PHYSIOLOGICAL, PHYTOCHEMICAL AND MOLECULAR STUDIES ON ABIOTIC STRESS MEDIATED ANTIOXIDANT ACCUMULATION IN *Amaranthus spinosus* Linn.

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KERALA, INDIA

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by

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(2012 - 11 - 132)

THESIS

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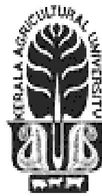

DEPARTMENT OF PLANT PHYSIOLOGY
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KERALA, INDIA

2014

DECLARATION

I, hereby declare that this thesis entitled **"Physiological, phytochemical and molecular studies on abiotic stress mediated antioxidant accumulation in *Amaranthus spinosus* Linn."** is a bonafide record of research done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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To

The God Almighty

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"Bowing My Head To Thy Supreme Intelligence "

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LIST OF ABBREVIATIONS

@	at the rate of
^{o}C	Degree Celsius
%	Per cent
m^{-2}	Per square metre
CD	Critical difference
cm	Centimetre
&	And
et al.	Co-authors/co-workers
CO_2	Carbon dioxide
Fig.	Figure
ml	Milli litre
M	Molar
g	Gram
i.e.	That is
ROS	Reactive oxygen species
E.C.	Enzyme Commission
ppm	Parts per million
^{o}	Degree
m	meter

α	Alpha
β	Beta
µ	Micro
UV	Ultra violet
CRD	Completely Randomised Design
RNA	Ribo nucleic acid
DNA	Deoxy ribo nucleic acid
Min.	minutes
rpm	Rotations per minute
g	gyration
OD	Optical Density
A_{663}	Absorbance at 663 nm
A_{645}	Absorbance at 645 nm
A_{480}	Absorbance at 480 nm
A_{510}	Absorbance at 510 nm
A_{520}	Absorbance at 520 nm
A_{460}	Absorbance at 460 nm
WDS	Water Deficit Stress

INTRODUCTION

1. INTRODUCTION

From the earliest times medicinal plants have been serving humanity as a source for traditional as well as herbal drugs. India being a herbal hub, has hosted the development of ancient and traditional method of treatments namely Ayurveda, Siddha, Unani and the modern system of medication, Allopathy. All these systems of medication flourished with knowledge about the ethnobotanical and phytochemical uses of medicinal herbs. The biomolecules in them are responsible for resistant action against human as well as plant biotic and abiotic stresses. Medicinal plants being natural and non-narcotic have no side effect and are safe, cost effective for preventive and curative therapies.

Medicinal plants occupied an important position in the socio-cultural, spiritual and medicinal arena of rural people of India. Their sustainable management and harvesting can conserve biodiversity, sustain human and environmental health, generate employment and enhance export earnings. The number and percentage of total medicinal plants among countries vary greatly (Schippmann, 2002). Estimates for the number of the species used medicinally include: 35000-70,000 worldwide (Schippmann, 2002); 10,000-11250 in China (He and Gu, 1997); 7500 in India (Shiva, 1996).

Studies reveal that over 9,000 plants have known medicinal applications in various cultures and countries, and this is without having conducted comprehensive research amongst several indigenous and other communities (Farnsworth and Soejarto, 1991). Medicinal plants are used at the household level by women taking care of their families, at the village level by medicine men or tribal shamans, and by the practitioners of classical traditional systems of medicine such as Ayurveda, Chinese medicine, or the Japanese kampo system. According to the World Health Organization, over 80% of the world's population

or 4.3 billion people rely upon such traditional plant based systems of medicine to provide them primary health care. Kerala with its abundance of luxuriant flora is the only state in the country where Ayurveda continues to be practiced in its purest form. There are more than 1000 pharmaceutical units in Kerala, out of which a dozen consume about 50% of total plant based materials produced and used for drug manufacture. The demand of the Ayurveda medicine in the state is growing 10-12% per annum (Kumar, 2003). Apart from having biodiversity hotspot in the state, many of the medicinal plants are under the stage of extinction and majority of the traditionally used herbs remain non-scrutinized and underexploited for their biomolecular activity. Hence there is an urgent need to study such crops to meet the ever increasing demand for medicinal herbs.

Amaranthus spinosus is a potent medicinal plant known for its antioxidant accumulation. It is a C4 plant, described as being competitive in situations of high light intensities and/or high temperature. *A. spinosus* Linn. (Amaranthaceae) is an annual herb found throughout India and also in many tropical countries. The roots are used as a laxative, as emollient poultice (Kirtikar and Basu, 1999), as antimalarial (Hilou *et al*., 2006), antioxidant (Amin *et al*., 2006), anti-inflammatory, antimicrobial, antidiuretic agent, and also in hepatic disorders (Olajide *et al*., 2004; Stintzing *et al*., 2004; Van Dunen, 1985). The plant is used for culinary as well as medicinal purposes in many countries (Mansfeld's Database, 2002).

Free radicals are a group of bioactive compounds produced during normal metabolism in a living system. They possess unpaired electrons that can start off harmful chain reactions by targeting stable molecules, resulting in lipid peroxidation, damage of DNA and proteins. Antioxidants are compounds that protect the cells against the free radicals. Plants are susceptible to damage caused by free radicals and thus develop numerous antioxidant defense systems resulting

in formation of numerous potent antioxidant components also called as plant secondary metabolites (PSMs). PSMs like flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, isocatechins, vitamins C and E, β-carotene and α-tocopherol are reported to possess antioxidative property (Aquil *et al*.,2006). Antioxidants are also known to prevent several chronic diseases caused by free radicals such as atherosclerosis, cancer, diabetes, arthritis, inflammation, cardiovascular and ageing related problems (Kaur and Kapoor, 2001). Recently there has been increasing interest in screening the phytochemical potency of medicinal plants having antioxidant property, scientifically evaluate them and to understand their mode of action for using them as natural antioxidant sources for drug manufacturing.

The biosynthesis of PSMs although controlled genetically, is affected largely by various endogenous and exogenous factors. Though secondary metabolites are specific to certain plants, its constituent of therapeutic value is best under selected environments rather than its habitat or other ecological zones (Lini, 2006). Hence understanding the effect of environmental factors on phytochemical production is of great importance for optimizing field growth conditions for maximum recovery of the phytochemical. Such information also aids in manipulating the habitat of the plant for its survival as well as for economic purpose.

The present investigation was conducted to study the environmental, biochemical and molecular mechanisms which up-regulate or down-regulate the total antioxidant accumulation in the plant *Amaranthus spinosus* Linn. with the objective to elicit information on the physiological, biochemical and molecular attributes associated to the antioxidant accumulation due to abiotic stresses viz. light stress, water deficit stress and CO_2 enrichment.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The accumulation of antioxidants although controlled genetically, is also largely influenced by various endogenous and exogenous factors. Abiotic stress has a major role in the increased production of antioxidants. Activation of specific secondary metabolite pathway in plants is also another consequence of abiotic stress. The plant defense mechanism against stress is characterized by the activation of secondary metabolism (Herms and Mattson, 1992). In this context the current programme studied the effect of shade stress, water deficit stress (WDS) and CO_2 enrichment on growth and the accumulation of antioxidants in *Amaranthus spinosus* Linn.

Amaranthus spinosus Linn. (Amaranthaceae) is a traditionally used medical plant which has numerous therapeutic uses. As a popular weed species, it is found extensively on road sides and neglected areas. It is commonly known as prickly amaranth, spiny amaramth or pig weed. It is an annual or perennial herb, native to tropical America and found throughout India as a weed in cultivated as well as fallow lands (Anon. 1988). Though whole plant is used as laxative (D'ymock, 1976), the roots are regarded as highly specific for colic by physicians in India (Sivarajan & Balachandran, 1994) and in Madagascar they are considered as laxative (Kirtikar and Basu, 1987).

During earlier days, the boiled leaves and roots of *A.spinosus* Linn. were given to children as laxative. It is also used traditionally as diuretic, antidiabetic, antipyretic, against snake venom, antileprotic, and anti-gonorrheal (Kirtikar and Basu, 1987). In Malaysia, *A.spinosus* Linn. is used as an expectorant and to relieve breathing difficulties in acute

bronchitis. Certain aborigins in India use the leaves of *A. spinosus* Linn. as abortifacient (Grubben and Denton, 2004). The plant is reported to have anti-inflammatory (Olumayokun *et al.*, 2004), antiandrogenic (Murgan *et al.*, 1993a) and anthelmintic properties (Assiak *et al.*, 2002). It is also known to have an effect on the biochemical changes in epididymis (Murgan *et al.*, 1993b).

The hepatoprotective activity by *A. spinosus* Linn. is due to the presence of antioxidant defense factors and phenolic constituents of the plant (Sanhita *et al.*, 2012). The presence of active constituents like, flavonoids, sterols and alkaloids in *A. spinosus* Linn. showed anti-hyperglycemic activity when tested in diabetes induced albino rats (Mishra *et al.*, 2012). In an ethnobotanical survey it was reported that *A. spinosus* Linn. was used as a diuretic, sudorific and febrifuge in tribal pockets of North Eastern Gujarat (Punjani, 2010). The ethanolic and aqueous extracts of *A. spinosus* Linn. roots, when investigated against ten bacterial strains including Gram-positive and Gram-negative bacteria, showed potent antibacterial activity (Vardhana , 2011).

A preliminary phytochemical investigation in methanolic and petroleum extracts of *Amaranthus spinosus* Linn. leaves revealed the presence of flavanoids, phytosterols, glycoside, tannins and phenolic compounds (Mathur *et al.*, 2010). Extracts of the plant were found to contain hydroxycinnamates, quercetin and kaempferol glycosides (Stintzing *et al.*, 2004). The antioxidant activity of the extracts of *A. spinosus* Linn. was reported due to the presence of flavonoids like rutin and quercetin (Kumar *et al.*, 2008). Rutin and Quercetin are flavanols having significant capacity to inhibit haemoglycolisation (Asgary *et al.*, 1999).

