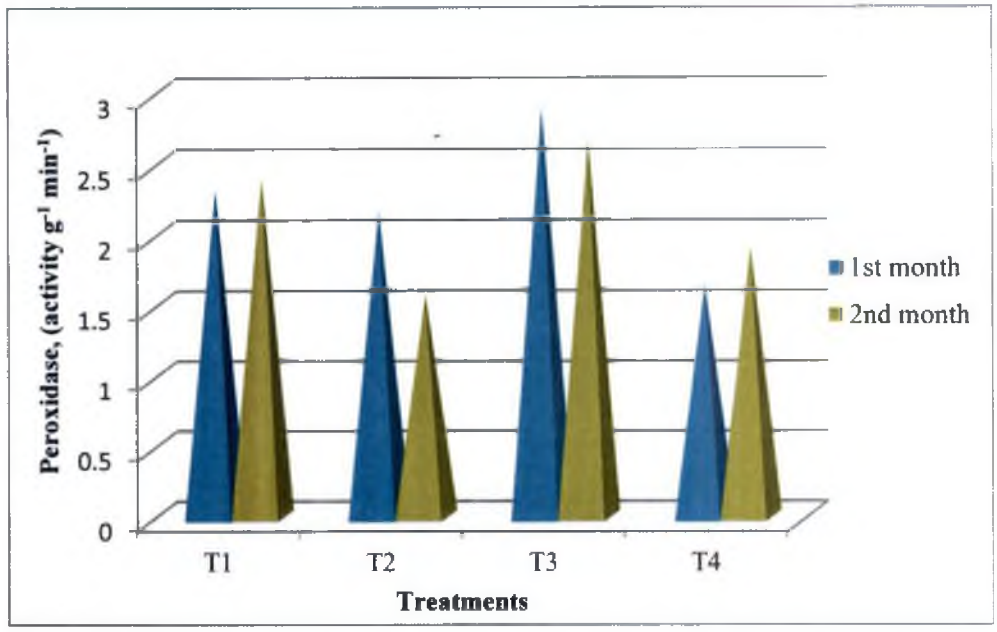
In the present study, during the initial phase of exposure free aminoacid content was found to be lower under elevated CO_2 and in trench system. This may be due to higher synthesis of protein observed during initial phase. The continuous exposure of elevated CO_2 caused significant increase in free aminoacid content to about 37.76%. The increasing amino acids content by CO_2

enrichment is in agreement with previous reports in soybean (Ainsworth *et al.*, 2007), tobacco (Geiger *et al.*, 1998), barley (Manderscheid *et al.*,1995).

5.3 EFFECT OF ELEVATED CO_2 ON ANTIOXIDANTS

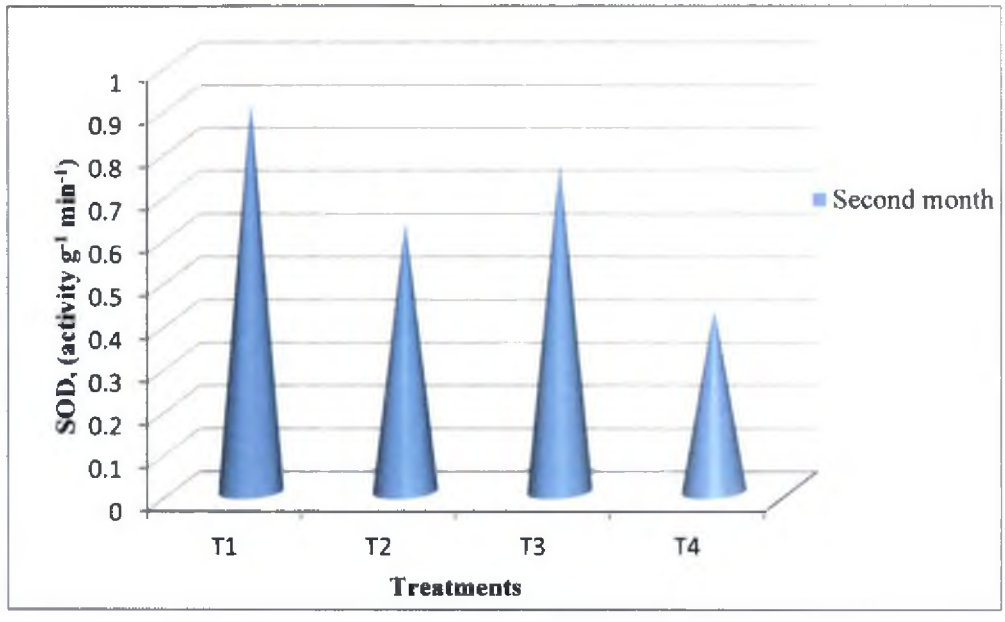
Various abiotic stresses lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress (Gill and Tuteja, 2010). Antioxidants are substances that protect cells from the oxidative damage and thereby reduce the risk of cell damage (Smitha and Sudha, 2011). Different non-enzymatic (ascorbate, glutathione, polyamines, phenols, α-tocopherol and carotenes) and enzymatic (SOD, ascorbate peroxidase, glutathione reductase and catalase) molecules are involved in scavenging excess ROS in plants (Yoshimura *et al.*, 2004).

The antioxidants studied in this experiment include peroxidase, catalase, superoxide dismutase (SOD) and ascorbic acid. A fluctuating range of enzyme activities under elevated CO_2 concentrations was reported. In the present study, peroxidase activity was observed to be higher for the plants under elevated CO_2 and trench system during the exposure period (Fig.13). During the first and second month of exposure, peroxidase activity increased to about 40.36% and to 25.52% under elevated CO_2. In trench system, around 75.30% and 39.06% increase was noticed during first and second month. Catalase activity was not found to have significant variation under elevated CO_2 whereas in trenches the activity was found to be significantly higher. In the case of SOD, 111.6% increase was observed in plants under elevated CO_2 and 79.06% increase in plants of trench system (Fig. 14). Vitamin C (Ascorbic acid) content was also found to be higher under elevated CO_2 during the initial phase of exposure but at the later stages no significant increase was noticed. The ascorbic acid content in plants of trench system was found to be enhanced at the end of the exposure period.



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system T4: Absolute control

Fig.13. Effect of elevated CO_2 on peroxidase activity, activity $g^{-1}min^{-1}$



T1: OTC (Ec), T2: OTC (Ac), T3: Trench system T4: Absolute control

Fig.14. Effect of elevated CO_2 on SOD activity, activity $g^{-1}min^{-1}$

When oxidative stresses do occur under high CO_2 conditions, the enhanced rates of photosynthesis and carbohydrate production resulting from atmospheric CO_2 enrichment can enable plants to better deal with such stresses by providing more of the raw materials needed for antioxidant enzyme synthesis. This may be the reason for higher production of antioxidants under such a situation. The results were in accordance with earlier findings of Niewiadomska *et al.* (1999), Schwanz and Polle (2001), Lin and Wang (2002) etc.

Several contradictory results were also reported about the production of antioxidants under elevated CO_2 conditions. Pritchard *et al.* (2000) reported that three months exposure to twice-ambient CO_2 concentrations reduced the activities of superoxide dismutase and catalase by an average of 23 and 39% respectively. In beech seedlings Polle *et al.* (1997) showed a reduction of catalase and superoxide dismutase activity under atmospheric CO_2 enrichment. Activities of superoxide dismutase, catalase and ascorbate peroxidase were declined under elevated CO_2 in *Catharanthus roseus* (Singh and Agrawal, 2015). It was reported that CO_2 enrichment reduced the activities of catalase and superoxide dismutase in oak trees (Schwanz and Polle, 1998).