Increased atmospheric carbon dioxide concentrations associated with increasing temperatures are predicted to have profound impacts on terrestrial ecosystems (Ward and Strain, 1999). Different species respond differently to elevated CO_2. Bazzaz *et al.* (1995) showed that even individual families might respond differently to elevated atmospheric CO_2, and suggested that future CO_2 levels would lead to increased intensity of natural selection. Literature pertaining to the influence of abiotic stresses *viz.* shade, water and CO_2 on the physiological, biochemical and molecular mechanisms responsible for the antioxidant accumulation in *A. spinosus* Linn. is reviewed in this chapter.

2.1 GROWTH PARAMETERS

Plants are exposed to almost all the environmental extremities. The unfavourable climatic conditions like water stress, salinity, extreme temperatures as well as irradiance have a definite impact on the various growth parameters like height of the plant, leaf area, shoot weight as well as dry matter production.

The growth performance of *Amaranthus spinosus* Linn. was observed to have a positive relation with soil water content. The height of the plant is reported to be decreased with decreasing field capacities (Chauhan and Abhugo, 2013).

The number of leaves and height of plants increased in diffused and partial light than in the full light, whereas the number of leaves and branches decreased in water-logged conditions in *Amaranthus viridis* L. Under water-logged conditions there was a general increase in height of shoots, floral axis and fresh weight (Farrukh *et al.*, 2003).

Water stress at an earlier vegetative growth stage resulted in significant reduction of plant height in *Amaranthus retroflexus*, a weed

species (Quasem and Biftu, 2010).

Natural shaded condition increased plant height in Mint species when grown as an intercrop in sugarcane in the subtropical climate (Singh *et al.*, 2002). An increasing trend in plant height with increasing shade intensity was reported in ginger (Ancy, 1992; Beena, 1992; Babu, 1992; Sreekala, 1999).

An increase in leaf area was reported under decreased irradiance in *Ageratum conyzoides*. (Sun *et al.*, 2012) In sorghum cultivars, leaf area decreased in all the lines studied under WDS (Rao, 1999). In cucumber plants, combination of UV-B radiation and water stress resulted in decreased leaf area as compared with the control (Yang *et al.*, 2000). An increase in total biomass of shoots was observed in *Amaranthus spinosus* Linn. with increased soil water content (Chauhan and Abhugo, 2013).

The effect of water availability is reported to have a pronounced effect on dry matter production and biomass yield as compared to fertilizer availability in *Amaranthus cruentus* (Ejieji and Adeniran, 2010) In *Aloe vera*, it was observed that plants grown under full sun produced twice the total dry mass than those grown under partial shade (Paez *et al.*, 2000). In Sugarcane, a reduction in dry matter production was reported under moisture deficit stress conditions (Ramesh, 2000).

Podophyllum hexandrum Royle, an endangered medicinal herb, when exposed to elevated levels of CO_2 (650 +/-50 $\mu molmol^{-1}$) showed increased levels of plant height, a slight increase in leaf area compared to the plants grown in ambient CO_2 conditions. The total biomass of roots alone increased whereas shoot biomass remained unaltered (Chaturvedi *et al.*, 2009). Total plant dry weight at bulbing was increased by 32-44% due to elevated CO_2 in two cultivars of *Allium cepa* L. (*cv*. Hyasm and Site)

(Daymond *et al.*, 1997). Leaf area and ratio of leaf fresh weight/ (stem + root) fresh weight, were greater in *Persea americana* Mill. grown with CO_2 enriched air (*de la* Vina *et al.*, 1999). There was higher biomass accumulation, leaf area and better growth when compared to ambient air grown plants, when *Hevea brasiliensis* was exposed to elevated concentration of CO_2 (700 +/-25 ppm) for 60 days (Devakuamr *et al.*, 1998). CO_2 had no effect on the relative growth rate, total biomass or partitioning of biomass in the C-4 species after 35 days exposure to elevated CO_2 in a comparative study of *Amaranthus retroflexus* (C-4) and *Abutilon theophrasti* (C-3) (Dippery *et al.*, 1995).

2.2. PHYSIOLOGICAL PARAMETERS

The changes in environment result in transient changes in various physiological features of the plant. Plants respond to the changes in the surrounding by changing its physiology there by adapting and acclimating to the new environment. Such physiological parameters that are influenced by abiotic stress are phytopigments, stomatal characteristics including stomatal frequency, stomatal conductance and stomatal index, specific leaf area as well as relative water content.

The study about the influence of light on growth in *Ageratum conyzoides* proved that under shaded conditions there is an increase in Chlorophyll a, Chlorophyll b and total chlorophyll (Sun *et al.*, 2012). Water deficit stressed plants showed a significant reduction in chlorophyll a, b and carotenoids than watered plants in Lettuce (Agami, 2013).

In shade grown plants of *Andrographis paniculata*, plants had more chlorophyll per unit dry weight (Pratima, 1999). A significant reduction

in chlorophyll 'b' was observed due to WDS in *Grevellia robusta* (Nautiyal *et al*., 1993) and a similar result was reported in tea (Rajasekar *et al*., 1991). In sorghum, WDS enhanced the chlorophyll content (Satbhai *et al*., 1998)

Specific leaf area (SLA) is the leaf area per unit leaf dry weight. Species that achieve the highest net CO_2 Exchange Rate per unit leaf area (CER) from the minimum leaf material are considered efficient (Rawson, 1987). On the contrary, Dornhoff and Shibles (1976) showed a negative relation in soyabean.

SLA has its positive and negative role through its link with photosynthetic volume (Charles, 1982). Thus lower SLA means more layer of mesophyll cells (Dornhoff and Shibles, 1970) and greater leaf area (Pettigrew *et al*., 1993). The genotypic differences in photosynthetic rate per unit leaf area have been correlated with genotype differences in SLA in many species including alfalfa (Pearce *et al*., 1969): soyabean (Dornhoff and Shibles, 1976) and in peanut (Rao and Wright, 1994).

In *Cryptantha flava*, shading did not reduce stomatal conductance proportionately to photosynthesis, which led to decreased water use efficiency for plants under shrubs (Forseth *et al*., 2001).

In *Impatiens flanaganae*, an increase in stomatal abnormalities was noticed in leaves of plants grown under high light intensities. These abnormalities include degradation of guard cells, super imposed and contiguous stomata, stomata with persistent intervening walls, cytoplasmic connections, single guard cells, guard cells without pores and persistent stomatal cells (Lall and Bhat, 1996). Carbon dioxide treatment on wild radish showed no significant variation in stomatal

characteristics compared to ambient CO_2 treatment (Case *et al.*, 1998).

A study carried out to investigate the degree of leaf wetness and its capacity to retain water droplets in relation to leaf morphological characteristics of *Valleriana jatamansi* grown under open and shade habitats revealed that leaves developed in open habitats had less wettability but higher capacity to retain water droplets and more number of stomata than those in shade habitats (Pandey and Nagar, 2002).

Relative water content (RWC) in lettuce was found to be reduced significantly under WDS condition than in irrigated plants (Agami, 2013). Under moisture stress, RWC was found to be decreased in *Cajanus cajan* (Nandwal *et al.*, 2001). In fully irrigated sun flower plants, a higher RWC was reported when compared to different irrigation regimes (Vijaykumar and Rao, 2001).

Elevated levels of CO_2 in *Podophyllum hexandrum* Royle showed decreased levels of stomatal conductivity and SLA (Chaturvedi *et al.*, 2009). A decrease in chlorophyll content was reported by Zhu *et al.*, (1997) when CO_2 enrichment was imposed for 30 days in pineapple. Specific leaf area showed a consistent decrease under elevated CO_2 treatment in a comparative study of C3 and C4 plant response to CO_2 (Garbutt *et al.,* 1990). There was no significant difference in RWC when alfalfa plants were grown under CO_2 enrichment (700 μmol mol^{-1}) under different levels of temperature (Aranjuelo *et al.*, 2005).

2.3 BIOCHEMICAL PARAMETERS

Biochemical pathways responsible for the synthesis of organic compounds in plants are highly influenced by various abiotic stress factors. The major biochemical compounds studied in the current

experiment are total soluble proteins, total starch, reducing sugars, free amino acids and proline.

During the course of stresses, active solute accumulation of compatible solutes like carbohydrates is reported to be an effective stress tolerance mechanism (Parthasarathy, 2004). Total soluble proteins and total sugars were found to be significantly lower under WDS in Lettuce plants (Agami, 2013). Carbohydrate changes are of particular importance because of their direct relationship with such physiological processes as photosynthesis, translocation, and respiration. Among the soluble carbohydrates, sucrose and fructans have a potential role in adaptation to these stresses. Hanson and Hitz, (1982) have reported that sugar content decreased under WDS.

The total soluble protein content significantly increased under WDS in palmarosa (Fathima *et al*., 2002). WDS significantly reduced soluble protein content whereas total leaf soluble protein increased 2.5 fold in *Vinca major* and nearly a 20 fold in pumpkin when shifted from shade to full sunlight (Logan *et al*., 1998).

In shade grown *Murcott tangor*, the starch concentration increased in leaves (Raveh *et al*., 2003). WDS reduced total soluble sugars and starch in chickpea, (Yadav *et al*., 1996), sugars in sorghum, (Satbhai *et al*., 1998) and soluble sugars in *Populus euramericana* (Marrison *et al*., 2002).

WDS significantly reduced starch and increased sugar content in moth bean at pre-flowering stage (Garg *et al*., 2001) whereas soluble sugars and starch were increased in wheat plants (Hamada, 2003).

Total reducing sugar and total starch content increased whereas a reduction in total soluble protein were reported under elevated CO_2 (550

ppm and 700 ppm) in black gram (Sathish *et al.*, 2014). The amounts of chlorophyll, soluble protein and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) per unit leaf area did not change with long-term exposure to elevated CO_2 concentration in winter wheat (Delgado *et al.*, 1994). Total soluble sugars and total protein content were enhanced with temperature, whereas total starch remained unchanged when Alfalfa plants were grown under CO_2 enrichment (700 $\mu molmol^{-1}$) under different levels of temperature (Aranjuelo *et al.*, 2005).

Total free amino acids indicate the physiological health status of the tissue (Sadasivam and Manickam, 2008). A reduction in soluble proteins and free amino acids under WDS condition in Barley was reported by El-Tayeb (2005). Drought stress caused organ specific increase in free amino acids in *Populus tremuloides* (Griffin *et al.*, 1991). In sorghum, water stress enhanced the free amino acid content (Satbhai *et al.*, 1998).

During drought, levels of citrulline and arginine increased up to 49 and 11% respectively of total free amino acids in the watermelon leaves (Kawasaki *et al.*, 2000). In chickpea, WDS reduced free amino acids content (Yadav *et al.*, 1996). Cry and his co-workers (1989) reported that the total amino acids level showed an increase as WDS increased in three species of *Pices marina*, white spruce, jack and pine.

In a comparative study of three species of Amaranthus namely, *A. hypocondriachus, A. tricolor* and *A.hybrids*; significant accumulation of proline was reported depending on the onset of WDS (Slabbert and Kruger, 2014). Agami (2013) reported enhanced production of osmoprotectants like proline and soluble sugars under WDS conditions in lettuce.

Osmotic adjustments by proline production in amaranth and tomato plants during the stress periods were reported by Umebese *et al.*, (2009). However a lower concentration of proline accumulation was reported in C4 plant, *Amaranthus cruentus* and sorghum when imposed with oxidative stress due to WDS (Brandt *et al.*, 2011). Exogenous application of proline under water deficit conditions was observed to decrease oxidative stress in cotton (Noreen *et al.*, 2013).

2.4. ANTIOXIDANTS

In plants, reactive oxygen species (ROS) are continuously produced predominantly in chloroplasts, mitochondria and peroxisomes. Production and removal of ROS must be strictly controlled in the plant system. However the equilibrium between production and scavenging of ROS may be perturbed by a number of adverse abiotic factors such as high light, drought, low temperature, high temperature and mechanical stress, which finally leads to oxidative stress (Elstner, 1991; Malan *et al.*, 1990; Prasad *et al.*, 1994; Tsugane, 1999).

ROS when present in excess concentration results in lipid peroxidation and DNA damage and eventually apoptotic death of the cell. (Sarowar *et al.*, 2005). Antioxidants are substances that protect cells from the oxidative damage and thereby reduce the risk of cell damage (Smitha and Sudha, 2011). Development of an antioxidant defense system in plants protects them against oxidative stress damage either by partial suppression of ROS production or by the scavenging of ROS already produced in plants (Murgia *et al.*, 2004).

Different non-enzymatic (ascorbate, glutathione, polyamines, phenols, α -tocopherol, and carotenes) and enzymatic (superoxide dismutase,

ascorbate peroxidase, glutathione reductase, and catalase) molecules are involved in scavenging excess ROS in plants (Yoshimura *et al*., 2004).

2.4.1. Enzymatic antioxidants

The enzymatic antioxidants studied in the experiment include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), peroxidase (POX) and polyphenol oxidase (PPO).

2.4.1.1. Superoxide dismutase activity (EC.1.5.1.1.)

Superoxide dismutases (SOD) are a family of metalloenzymes that catalyze the disproportionation of $O^{.-2}$ radicals (super oxide) into H_2O_2 and O_2, and play an important role in protecting cells against the toxic effects of superoxide radicals produced in different cellular compartments. SODs are distributed in different cell loci, mainly chloroplasts, cytosol, and mitochondria.

SOD

$$.O^{2-} + .O^{2-} + 2H^{+} \xrightarrow{\hspace{2em}} H_2O_2 + O_2$$

Therefore, the activity of this enzyme determines the relative proportions of the two constituents of the Haber-Weiss reaction that generates hydroxyl radicals. Since SOD is present in all aerobic organisms and most subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defense against oxidative stress.

There are three distinct types of SOD classified on the basis of the metal cofactor: the copper/zinc (Cu/Zn - SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) isozymes. The Mn-SOD is found in the mitochondria of eukaryotic cells; some Cu/Zn-SOD isozymes are found

in the cytosol, others in the chloroplasts of higher plants. The Fe-SOD isozymes are often not detected in plants, but when detected, Fe-SOD is usually associated with the chloroplast compartment.

Several forms of SOD have been cloned from a variety of plants. It has been shown that SOD activity increased in cells in response to diverse environmental and xenobiotic stresses like high light, salinity, water logging and drought. Apparently, each of the SOD isozymes are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments. This role may be served by unique lipid peroxidation products from each organelle that diffuse from the site of oxidative damage to the nucleus where they would enhance transcription of specific SOD genes (Buchanan *et al.*, 2000).

A significant increase in antioxidant enzymes (SOD, POD, CAT, APX and GR) was reported in high light intensities and a decrease in the same in low light intensities under drought conditions in *Picea asperata* seedlings (Yang *et al.*, 2008). A fluctuating range of activities with the progress of stress were reported in a study of three genotypes of *Radix astragali* seedlings under WDS (Yong *et al.*, 2006).

Gogorcena *et al.* (1995) reported that SOD activity decreased in drought stressed pea nodules and a similar reduction was reported in rice (Boo and Jung, 1999). In water stressed wheat plants, SOD activity remained unchanged (Bartoli *et al.*, 1999).

WDS had no influence on SOD activity in sorghum (Zhang and Kirkham, 1996). Sairam *et al.* (1998) reported that SOD activity was decreased with osmotic stress in upland rice. Elevated CO_2 treatment in soybean showed decreased activity of SOD (Booker and Fiscus, 2005).

2.4.1.2 Catalase Activity (EC.1.11.1.6)

Catalase is a heme-containing enzyme that catalyses the dismutation of hydrogen peroxide (H_2O_2) into water and oxygen. The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in ß-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. Catalase is one of the first enzymes to be isolated in a highly purified state. Multiple forms of catalase have been described in many plants. Plants have three isoforms of catalase termed cat-1, cat-2 and cat-3 that are on separate chromosomes and are differentially expressed and independently regulated. The cat-1 and cat-2 are localised in peroxisomes and the cytosol, whereas cat-3 is mitochondrial.

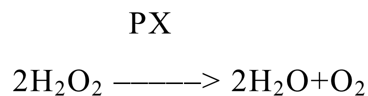
Catalase is very sensitive to light and has a rapid turnover rate similar to that of the D1 protein of PSII, as a result of light absorption by the heme. Stress conditions, which reduce the rate of protein turnover, such as salinity, heat shock or cold cause the depletion of catalase activity. This may have significance in the plant's ability to tolerate the oxidative components of these environmental stresses. Catalase is critical for maintaining the redox balance during oxidative stress. Such control may not be limited to peroxisomal H_2O_2 production.

Moran *et al*. (1994) have reported that catalase activity was decreased in pea plants under water stress. An initial increase in CAT activity was found in *Radix astragali*, with the onset of WDS, which decreased on subsequent days of stress treatment (Yong *et al*., 2006). Lower CAT activity was observed in low light intensities under WDS condition in *Picea asperata* (Yang *et al.*, 2008).

Catalase activity was not affected by mild drought in sorghum (Zhang and Kirkham, 1996). Overexpression of catalase in transgenic rice resulted in enhanced tolerance for oxidative stress produced by chilling (Saruyama *et al.*, 2005).

2.4.1.3. Peroxidase Activity (EC.1.11.1.7)

Peroxidase isoenzymes play an important role in eliminating H_2O_2 and are distributed in at least four distinct cell compartments, the stroma (PX) and thylakoid membrane (PX) in chloroplasts, the microbody (mPX), and the cytosol (cPX).



$$2H_2O_2 \xrightarrow{PX} 2H_2O + O_2$$

Enhanced peroxidase activity due to overexpression of *CAPOA1* gene in transgenic Tobacco resulted in high tolerance to oxidative stress imposed by methyl viologen (Sarowar *et al.*, 2005). Enhanced tolerance of transgenic tobacco plants overexpressing both chloroplastic SOD and PX against methyl viologen mediated oxidative stress was reported by Kwon and his co-workers (2002).

An analysis of knockout plants deficient in cytosolic APX1 (KO-Apx1) revealed that in the absence of APX1 (acsorbate peroxidase) photosynthetic activity was suppressed, suggesting that cytosolic APXs might be essential for chloroplast protection during light stress (Pnueli *et al.*, 2003; Mittler *et al.*, 2004).

The plants under stress enhance the activities of enzymatic antioxidants especially peroxidase (Scandalios, 1993). The transcript level of pea peroxidase increased four fold in response to drought stress and was dramatically enhanced (15 fold) following recovery from stress

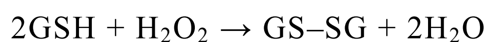
(Mittler and Zilinskas, 1992). The increased peroxidase activity was reported in water deficit stressed Marigold (Karup *et al.*, 1994) and same result was reported in sugarcane (Yang et al. 1995). The transgenic tobacco with higher expression of peroxidases had increased tolerance to photo oxidative damage (Mano *et al.*, 1994).