5.4 MOLECULAR STUDIES

CO_2 enrichment causes imbalance in the supply and demand of carbohydrates resulting in their increased accumulation in the leaves (Stitt, 1991). Sugar accumulation in the leaves has been shown to down regulate the expression of photosynthetic genes in higher plants under elevated CO_2 (Prentice *et al.*, 2001). In this study, the electrophoretic analysis of proteins using SDS PAGE revealed that elevated CO_2 induced the production of a few new proteins but suppressed certain others. The protein content and profile varied with different varieties in response to elevated CO_2 level. Sathish *et al.* (2014) reported a significant variation in protein profile nature of black gram genotype (*Vigna mungo* (L.) Hepper) under conditions of elevated CO_2. But in the present study, even with the down regulation of Rubisco subunits, there was enhancement in the accumulation of photosynthates and drymatter production under elevated CO2

conditions. This can be due to the enhanced CO2: O2 ratio which can contribute to better carboxylation reaction. Further qualitative and quantitative studies are to be done on the modified protein profile in the case of black pepper.

The most pronounced change in the photosynthetic apparatus with prolonged growth under elevated CO_2 is a decrease in Rubisco activity and amount (Long *et al.*, 2004). This is often associated with decreased expression of genes encoding the small subunits of Rubisco (*rbcS*) (Drake *et al.*, 1997; Moore *et al.*, 1999). In this experiment the expression levels of both the large and small subunits of Rubisco were slightly brought down by CO_2 enrichment. The reduction in Rubisco expression under elevated CO_2 has also been observed in many preceding studies (Drake *et al.*, 1997; Long *et al.*, 2004). Pandurangam *et al.* (2006) reported that photosynthetic acclimation to elevated CO_2 concentration due to down regulation of Rubisco is through the limitation imposed on Rubisco small subunit gene expression as a consequence of high sugar content.

SUMMARY

6. SUMMARY

Climate change is widely recognized as the major environmental problem facing the Earth. Around the globe, seasons are shifting, temperatures are climbing and sea levels are rising. Despite technological advances such as improved varieties, genetically modified organisms and irrigation systems, weather is still a key factor in agricultural productivity. Climate change affects agriculture in a number of ways, including the changes in average temperatures, rainfall, changes in pests and diseases and also through the changes in atmospheric carbon dioxide concentrations.

Carbon dioxide is the most important greenhouse gas that absorbs the Earth's radiation resulting in an enhanced greenhouse effect leading to climate change (i.e. global warming). According to NOAA 2014, CO_2 concentration in the atmosphere has reached about 400 ppm and the rise in CO_2 concentration is at a rate of 2 ppm per year (IPCC, 2007). Since CO_2 is a major plant nutrient, the rise in CO_2 levels in the atmosphere will have direct and indirect effects in plant system. But there exists a spatial and species 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. Currently, technologies such as FACE (Free Air Carbon dioxide Enrichment), OTC (Open Top Chamber), SPAR (Soil Plant Atmospheric Research) etc. are developed and are being used to study the impact of elevated CO2 in plant systems.

Kerala is well known as the land of spices. Black pepper is indigenous to Kerala or the Malabar Coast from where it spread to rest of the world. The advance estimate of National Horticultural Board indicated that at present (2013-14) pepper has an area of 1, 17,770 ha with a production of 45, 000 tonnes in India. Black pepper (*Piper nigrum* L.) which is the deliberated and lushest spice crop of Kerala is showing a never before declining trend in its production and productivity. Weather aberrations like monsoon uncertainties, floods, droughts and heat load during summer are one among the vital factors that leads to declining production of black pepper. In this context, the current programme

attempts to study the physiological, molecular and biochemical basis of growth responses in black pepper (*Piper nigrum* L.) under elevated CO_2 conditions. This study is the first attempt to disclose the growth responses in black pepper under elevated CO_2 condition.

The study was conducted with 8 month old black pepper varieties like Panniyur 1, Panniyur 5 and Karimunda. Rooted cuttings and bush types of these varieties were exposed to elevated CO_2 conditions for a period of two months. Technologies used for CO_2 enrichment were Open Top Chamber and Trench systems. CO_2 was released from CO_2 cylinders to one of the two OTCs and a level of 500ppm was maintained. The second OTC worked as a control at ambient CO_2 for chamber effect. In trench system coirpith which was found to be the best organic material among the tested ones in terms of CO_2 release on soil microbial action was used for elevating the CO_2 level and an enhanced level of about 480ppm was achieved. Observations on growth parameters and molecular studies were done at the end of exposure period whereas the physiological and biochemical parameters were taken at monthly intervals.

The observations on growth parameters revealed a general increase in plant height (18%), no of leaves (84.5%) and leaf area (174.4%) in all varieties under elevated CO_2 condition compared to absolute control. Specific leaf area marked a reduction of 13.3% under elevated CO_2. An increase of 147.8% and 51.1 % were noticed in net assimilation rate and relative growth rate respectively under elevated CO_2. Root and shoot dry weights were also found to be higher under elevated CO_2 resulting an increase in root shoot ratio of 42.1%. Dry matter production was maximum for the varieties kept under elevated CO_2.

Among the physiological parameters studied, CO_2 enrichment significantly lowered the stomatal frequency, stomatal conductance and transpiration rates of 23.3%, 29.96% and 14.28%, respectively at the end of exposure. On an average, significant increases in chlorophyll a (1.20 mg g^{-1}), chlorophyll b (0.52 mg g^{-1}), total chlorophyll (1.45 mg g^{-1}) and carotenoid (0.55

mg g^{-1}) contents were registered in varieties under elevated CO_2 condition at the end of exposure period.

Elevated CO_2 was found to have a promotional response in biochemical parameters like total soluble protein, starch, reducing sugar, phenol content and free amino acids. Total soluble proteins were higher under elevated CO_2 condition holding the values of 4.22 mg g^{-1} and 6.40 mg g^{-1} during the first and second month respectively. A general increase in starch content was registered under elevated CO_2 condition. During the first and second month of exposure, starch content showed average values of 31.91 mg g^{-1} and 29.92 mg g^{-1} under elevated CO_2. Elevated CO_2 enhanced the reducing sugars about 66.4% at the end of exposure.

Though the phenol content was reduced to a level of 0.664 mg g^{-1} at the end of first month of exposure, and increase of 14.7% was observed with a value of 0.70 mg g^{-1} at the end of the exposure period. Free aminoacid status of the plants was also found to be lower during the initial phase of exposure but at the later stages an enhancement of 37.7% was noticed under elevated CO_2. There occurred a significant increase in epicuticular wax production in CO_2 enriched varieties during the exposure period. Though there was a reduction in membrane integrity under elevated CO_2 at the initial stages, it got stabilized at later stages. The isotope discrimination value was found to be higher under elevated CO_2.

Enzymatic and non-enzymatic antioxidants were found to have significant changes with the CO_2 enrichment given. The peroxidase activity was higher under elevated CO_2 conditions and had values of 2.33 and 2.41 activity g^{-1} min^{-1} during the first and second month of exposures. SOD was also found to be enhanced under elevated CO_2 condition (0.91 activity g^{-1} min^{-1}). Elevated CO_2 had a positive influence on catalase and ascorbic acid contents.

The electrophoretic analysis of proteins using SDS-PAGE revealed that elevated CO_2 induced the production of few new proteins in varieties while suppressing certain others. A varietal variation in protein profile was observed.

The expression levels of both the large and small subunits of Rubisco were slightly brought down by CO_2 enrichment.

The varieties kept in Trench system also responded in a similar manner but to a lesser extent.

6.1 CONCLUSION

The present investigation was carried out with the objective to study the physiological, molecular and biochemical basis of growth responses in black pepper (*Piper nigrum* L.) under elevated CO_2 conditions. Considering all the physiological, biochemical and molecular studies conducted, it can be concluded that elevated CO_2 had a positive influence on growth and development of black pepper. Elevated CO_2 had pronounced effects on pepper cuttings. Among different varieties studied, Panniyur 1 was found to have better performance under elevated CO_2 condition in terms of growth and development. The same variety was the best performing one, among the selected bush pepper varieties. Considering the factors that contribute towards water stress tolerance, Karimunda was found to be the best one. This variety exhibited better root characters like root weight and root shoot ratio, high mesophyll efficiency and low transpiration rate which can contribute to higher water use efficiency. This attributes Karimunda suitable for the predicted water limited situations.