In tobacco plants, oxidative stress due to salinity lead to two to three fold increase in the activity of SOD and peroxidases (Vancamp *et al.*, 1996). Zhang and Kirkham (1996) reported a decrease in activity of peroxidase in sunflower and sorghum seedlings under water deficit stress.

In spinach leaves, the activities of the peroxidase isoforms decreased as the intensity of light increased, despite the fact that the transcription and protein levels of these isoforms were not altered (Yoshimura *et al.*, 2000).

2.4.1.4. Glutathione peroxidase activity (EC 1.11.1.9)

Glutathione peroxidase (GPx) is a seleno-protein enzymatic antioxidant that removes H_2O_2 and organic hydroperoxides. Several isozymes are encoded by different genes, which vary in cellular location and substrate specificity. Glutathione peroxidase 1 (GPx1) is the most abundant version, found in the cytoplasm. The main reaction that glutathione peroxidase catalyzes is:



$$2GSH + H_2O_2 \rightarrow GS\text{–}SG + 2H_2O$$

where GSH represents reduced monomeric glutathione, and GS–SG represents glutathione disulfide. The mechanism involves oxidation of the selenol of aselenocysteine residue by hydrogen peroxide. This process gives the derivative with a seleninic acid (RSeOH) group.

Generally, in plants, GPxs are localized at different cell organelles as mitochondria, chloroplast, endoplasmic reticulum/cytosol (Ramos *et al*., 2009). The major two functions of GPXs in plants are to protect cell membranes from peroxidative damage and involve in redox transduction under stress (Gueta-Dahan *et al*., 1997; Miao *et al*., 2006; Gill and Tuteja 2010; Suzuki *et al*., 2011).

When treated with stresses including salinity, heavy metal toxicity, drought, heat, cold and hormone, the expression of many GPX genes were enhanced dramatically (Holland *et al*., 1993; Avsian-Kretchmer *et al*., 2004; Ramos *et al*.,2009; Chang *et al*., 2009; Faltin *et al*., 2010).

The expression profiles of CsGPX2 (gene encoding GPx) showed that the enzyme activity was ubiquitous in tea plant organelles and was regulated by different abiotic stresses, which indicates this gene has contributed to the defense against oxidative damage caused by normal plant metabolism (Milla *et al*., 2003) .

The CsGPX2 expression was induced by abiotic stresses including salinity, heavy metal toxicity, drought and heat in Arabidopsis and Citrus (Holland *et al*., 1993; Milla *et al*., 2003; Miao *et al*., 2006). Expression analysis of cloned gene encoding GPx from Tea plant under abiotic stress also showed similar results (Fu, 2014).

2.4.1.5. Polyphenol oxidase activity (E.C. 1.14.18.1)

Polyphenol oxidases (PPOs) are ubiquitous copper-containing enzymes which use molecular oxygen to oxidize common ortho-diphenolic compounds such as caffeic acid and catechol to their respective quinones. PPO-generated quinones are highly reactive and may cross-link or alkylate proteins, leading to the commonly observed brown pigments in damaged plant tissues and plant extracts (Constabel *et al*.,

2008).

Polyphenol oxidase activity was inhibited in Aswagandha under fluoride as a soil pollutant (Vyas and Mavani, 2001). The activation of the oxidative enzymes catalase, peroxidase, polyphenol oxidase increased up to optimum levels and thereafter gradually decreased in *Salicornia brachiate* under different levels of exogenous addition of NaCl (Manikandan and Venkateswaran, 2003).

2.4.2. Non- enzymatic antioxidants

The non – enzymatic antioxidants studied in the experiment includes Vitamin A, Vitamin E, ascorbic acid, flavonoids and phenolic compounds.

2.4.2.1 Vitamin A (β carotene)

It is a fat soluble vitamin which scavenges O_2^{-2} and reacts directly with peroxy radical (Somannavar and Kodliwadmath, 2011). Drought stress has been found to increase β-carotene and α -tocopherol in wheat seedlings and lavender plants, respectively (Keles and Oncel, 2002; Munne-Bosch *et al.*, 2001). Arabidopsis plants when induced with high light stress resulted in rapid accumulation of oxidation products of β-carotene (Ramel *et al.*, 2012).

2.4.2.2 Vitamin E (α-tocopherol)

It is a fat soluble vitamin which converts O_2^- , $.OH^-$ and lipid peroxyl radicals to less reactive forms and acts as a chain breaking antioxidant (Somannavar and Kodliwadmath, 2011). α-Tocopherol is synthesised in the envelope of plastids (Soll *et al.*, 1985; Arango and Heise, 1998), and is stored in plastoglobuli of the chloroplast stroma, (Lichtenthaler *et al.*, 1981; Grumbach, 1983) and in thylakoid membranes (Fryer, 1992; Havaux, 1998).

Transgenic tobacco plant overexpressing the genes involved in tocopherol biosynthesis alleviated stress induced leaf damage (Espinoza *et al*., 2013). Tocopherol mutant of Arabidopsis (*vte1*) when exposed to high light intensity under conditions that lowered photosynthetic activity, chlorophylls and lipids were actively photooxidised compared to the wild type (Havaux *et al*., 2005).

2.4.2.3 Ascorbic acid

Ascorbic acid (Asc) functions as an antioxidant, an enzyme cofactor, and a precursor for oxalate and tartrate synthesis in plants. It participates in a variety of processes, including photosynthesis, photoprotection, the cell cycle, cell-wall growth and cell expansion, synthesis of ethylene, gibberellins, anthocyanins, and hydroxyproline, and resistance to environmental stresses. Ascorbic acid is synthesized partly in the cytosol and in the mitochondria, and it is also found in the apoplast, vacuoles, peroxisomes and chloroplasts. Ascorbic acid has a major role in photo protection as a cofactor in the xanthophyll cycle (Eskling *et al*., 1997).

The ascorbate content is found to be increased in the seedlings of *Picea asperata* under high light intensities (Yang *et al*., 2008). The ascorbate content in plants increased in response to excess level of Zn and high irradiance stress, indicating that ascorbate is one of the effective defense mechanisms against stresses in plants (Michael and Krishnaswamy, 2011). The ascorbate deficient *vtc1* mutants of Arabidopsis showed more sensitivity to drought stress than the wild type (Niu *et al*., 2013). Cucumber seedlings when exposed to short term water deficit a decreased activity of ascorbate was observed (Fan *et al*., 2014).

In *Triticum durum* seedlings grown at an elevated temperature (~30 °C) for 34 days the Asc content declined significantly (Paolacci *et*

al., 1997). Low Asc in the *vtc-1* mutants of Arabidopsis increased the oxidative stress in chloroplasts of water deficit stressed plants (Bosch and Alegre, 2002). In turfgrass, water deficit stress did not induce any increase of ascorbic acid concentration (Xunzhong and Schmidt, 2000). Elevated CO_2 tretament in soybean showed a considerable increase in ascorbate activity (Booker and Fiscus, 2005).

In the early studies of Barbale (1970) and Madsen (1971, 1975), a tripling of the atmospheric CO_2 concentration produced a modest (7%) increase in this antioxidant in the fruit of tomato plants. Kimball and Mitchell (1981), however, could find no effect of a similar CO_2 increase on the same species, although the extra CO_2 of their study stimulated the production of vitamin A. In bean sprouts, on the other hand, a mere one-hour-per-day doubling of the atmospheric CO_2 concentration actually doubled plant vitamin C contents over a 7-day period (Tajiri, 1985).

2.4.2.4 Total flavonoids and phenolic compounds

Secondary metabolites, particularly phenylpropanoids, have long been recognized as serving multiple roles in the responses of higher plants to a wide range of environmental stimuli (Baker and Orlandi, 1995; Dixon and Paiva, 1995; Caldwell *et al*., 2003). Evidence for a key antioxidant function of flavonoids in response to excess UV-B or sunlight irradiance has been suggested (Ryan *et al*., 1998; Gould *et al*., 2000; Jordan, 2002; Neill *et al*., 2002).

Secondary metabolism has been shown also to be upregulated by drought-induced oxidative load, while photosynthetic processes are concomitantly down regulated (Smirnoff, 1993; Mackerness *et al*., 2001; Rizhsky *et al*., 2002; Casati and Walbot, 2003). In ginger, high light

intensity favored accumulation and partioniong of phenolic compounds whereas low light intensity promoted the accumulation of flavonoid compounds (Ghasemzadeh and Ghasemzadeh, 2011).

Drought stress significantly decreased the polyphenol concentration of leaves of *Ligustrum vulgare* developed at 35 or 100% solar radiation, whereas it did not change that of leaves developed at the shade site (Tattini *et al.*, 2004). Light limitation and drought stress resulted in reduced phenolic concentrations (chiefly ferulic and p-coumaric acid) in *Avena fatua* L. seeds (Robert *et al.*, 2010).

Flavonoids exhibit a fluence rate dependence, typical of high irridance response of plant photo morphogenesis (Begg and Wellman, 1994). High irradiation induces flavonoids (particulary Kaempferol derivatives) and its esters in Arabidopsis and isoflavonoids and psovalens in other species (Lois, 1994).

According to Takahama and Oniki, (1997) the condensed tannins (flavonols), as measured by high performance liquid chromatography, were 1000 times higher in normal Pseudocorus rhizomes than in those of stressed one. The effect of stress on flavonol content (a decrease after 35 days of treatment) suggests their participation in the antioxidant defense in normal ones.

Elevated CO_2 improved antioxidant activity and flavonoid content in strawberry fruit (Wang *et al.*, 2003). A 180-ppm increase in the air's CO_2 content increased the foliar concentrations of flavonoids, in field-grown spring wheat by 11 to 14% (Caldwell, 2005). In an experiment conducted under very high atmospheric CO_2 concentrations, Ali *et al.* (2005) found that CO_2 levels of 10,000 ppm, 25,000 ppm and 50,000 ppm increased total flavonoid concentrations of ginseng roots by 228%, 383% and 232%,

respectively, total phenolic concentrations by 58%, 153% and 105%, cysteine contents by 27%, 65% and 100%, and non-protein thiol contents by 12%, 43% and 62%, all of which substances are potent antioxidants.