6.2. FUTURE LINE OF WORK

Black pepper is the oldest and a highly valuable medicinal spice in India playing a pivotal role in India's international trade. The black pepper and its active component piperine can be used as a spice drug, preservatives and larvicidal control agents. Black pepper is also important for its medicinal value. Incredibly popular black pepper is showing a declining trend in its production and productivity in the present scenario of climate change. CO_2 is the most dominant greenhouse gas responsible for global warming and the associated changes in weather factors. Thus further studies on black pepper are needed to know the impact of foreseen rise in CO_2 on the flowering, yield attributes and quality

characters of black pepper. Efforts should also be taken towards developing technologies for exploiting the positive impacts of elevated CO_2 environment.

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PHYSIOLOGICAL AND MOLECULAR ANALYSES OF GROWTH RESPONSES IN BLACK PEPPER (*Piper nigrum* L.) UNDER ELEVATED CARBON DIOXIDE ENVIRONMENTS.

MINU M

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ABSTRACT

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Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT PHYSIOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

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ABSTRACT

A study entitled 'Physiological and molecular analyses of growth responses in black pepper (*Piper nigrum* L.) under elevated carbon dioxide environments' was carried out with the objective to study the physiological, molecular and biochemical basis of growth responses in black pepper (*Piper nigrum* L.) under elevated CO_2 conditions. In this study, 8 month old rooted cuttings and bush pepper plants of 3 varieties viz. Panniyur 1, Panniyur 5 and Karimunda were used. Technologies used for CO_2 enrichment were Open Top Chamber (OTC) and Trench Systems. CO_2 was released from CO_2 cylinders to one of the two OTCs bringing the CO_2 level to 500ppm (OTC Ec) and the second OTC worked as a control at ambient CO_2 for chamber effect (OTC Ac). In trench system, CO_2 concentration was enhanced to a level of 480 ppm. The plants were maintained under high CO_2 level for a period of two months in both the systems. The experiment was laid out in CRD with 4 treatments and 3 replications. Observations on growth parameters and molecular studies were conducted at the end of exposure period and physiological and biochemical parameters were taken at monthly intervals.

Elevated CO_2 was found to have a promotional effect on all the growth parameters compared to absolute control. An increase of 18% was noticed in plant height, 84% in leaf production, 174.4% in leaf area, 147.8% in net assimilation rate, 51.1% in relative growth rate, 87.5% in root weight, 25.3% in shoot weight, 42.1% in root-shoot ratio and 62.27% in dry matter production. Specific leaf area was found to have a 13.3% reduction under elevated CO_2. Under elevated CO_2, the variety Panniyur 1 bush recorded highest net assimilation rate (0.066 mg cm^{-2} day^{-1}), relative growth rate (0.0078 mg g^{-1} day^{-1}), shoot weight (13.03 g) and dry matter production (10.07 g). Highest root production, root shoot ratio and lowest transpiration rate were noticed in Karimunda variety.

CO_2 enrichment significantly lowered the stomatal frequency (1311 No. cm^{-2}), stomatal conductivity (28.70 mmoles m s^{-1}) and transpiration rate (0.72 mmoles m^{-2} s^{-1}) in all the varieties except for Panniyur 5 bush pepper resulting in

better relative water content. Photosynthetic pigments in all varieties (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) were increased under elevated CO_2. Elevated CO_2 had significant positive influence on biochemical parameters like total soluble protein (6.40 mg g^{-1}), starch (29.92 mg g^{-1}), reducing sugars (15.06 mg g^{-1}), phenol content (0.70 mg g^{-1}), free amino acids (2.59 mg g^{-1}) and wax concentration (2.65mg cm^{-2}). Though there was a reduction in membrane integrity during the initial phase, it got stabilized at later stages. Isotope discrimination values were enhanced under elevated CO_2 in all the varieties except Panniyur 5. CO_2 enrichment was found to have significant influence on peroxidase (2.41 activity g^{-1} min^{-1}) and superoxide dismutase (0.91 activity g^{-1} min^{-1}) activities but in the case of catalase and ascorbic acid no significant variation was observed. Protein profile revealed that elevated CO_2 induced the production of a few new proteins but suppressed certain others. The expression levels of both the large and small subunits of rubisco were slightly brought down by CO_2 enrichment. The plants kept in trench system also responded in a similar manner but to a lesser extent.