2.5 GENE EXPRESSION STUDY

2.5.1 Phenyl Propanoid pathway (PP Pathway)

Plants produce a large variety of secondary compounds containing a phenol group. These phenolic compounds are synthesized *via* two different routes: the shikimate pathway and the acetate-malonate pathway, and thus represent a heterogeneous group. The shikimate pathway participates in the synthesis of most plant phenolics, whereas the malonate pathway is of less significance in higher plants, although it is an important source of phenolic products in fungi and bacteria (Taiz and Zeiger, 2010). Secondary metabolites have potent antioxidant properties that have received considerable attention in food science and biomedicine (Kaufman *et al.*, 1999).

Phenylpropanoids are a large group of polyphenolic compounds that comprise an important class of secondary metabolites such as flavonoids, phenols, anthocyanin and lignin in plants (Ververidis *et al.*, 2007). Several studies have suggested that the PP pathway is regulated by interactions between plant roots and soil microorganisms (Jetiyanon, 2007; Jones *et al.,* 2007; Jain *et al.*, 2012; Singh *et al.*, 2013). Phenylpropanoids contribute to all aspects of plant responses towards biotic and abiotic stimuli. They are not only indicators of plant stress responses upon variation of light or mineral treatment, but are also key mediators of the plants resistance towards pests (La Camera *et al.,* 2004). Mole *et al*. (1998) explained that high light activates the phenyl propanoid pathway. Phenolic compounds are classified into several

groups, including anthocyanins, flavonoids, isoflavonoids (phytoalexins), lignin, tannins and polymeric phenolic compounds.

The plant shikimate pathway is the entry to the biosynthesis of phenylpropanoids. The shikimate pathway begins with a coupling of phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate to give the seven-carbon 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP). The shikimate pathway intermediate compounds and the enzymes involved are detailed in Fig.1. The enzyme chorismate mutase converts chorismate to phenylalanine which is the substrate for PP pathway. In PP pathway, phenyalnine is first converted to cinnamic acid by the activity of the enzyme Pheyl Alanine Lyase (PAL). The subsequent reactions of the PP pathway involves transformation of cinnamic acid to coumaric acid through hydroxylation, coumaric acid is further hydroxylased to form the phenolic compounds like caffeic, ferulic acid, lignins and lignans. Coumaric acid forms coumaryl CoA by CoA ligases, which proceeds to other phenolic compounds as flavanoids (Buchanan *et al.*, 2000). The intermediate compounds in PP pathway and enzymes involved are given in Fig.2.

Flavanoids constitute enormous class of phenolic natural products with more than 4500 different representatives. The first committed step in flavonoid synthesis pathway is catalyzed by Chalcone Synthase (CHS). The CHS enzyme comes under the multigene family of type III polyketide synthase enzyme (PKS). In plants, CHS is activated by a wide range of environmental and developmental stimuli. Chalcone synthase and PAL, the key enzyme in phenolic biosynthesis, were upregulated by drought stress in Arabidopsis (Bray, 2002). Advanced studies of the responds to

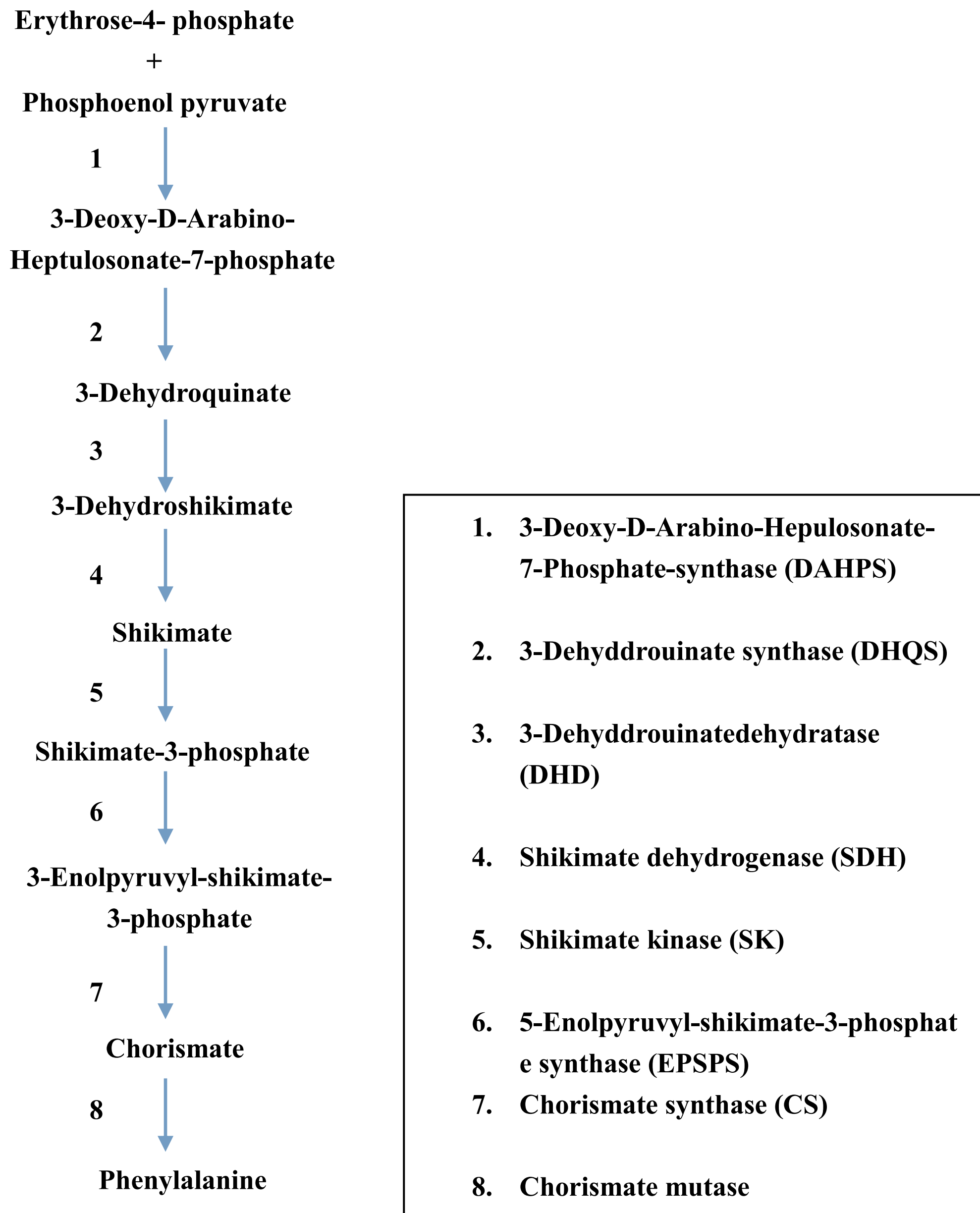


Fig.1. Shikimate pathway. Enzymes involved in each reaction are numbered.

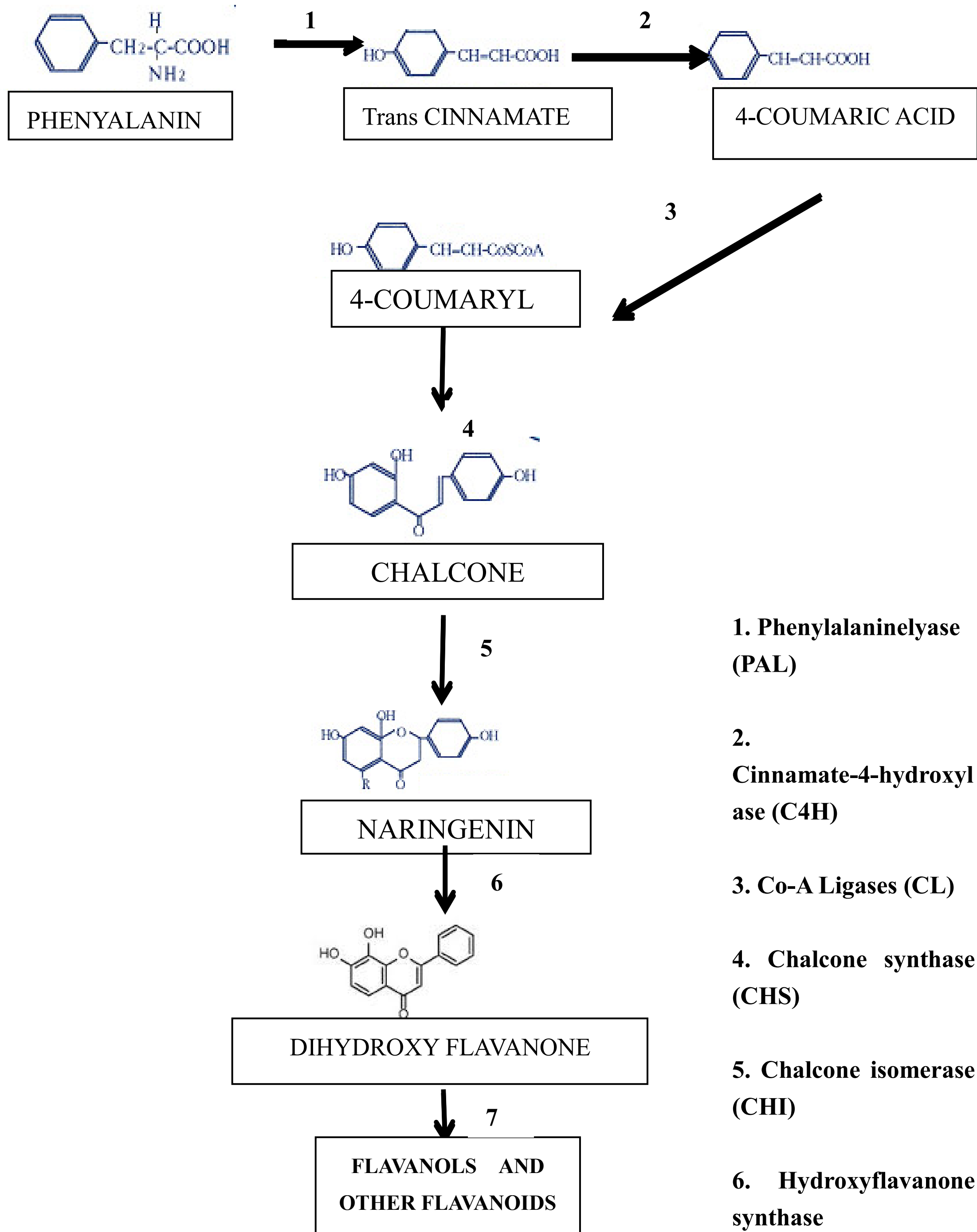


Fig.2.Phenyl Propanoid Pathway

UV-B and UVR-8 in Arabidopsis showed a rapid upregulation of CHS gene (Xing *et al*., 2014).

Relationship between phytochemicals and high light or UV-B radiation has been well established. UV-B has been shown to induce a broad range of phytochemicals, which absorb the harmful radiation and protect plants against damaging ROS (Bieza and Lois, 2001; Burritt and Mackenzie, 2003; Gitz III *et al*., 2004). UV-B radiation can also increase PAL activity which can facilitate phenolic accumulation and thus, induce stress tolerance in plants (Teklemariam and Blake, 2004). Similarly, high temperature has been shown to induce phytochemical accumulation in several plant species as a defense strategy (Sayre *et al*., 1953; Lefsrud *et al*., 2005).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The objective of the programme was to elicit information on the physiological, biochemical and molecular attributes associated to the antioxidant accumulation due to abiotic stress and CO_2 enrichment in *Amaranthus spinosus* Linn. For this, a pot culture experiment was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani. The work was implemented in two separate experiments. In one experiment, *Amaranthus spinosus* Linn. plants were raised in pots and the required treatments *viz.* shade stress and water deficit stress were given, while in the other experiment *A. spinosus* Linn. plants were grown in pots and kept in Open Top Chamber (OTC) facility for studying the effect of CO_2 enrichment. Biometric observations were taken and physiological, biochemical as well as molecular analysis was done after subjecting the plant to stress for a period of 30 days.

3.1. EXPERIMENTAL DETAILS

3.1.1 Location

The pot culture experiment was conducted at College of Agriculture, Vellayani, situated at 8^o5'N latitude and 76^o9'E longitude at an altitude of 29 m above mean sea level. The soil of Instructional Farm, which was used for the study, was red loam, belonging to the order Oxisol and taxonomic class loamy Kaolnite Rhodic haplastox (Vellayani Series). The soil was acidic with a pH of 5.2 and with an EC of 0.002 d Sm-1.

3.1.2 Season

The experiments were conducted from April 2014 to June 2014 and weather data during the treatment period is given in appendix I.

3.1.3 Planting Material

Seeds of *Amaranthus spinosus* Linn. (Plate 1) were procured from NBPGR.

Plate 1. *Amaranthus spinosus* Linn.

(Accession No. IC- 547517).

3.1.4 Layout of the Experiment

The Experiment was laid in CRD. There were nine treatments and three replications in one experiment and six treatments and four replications in the other, conducted at OTC.

3.1.5 Outline of Technical Programme

The whole research program was classified into two experiments. Each experiment was carried out in different locations simultaneously *viz.* the first experiment comprised of nine different treatments which had combinations of water deficit stress and shade stress and second experiment comprised of six different treatments with combinations of water level and CO_2 concentrations. The treatments included three levels each of water deficit stress and shade. The water deficit stress levels were 100 %, 75 % and 50 % of field capacity. The shade levels were 50% and 25 % and open condition. Seeds were sown in pots and thinning was done one month after planting. Treatments were imposed for a period of 30 days, one month after sowing. The treatment combinations are given in the Tables 1 and 2. In experiment no.1, the treatments 1, 2, and 3 were given 100 %, 75 % and 50 % of field capacity under 50 % shaded condition. Treatments 4, 5, and 6 were given 100 %, 75 % and 50 % of field capacity under 25 % shaded condition and treatments 7, 8 and 9 were given 100 %, 75 % and 50 % of field capacity under open condition where as in the second experiment, water deficit stress combined with CO_2 enrichment was given. There were a total of six treatments and four replications and all the treatments were represented with the prefix ‘O’, to identify it as plants kept in OTC.

Table 1. Details of shade and water deficit stress treatments imposed in *Amaranthus spinosus* Linn.

Sl. No.	Treatment	Name of the treatment
1.	T1	50% shade + 100 % Field Capacity
2.	T2	50% shade + 75 % Field Capacity
3.	T3	50% shade + 50% Field Capacity
4.	T4	25% shade + 100% Field Capacity
5.	T5	25% shade + 75% Field Capacity
6.	T6	25% shade + 50% Field Capacity
7.	T7	Open + 100 % Field Capacity
8.	T8	Open + 75 % Field Capacity
9.	T9	Open + 50 % Field Capacity

Table 2. Details of water deficit stress and CO_2 enrichment treatments imposed in *Amaranthus spinosus* Linn.

Sl. No.	Treatment	Name of the treatment
1.	OT1	100 % Field Capacity + 500 ppm CO_2
2.	OT2	50 % Field Capacity + 500 ppm CO_2
3.	OT3	100 % Field Capacity + chamber without CO_2
4.	OT4	50 % Field Capacity + chamber without CO_2
5.	OT5	100 % Field Capacity + Open field
6.	OT6	50 % Field Capacity + Open field

3.1.6 Preparation and Planting

The experiment was conducted in pots of 10 litre volume filled with potting mixture consisting of farm yard manure, sand and soil in ratio of 1:1:1. The experiment was laid out in CRD. The seeds were sown in pots. After 30 days thinning was done and one healthy seedling per pot was retained (Plate 2).

3.1.7 Artificial shading/Shade stress

The shade levels (50 % and 25% shade) were provided by using high-density polyethylene nets purchased from Kerala Agro Industries Corporation, which was spread over pandals (Plate 3).

3.1.8 Water stress

For imposing water deficit stress, the potting mixture was taken in pots. Prior to the treatment, the pots were irrigated at evening and left over night to bring them to field capacity. The exposed part of the soil on the pot was completely covered with the broken pieces of pot. The drainage holes were closed with cement. The weight of the individual pot with soil, pot pieces and plant was recorded with the help of an electronic digital top loading weighing balance. The water holding capacity of the soil was calculated gravimetrically. All the pots in each shade level were grouped into three sets with one set at FC and others at 75% and 50 % FC. The weight of pots at 75% and 50% was calculated based on the water holding capacity of the soil using the equation given below.

Pot Weight at 100% FC (W100) = A+B+Q100

Pot Weight at 75% FC (W100) = A+B+Q75

Pot Weight at 50% FC (W100) = A+B+Q50

Where, A= soil dry weight with pot

B= Broken pot pieces (spread on each pot surface to prevent evaporation) Whereas Q100, Q75 and Q50 =Quantity of water present at 100 % FC, 75 % FC

Plate 2. One month old seedlings

Plate 3. Plants maintained under artificial shade

and 50 % FC respectively.

The plants were kept at respective soil moisture level by adding water daily, which was lost by transpiration in the previous day. The transpiration loss was calculated by weighing the pots daily and finding the difference in weight from the respective reference weight.

3.1.9 CO_2 enrichment study

Open Top Chambers (OTCs) are structures constructed to maintain near natural conditions and elevated CO_2 conditions for plants for experimental purpose. For the conduct of current study, the OTC constructed in the Department of Plant Physiology was utilized. The chamber built of metal frame covered with white UV poly sheet. The chamber is 3 x 3 x 3 m dimension, 45° slope and $1m^2$ opening at the top. Two such chambers were built; one serves to impose CO_2 enrichment and the other serves as ‘control chamber’ to study the chamber effect.

Pots were standardized for 100% field capacity following the same methodology explained for experiment 1. Seeds were sown in pots and allowed to germinate. Thinning was done 30 days after sowing and pots along with plants were kept in respective chambers as described in treatment details (Table 2). The weather data from OTC during the period of study is given in Appendix II.

3.2 OBSERVATIONS

3.2.1 Growth Parameters

3.2.1.1 Plant Height

Plant height was measured from the base of the plant to the apex of the plant using a scale.

3.2.1.2 Leaf Area

Leaf area was measured by plotting the leaves of the treatments plants in a graph paper and counting the number of milli squares covered by the leaf.

3.2.1.3 Shoot Weight

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

3.2.1.4 Dry matter production

Whole plants were uprooted and dried to constant weight at 80^oC in oven. The sum of the dry weight of all the plant parts constituted the total dry matter yield.