The present study revealed that elevated CO_2 environment had a positive influence on growth and development of pepper. Panniyur 1 was the best performing bush pepper. Pepper plants of the variety Karimunda had the best root characters, high mesophyll efficiency and low transpiration rate under elevated CO_2 environments which makes it suitable for water limited situations with higher water use efficiency.

APPENDICES

APPENDIX-I

Weather data during the treatment period
(September 25th –December 31st, 2014)

Date	Temp (° C)		RH (%)		Wind Velocity (km/h)	Sunshine hours	Rain (mm)	Evpn. (mm)
	Max.	Min.	I	II				
25-Sep	31.2	24.9	91	86	3.24	7.3		6.1
26-Sep	31.9	23.4	99	86	2.16	6.4	9.0	6.2
27-Sep	31.8	23.4	99	79	6.12	7.2	59.4	6.1
28-Sep	29.9	24.0	97	82	7.20	7.4	4.0	2.5
29-Sep	30.8	23.7	93	89	3.96	8.2	2.0	2.5
30-Sep	30.6	23.6	92	89	4.32	8.5		2.0
1-Oct	29.0	23.4	96	71	3.60	8.1		2.0
2-Oct	30.8	23.6	93	70	5.76	9.6		3.5
3-Oct	31.7	24.6	98	81	6.12	9.2	1.0	3.1
4-Oct	30.6	23.4	97	72	7.56	7.5	7.0	4.0
5-Oct	30.5	24.0	98	82	4.32	9.4		3.2
6-Oct	31.0	24.6	99	71	3.60	7.1		2.4
7-Oct	31.1	23.8	87	68	5.04	9.4	1.0	2.0
8-Oct	30.9	23.2	83	89	6.84	9.3	18.6	4.6
9-Oct	30.6	24.2	73	90	6.84	7.3		3.8
10-Oct	29.4	24.8	76	83	6.48	8.3	1.0	4.0
11-Oct	30.8	25.3	72	82	14.40	9.3		4.2
12-Oct	31.2	23.8	68	85	6.84	9.6		3.2
13-Oct	31.2	23.6	74	83	6.48	9.3		3.4
14-Oct	30.7	24.5	69	88	5.04	9.5	1.0	3.0
15-Oct	31.5	23.6	72	91	7.20	8.6	68.0	2.8
16-Oct	31.4	24.3	79	96	3.96	7.2	1.0	3.5
17-Oct	31.3	24.3	82	96	4.68	7.1	8.0	2.9
18-Oct	29.7	23.6	81	89	6.48	6.2	13.0	3.5
19-Oct	28.7	22.7	78	96	3.96	8.2	51.0	7.0
20-Oct	28.9	23.4	90	83	6.84	9.4	11.0	7.0