3.2.2 Physiological Parameters

3.2.2.1 Estimation of Chlorophyll and Carotenoids (DMSO method)

A weighed quantity of sample (0.5g) was taken and cut 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 510 nm using a spectrophotometer. The chlorophyll content was measured by substituting the absorbance values in the given formulae.

$$\text{Chl a} = (12.7 \times A_{663} - 2.69 \times A_{645}) \times V/1000 \times 1/\text{Fresh weight}$$

$$\text{Chl b} = (22.9 \times A_{645} - 4.68 \times A_{663}) \times V/1000 \times 1/\text{Fresh weight}$$

$$\text{Total Chl (a + b)} = (8.02 \times A_{663} + 20.2 \times A_{645}) \times V/1000 \times 1/\text{Fresh weight}$$

$$\text{Carotenoid} = [(7.6 \times A_{480}) - (1.49 \times A_{510}) \times V] / (w \times 1000)$$

3.2.2.2 Specific Leaf Area (SLA)

From each pot, fully expanded third leaf (from main stem apex) was collected. Leaflets were separated and the petioles were discarded and area was measured. Leaflets were dried at 80^oC for 2 days and the dry weight was taken. SLA was calculated using the formula.

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

3.2.2.3 Stomatal Characteristics

3.2.2.3.1 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 40 x objective and 10 x eyepieces. The field of the microscope was measured using a stage micrometer and stomatal frequency per unit area was calculated.

Stomatal frequency = No. of stomata / Area of the microscopic field

3.2.2.3.2 Stomatal Index

Stomatal Index was calculated based on the stomatal frequency and the number of epidermal cells surrounded by the stomata per unit area of the microscopic field, observed under microscope and counted using a 40 x objective and 10 x eyepieces.. Stomatal index was calculated using the following formula.

Stomatal Index = Stomatal number per unit microscopic field ÷ no. of epidermal cells

3.2.2.3.3 Stomatal Conductance

Stomatal conductance was measured using the SAI-1 Porometer of company Delta T Devices and expressed as milli moles/m^2/s.

3.2.2.4 Relative Water Content (RWC)

Relative water content was calculated by measuring the fresh weight, dry weight and turgid weight of known number of leaf discs from the treatment 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 ^{o} C for 3 consecutive days. The RWC of the treatments was calculated using the following formula.

RWC = [Fresh weight – dry weight) / (turgid weight –dry weight)] x 100

3.2.3 Biochemical parameters

3.2.3.1 Estimation of Total Soluble Protein (Bradford method)

The totals soluble proteins were estimated following the Bradford method (1976). A series of protein samples were prepared in PBS. The experimental samples were prepared in 10 microliter of PBS. A known volume (5 ml) of diluted dye binding solution was added to each tube. The solution was mixed well and allowed to develop a blue colour for at least 5 min but no longer than 30 min. The red dye turns blue when it binds protein and its absorbance was measured at 596 nm. A standard curve was plotted using the field plot absorbance verses concentration. The protein in the experimental sample was calculated using the standard curve.

3.2.3.2 Estimation of Starch

The estimation of starch in plants was done following the Anthrone method (Sadasivam and Manickam, 2008). A known quantity of plant sample (0.1 g) 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 to 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^oC 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.

3.2.3.3 Estimation of reducing sugars (DNS Acid Method)

The estimation of reducing sugars in plants was done following Dinirto Salicylic acid (DNS) method (Sadasivam and Manickam, 2008). 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^{0}C. The sugars were dissolved by adding 10 ml water. Aliquots of 0.5 to 3 ml were pipetted out into test tubes and the volume was equalized to 3ml with distilled water in all the test tubes. To this 3 ml of DNS reagent was added. The test tubes were heated in a boiling water bath for 5 minutes. Rochelle salt solution (40%, w/v) (1 ml) was added to the test tubes when the contents were hot. Then the test tubes were cooled and the intensity of dark red colour was read at 510 nm. A series of the standard, Glucose, (0 to 500 µg) was run and a standard curve was plotted. The amount of reducing sugars in the sample was calculated from the standard graph.

3.2.3.4 Estimation of total free amino acids

The total free amino acids were estimated following the Ninhydrin method (Sadasivam and Manickam, 2008). 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. The intensity of the purple color was read at 570 nm, in a colorimeter, 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.

3.2.3.5 Estimation of Proline

The proline content in the plant samples was estimated following the method described by Sadasivam and Manickam (2008). The plant sample (0.5 g was extracted by homogenizing in 10 ml of 3% aqueous sulphosalycylc acid. The homogenate was then filtered through Whatmann No. 2 filter paper. Aliquot of 2ml was drawn from the filtrate in a test tube and 2 ml of glacial acetic acid and 2 ml of acid ninhydrin was added to it. The mixture was heated in a boiling water bath for one hour. The reaction was *terminated* by placing the tube in ice bath. Toluene (4 ml) was added to the reaction mixture and stirred well for 20- 30 seconds. The toluene layer was then separated and warmed to room temperature. The red colour intensity was read at 520 nm. A series of standard with pure proline was run in a similar method to prepare the standard curve. The amount of proline in the sample was calculated from the standard curve. The proline content is expressed as follows.

$$\mu \text{ moles per g tissue} = \{[(\mu\text{g proline / ml}) \times \text{ml toluene}] \div 115.5\} \times (5/\text{ g sample})$$

where 115.5 is the molecular weight of proline.

3.2.4 Anti-oxidant Assays

3.2.4.1 Non- Enzymic Antioxidant Assays

3.2.4.1.1 Estimation of Vitamin E

The estimation of Vitamin E was done following the method described by Rosenberg (1992). Equal volumes (1.5 ml each) of test, standard and blank (water) were pipetted out into 3 centrifuge tubes and closed. Ethanol (1.5ml) was added to the test and blank, while 1.5 ml of water was added to the standard and then centrifuged. Xylene (1.5 ml) was added to each tube, followed by a centrifugation. One milliliter of xylene layer was transferred into another stopper tube, taking care not to include any ethanol or protein. 2, 2- dipyridyl reagent (1 ml) was added to each tube and mixed well. An aliquot of 1.5 ml was pipetted out into a cuvette and the extinction coefficient of test and standard were read at 460 nm against the blank. Ferric chloride, 0.33 ml, was added to each tube beginning with blank and mixed well. The absorbance of test and standard was read at 520 nm after exactly 15 minutes of incubation. The amount of tocopherol was calculated as follows:

Amount of tocopherol (µg/g) = [(A_{520} –A_{460})/A_{520} of std.]÷ 0.29 x 15 x (total volume of homogenate/ vol. used x weight of tissue)

3.2.4.1.2 Estimation of Vitamin A

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

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

Amount of β carotene = (OD of sample x 13.9 x 104 x 100) ÷ (weight of sample x 560 x 1000)

3.2.4.1.3 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 (5 ml) was pipetted out into a 100 ml conical flask. 4% oxalic acid was added to it and titrated against the dye (V_1 ml). End point was noted on appearance of pink colour which persisted for a few minutes. The sample (0.5- 5g) was weighed and ground in a mortar with pestle using 15 ml 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 25 ml using oxalic acid. 5.0 ml aliquot was pipetted into a conical flask to which 10 ml 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 acid (mg/100g) = (0.5 mg /V1 ml) x (V2/ 5 ml) x (100/ weight of sample).

3.2.4.1.4 Estimation of flavonoids

The total flavonoids in plants were estimated following the method described by Cameron *et al.* (1943). The sample (0.5g) was first extracted with methanol; water mixture (2:1) and secondly with the same mixture in the ratio 1:1. The extracts were shaken well and allowed to stand overnight. The supernatants were pooled and the volume was measured. This supernatant was concentrated and

then used for the assay. A known volume of the extract was pipetted out and evaporated to dryness. Vanillin reagent (4 ml) was added and the tubes were heated in a boiling water bath for 15 minutes. Varying concentrations of the standard were also treated in the same manner. The optical density was read using a spectrophotometer at 340 nm. A standard curve was constructed and the concentration of flavonoids in each sample was calculated. The results were expressed as mg/g sample.

3.2.4.1.5 Estimation of phenols

Quantification of phenols was done by Folin-Ciocalteau method (Mayr *et al.,* 1995). Phenol was estimated from 0.5 g sample 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.

3.2.4.2 Enzymatic Antioxidant Assays

3.2.4.2.1 Superoxide dismutase (SOD)

SOD activity of plants was quantified following the method described by Kakkar *et al*. (1984). The samples (0.5 g), were ground with 3.0 ml of potassium phosphate buffer, centrifuged at 2000 g for 10 minutes and the supernatants were used for the assay. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml 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 0.2 ml of

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

3.2.4.2.2 Estimation of Catalase (CAT)

The CAT activity in plants was quantified following the method described by Luck (1974). A 20% homogenate of the sample was prepared in phosphate buffer. The homogenate was centrifuged and the supernatant was used for the enzyme assay. The H_2O_2-phosphate buffer (3.0 ml) was taken in an experimental cuvette, followed by the rapid addition of 40μl of enzyme extract and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm in a spectrophotometer. 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 24 0nm by 0.05 units.

3.2.4.2.3 Estimation of Peroxidase (POX)

The peroxidase activity in plants was estimated following the method described by Reddy *et al*. (1995). A 20% homogenate was prepared in 0.1 M phosphate buffer (pH 6.5) from the sample, clarified by centrifugation and the supernatant was used for the assay. To 3.0 ml of pyrogallol solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5 ml of H_2O_2 was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer. One unit of peroxidase is defined as the change in absorbance/minute at 430 nm.

3.2.4.2.4 Estimation of Glutathione Peroxidase (GPx)

The activity of GPx in plants was estimated following the method described by Sadasivam and Manickam (2008). Tissue homogenate (10%) was prepared in phosphate buffer (pH 7.0) and centrifuged at 10,000 rpm for 15 min. at 4 ºC. the supernatant was used for the assay of GPx. The reaction mixture was prepared by adding 0.4 ml buffer, 0.1 ml sodium azide, 0.2 ml reduced glutathione, 0.5 ml enzyme extract and 0.1 ml H_2O_2 into a test tube. The final volume was made up to 2.0ml with distilled water. The tube was incubated at 37ºC for 5- 30 min. the reaction was stopped by adding 0.5 ml of 10% Trichloroaceticacid. The content was centrifuged and the supernatant was collected. Disodium hydrogen phosphate (3 ml) and DTNB reagent (1 ml) was added to the supernatant. This was done to determine the unused or residual glutathione content. The colour developed was measured at 412 nm using a spectrophotometer. Blank was prepared with disodium hydrogen phosphate and 1ml of DTNB reagent. Aliquots of standard solution were also treated in the above described method. The activity is expressed as µg of glutathione consumed / minute/mg protein.