21-Oct	30.9	24.3	95	96	1.80	2.4	11.0	3.9
22-Oct	26.4	23.4	78	96	4.32	7.5	9.0	1.4
23-Oct	30.2	23.5	89	96	5.04	6.3	8.4	1.4
24-Oct	30.7	23.9	73	93	4.32	8.9	11.0	2.9
25-Oct	31.1	23.6	74	93	7.20	7.3		5.0
26-Oct	30.4	23.4	68	90	5.76	9.0		3.8
27-Oct	31.8	23.2	92	91	9.00	7.5	7.0	5.7
28-Oct	31.0	23.8	92	96	4.32	7.3	0.2	4.5
29-Oct	31.3	23.5	81	96	4.68	8.2		3.4
30-Oct	30.9	23.6	77	88	2.88	5.3	2.0	2.0
31-Oct	30.7	23.6	71	94	2.88	6.6		4.6
1-Nov	30.5	23.6	95	72	3.6	8.6	2.0	3.8
2-Nov	31.1	23.0	94	79	2.16	9.1	10.0	4.0
3-Nov	29.0	24.4	92	91	2.88	6.2	1.0	4.2
4-Nov	30.0	23.1	93	76	7.20	8.2	9.0	3.2
5-Nov	31.1	23.6	92	72	8.64	7.6	1.0	3.4
6-Nov	30.6	23.7	96	72	9.00	9.5		3.0
7-Nov	30.9	22.7	94	68	4.68	9.3		2.8
8-Nov	30.7	23.1	98	72	7.92	9.2		3.5
9-Nov	30.5	21.8	94	66	6.12	9.3		2.9
10-Nov	30.1	22.9	88	80	6.84	9.3		3.5
11-Nov	31.0	23.9	90	84	1.80	8.4		7.0
12-Nov	31.5	23.8	94	71	10.44	8.5	2.0	7.0
13-Nov	31.7	24.2	96	74	4.32	9.5	1.0	2.0
14-Nov	31.7	23.2	98	77	3.60	8.2	11.0	4.6
15-Nov	31.4	23.8	95	81	5.04	8.3	2.8	3.8
16-Nov	30.6	23.2	71	74	5.04	9.4		4.0
17-Nov	30.8	23.6	89	69	6.48	7.5	3.2	4.2
18-Nov	31.0	24.0	90	74	1.80	7.3	6.4	3.2
19-Nov	31.2	23.4	97	68	1.44	8.6	1.0	3.4
20-Nov	30.8	23.9	92	60	6.84	8.6		3.0

21-Nov	31.8	23.6	96	95	3.24	0.1	23.0	1.0
22-Nov	26.5	22.6	96	94	0.72	3.3	5.0	1.9
23-Nov	26.5	23.0	99	91	5.40	6.5	1.0	1.5
24-Nov	28.3	23.8	95	66	4.32	8.5	16.9	1.5
25-Nov	31.0	23.5	96	80	2.88	7.4		2.0
26-Nov	30.4	23.1	94	74	3.96	8.8		1.0
27-Nov	29.4	23.4	97	85	3.60	6.6	1.0	1.9
28-Nov	28.9	23.3	96	83	5.40	6.4	11.0	1.9
29-Nov	27.8	23.2	93	92	6.12	7.4	28.0	2.0
30-Nov	28.7	23.0	94	77	6.12	6.5	1.0	1.5
1-Dec	29.5	23.3	96	92	3.96	8.8		1.5
2-Dec	29.0	22.5	85	86	6.84	7.5	0.4	1.0
3-Dec	29.9	22.7	94	62	5.04	9.2	1.0	1.9
4-Dec	30.7	23.1	84	71	4.68	9.2	0.4	1.9
5-Dec	30.7	21.0	92	67	5.76	9.1		2.0
6-Dec	30.1	22.0	94	62	6.48	9.3		2.0
7-Dec	30.7	21.9	86	61	5.40	9.2		2.0
8-Dec	30.4	23.4	92	76	3.24	8.4		2.0
9-Dec	31.6	24.3	89	76	6.84	8.4	14.0	2.9
10-Dec	31.3	24.2	92	71	2.16	4.14	2.0	3.5
11-Dec	29.3	22.5	84	78	2.52	6.29	85.0	1.0
12-Dec	29.6	22.9	89	82	2.16	8.18	1.0	5.0
13-Dec	28.7	23.0	92	84	9.36	8.34	9.0	2.0
14-Dec	29.9	23.1	86	75	6.48	9.25		4.6
15-Dec	30.1	24.0	93	79	6.12	8.42		3.8
16-Dec	30.2	23.2	91	70	3.24	9.13		3.2
17-Dec	31.6	24.2	95	92	3.96	5.11		2.6
18-Dec	28.9	24.3	87	77	3.96	7.48		2.9
19-Dec	31.9	23.7	90	80	3.24	6.49	1.0	3.5
20-Dec	28.7	22.4	88	73	5.04	7.51	12.0	1.0
21-Dec	31.3	22.4	98	66	4.68	8.33	1.7	2.0

22-Dec	31.3	23.9	99	89	6.48	9.31		2.0
23-Dec	30.8	23	98	75	5.4	9.4		4.6
24-Dec	30.4	24.2	99	77	3.96	7.3		2.0
25-Dec	29.3	23.8	89	77	8.28	9.8		2.0
26-Dec	29.3	23.7	85	75	3.24	7.11		2.0
27-Dec	29.1	23.6	86	65	1.08	8.34		2.0
28-Dec	29.6	23.4	99	96	2.16	7.2		2.0
29-Dec	29.8	23.9	97	67	4.32	8.29	6	2.0
30-Dec	31.3	24.3	87	78	9.72	7.57		2.0
31-Dec	31	24.1	93	70	7.56	9.13		2.0

APPENDIX-II

Weather data from OTC during the treatment period
(September 25th – November 25th, 2014)

Date	CO_2 (ppm)	Air temp A (°C)	Air temp B (°C)	Rh A (%)	Rh B (%)	Sun shine duration (min.)	Solar Incidence (micro Enst.)
25-Sep	543.79	39.72	38.26	56.51	57.37	902.34	491.42
26-Sep	547.22	34.66	34.68	69.86	72.27	2640.00	196.80
27-Sep	550.33	36.99	35.38	63.36	65.12	2031.72	260.90
30-Sep	554.87	35.47	34.27	65.25	66.20	1120.62	247.07
1-Oct	541.48	39.02	38.60	54.81	54.24	1451.52	386.43
2-Oct	542.79	34.38	35.19	72.54	74.04	1762.67	241.25
3-Oct	548.22	33.27	33.61	71.40	73.13	1762.56	230.14
7-Oct	550.33	40.52	40.38	46.07	44.00	1610.65	616.71
9-Oct	551.17	41.20	40.27	45.65	45.37	2031.72	624.43
11-Oct	546.38	37.25	36.52	60.61	61.30	1120.62	591.33
14-Oct	542.19	34.26	35.20	58.51	59.55	681.52	261.73
15-Oct	546.22	36.68	37.25	61.26	60.13	1582.67	351.07
18-Oct	550.33	35.15	35.26	63.46	60.42	1762.56	310.43
20-Oct	550.87	38.94	37.68	63.20	61.00	792.34	446.45
23-Oct	541.78	34.39	34.38	54.00	55.24	2640.00	287.74
26-Oct	543.79	33.28	33.27	70.48	71.88	2871.72	286.63
28-Oct	544.29	40.43	41.60	71.15	69.24	1120.62	527.28
31-Oct	549.22	40.61	40.19	49.35	48.55	651.52	565.81
1-Nov	557.33	34.38	35.90	47.25	45.26	1762.67	591.33
3-Nov	550.81	33.27	35.31	59.71	59.00	1762.56	614.72
5-Nov	546.47	40.52	39.22	57.21	56.48	1610.65	620.44
7-Nov	547.74	41.20	41.54	69.86	70.15	3707.70	596.34
9-Nov	548.22	37.25	37.61	63.36	60.37	1798.87	341.52
11-Nov	551.33	34.26	34.38	64.45	65.78	982.34	381.87
13-Nov	557.87	36.68	37.72	55.11	56.51	2031.72	318.85
15-Nov	541.88	34.38	33.66	70.54	70.01	1202.54	440.47
17-Nov	544.09	33.27	32.99	71.40	73.28	854.51	280.99
18-Nov	545.69	35.60	35.47	44.00	45.91	1702.58	387.33
19-Nov	548.28	34.19	35.02	55.51	55.51	1772.26	427.21
20-Nov	551.35	39.90	38.38	65.26	65.35	1500.67	465.88
21-Nov	556.87	35.31	35.52	66.16	68.98	2001.42	698.26
22-Nov	548.49	33.22	34.20	64.27	62.57	1148.67	416.47
25-Nov	544.75	41.54	40.25	53.11	55.27	1151.96	524.33

APPENDIX III

Protein denaturing solution

10 M urea	- 80 ml
1 M $NaH_2PO_4.2H_2O$ (pH 8)	- 5 ml
5 M sodium chloride	- 2 ml
1 M Tris (pH 8)	- 1 ml

Make up volume to 100 ml by adding 12 ml of distilled water.