3.2.4.2.5 Estimation of Polyphenol Oxidase (PPO)

Polyphenol activity was determined as per the procedure given by Mayer *et al.,* (1965). The enzyme extract was prepared in 0.1 M phosphate buffer (pH 6.5). The reaction mixture contained 0.1 M sodium phosphate buffer (1 ml) (pH 6.5) and 50 µl of enzymes extract. The reaction was initiated by adding 1 ml of 0.01M catechol. The change in absorbance was recorded at 495 nm and PPO activity was expressed as change in the absorbance of the reaction mixture per minute per gram on fresh weight basis.

3.2.5 Gene Expression

Gene expression studies were carried out using the facilities of Department of Plant Physiology, College of Agriculture, Vellayani and Rajiv Gandhi Centre

for Biotechnology, Thiruvananthapuram.

3.2.5.1 RNA Isolation

3.2.5.1.1 DEPC Treatment of Glasswares and Milli-Q water

Diethylpyrocarbonate (DEPC) was added to Milli-Q water to a final concentration of 0.02%, mixed well using a magnetic stirrer and kept overnight. The residual DEPC was destroyed by autoclaving the treated water at 121ºC at 1.05 kg/cm^2 for 20 min. Glasswares and plastic wares used for RNA isolation and cDNA synthesis were treated with DEPC water and autoclaved as mentioned above. Glass wares for reagent preparations were baked further at 180ºC in a hot air oven.

3.2.5.1.2 Total RNA Isolation from A. spinosus Linn. leaves

Total RNA was isolated from 100 mg leaf tissue using TrizolTM reagent. Hundred milli gram of young leaves was frozen in liquid nitrogen. Frozen tissue was ground to fine powder in an RNase free mortar using pestle. One mL TrizolTM reagent was added to the ground tissue and was kept at room temperature for 5 min. The homogenate was transferred to a 2 mL RNase free microfuge tube. The sample was centrifuged at 12000 rpm for 10 min at 4ºC. The supernatant was transferred to an RNase free centrifuge tube and 200 µL of chloroform was added per 1 ml TrizolTM reagent used. The tube was shaken in hand for 15 seconds and incubated for 3 minutes at 28 ^{o}C. It was then centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was collected in a new centrifuge tube and 500 µL chilled isopropanol was added and kept at room temperature for 45 minutes to precipitate the total RNA. The sample was centrifuged at 12000 g for 10 min at 4ºC. The supernatant was discarded and the pellet was washed in 10 mL of 75% ethanol by centrifugation at 7500 g for 5 min at 4 ºC. After centrifugation ethanol was decanted and the gel like RNA pellet was washed two times with 75% ethanol and centrifuged at 7500 rpm for 5 minutes a

4 ^{o}C. The RNA pellet was dried at room temperature for 10 minutes. The RNA pellet was dissolved in 100 µL of nuclease free water and stored at -80ºC.

3.2.5.1.3 DNase I treatment of total RNA

The total RNA was treated with DNase I to remove any traces of genomic DNA present. The reaction mix contained total RNA, 40 mM Tris-HCl (pH 7.5), 6mM $MgCl_2$ and 5 units of DNase I set to a final volume of 200 µL. The reaction was incubated at 37 ºC for 10 min followed by incubation at 65 ºC for 15 min to denature DNase I. The treated RNA was extracted with phenol: chloroform: isoamyl alcohol mixture (25: 24: 1) by centrifugation at 10,000 g for 10 min at room temperature. To the aqueous phase, 20 µL of 3 M sodium acetate and 200 µL of isopropanol were added and incubated at room temperature for 10 min. The sample was centrifuged at 10,000 g for 10 min at room temperature. The RNA pellet was washed with 1 ml of 75 % ethanol at 7500 g for 5 min. The pellet was air dried and dissolved in 100 µL of nuclease free water. The quality and integrity of total RNA was checked in a 2 % agarose gel by loading 5 µL samples along with loading dye.

3.2.5.1.4 Quantitation of Total RNA

Total RNA was quantitated spectrophotometrically at 260 nm. The concentration of RNA in 1 µL of the RNA sample was calculated by the following equation.

$$\text{Conc.of RNA } (\mu g\ ml^{-1}) = (40 \times OD_{260} \times \text{Dilution factor}) \div 1000$$

3.2.5.1.5 cDNA Synthesis

First strand cDNA was synthesized from mRNA by reverse transcription using RevertAid M-MuLV Reverse Transcriptase (M-MuLV RT) provided with the Thermo Scientific ReverAid First Strand cDNA Synthesis Kit manufactured by Thermo Fisher Scientific Inc. The reaction mix was prepared for 20 µL volume

with 1.5 µg of mRNA, 1 µL oligo (dT)18 primer, 5X Reaction Buffer, 1 µL RiboLock RNase Inhibitor, 20 Units 10mM dNTP mix and 200 Units of RevertAid M-MuLV RT. Total RNA, oligo dT18 primer and nuclease free water were added to a nuclease free microfuge tube and denatured to remove secondary structure from RNA by incubating at 65 ºC for 5 minutes and snap cooling on ice. The sample was spun down to remove any condensed reagent at the microfuge lid and rest of the components were added to the denatured RNA. Reverse Transcriptase reaction was incubated at 42 ^{o}C for 60 min. followed by heat inactivation of the enzyme at 70°C for 5 min on a PCR machine. Sample was stored at -20 ^{o}C. Reaction mixtures for positive control, negative control and no template control were also prepared and the same procedure was followed subsequently.

3.2.5.1.6 Reverse Transcriptase - PCR (RT-PCR)

Reverse Transcriptase Polymerase Chain Reaction was carried out to determine the expression level of the Chalcone synthase (CHS) genes in the selected treatments of the plant. The cDNA synthesized was used for PCR amplifications with primers of CHS genes. The 20 µL PCR reactions contained 1 µL cDNA template 1 µL each of primers, 10 µL (units) of Orion Master Mix and remaining units of nuclease free water. A primary PCR was carried out using the primers CHS1 and CHS4, followed by nested PCR using the primers CHS2 and CHS3 using 3 µL of the primary PCR product as the template (Radhakrishnan and Soniya, 2009). The annealing temperature, details of the primers and number of PCR cycles are detailed in Table 3.

Table 3.Primers, Temperature and number of PCR cycles used in RT-PCR.

Gene	CHS	CHS (Nested)
Forward Primer	CHS1	CHS2
Reverse Primer	CHS4	CHS3
Tm (^{o}C)	42	42
No. of PCR cycles	35	35

CHS1 5' - RARGCIITIMARGARTGGGGICA- 3'

CHS2 5' - GCIAARGAYITI-GCIGARAAYAA- 3'

CHS3 5' - CCCMWITCIARICCITCICCIGTIGT -3'

CHS4 5' - TCIAYIGTIARICCIGGICCRAA -3'

3.3 STATISTICAL ANALYSIS

Statistical analysis was done using WASP 2.0 (Web Agri Stat Package 2.0), a web based agricultural statistics package available at www.icargoa.res.in.

RESULTS

4. RESULTS

The current investigation was carried out to study the effect of abiotic stress factors like water deficit stress and shade stress and CO_2 enrichment on the accumulation of antioxidant in *Amaranthus spinosus* Linn. One set of plants were imposed with a combination water deficit stress at three different levels including 100%, 75% and 50% field capacities and shade at three different intensities including 50% and 25% shade and at open conditions (Plate 4). Another set of plants were given CO_2 enrichment along with water deficit stress and two different levels, i.e. 100% and 50% field capacities (Plate 5). Various growth parameters, physiological and biochemical parameters were recorded to study to relate the antioxidant characteristics of the plant under conditions of abiotic stress as well as CO_2 enrichment. Plants after CO_2 enrichment is given in Plate 6. The results of the observations and experiments done to address the above mentioned objective is given below.

4.1 GROWTH PARAMETERS

4.1.1 Plant Height

The effect of abiotic stress on plant height of *Amaranthus spinosus* Linn. is presented in Table 4. Among the treatments a significant variation in plant height was observed. The height of plants kept under shade was higher compared to the plants kept in open condition. The plant was tolerant upto 50% shaded condition. The plants in treatment T6 recorded a maximum height of 62.83 cm. Increased plant height was also observed for the treatments T5 (62.13 cm), T4 (61.93 cm) and T1 (58.07 cm). Plants kept in open condition showed reduction in height. A significant reduction in height of the plant was observed in treatment T9 (46 cm). Plants which were given treatment OTI showed a maximum plant height of 52.83 cm when compared with the plants under CO_2 enrichment as shown in Table 5.

(A)

(B)

(C)

Plate 4. Plants after treatment (A) T1,T2, T3; (B) T4,T5,T6; (C) T7,T8, T9

Table 4. Effect of abiotic stress on growth parameters of *Amaranthus spinosus* Linn.

Treatments	Treatment means of			
	Plant Height (cm)	Leaf Area (cm^2)	Shoot weight (g)	Dry Matter Production (g)
T1	58.07	35.47	48.34	20.43
T2	44.67	40.33	40.99	18.65
T3	44.70	40.57	25.51	17.46
T4	61.93	32.36	38.89	19.41
T5	62.13	32.54	30.32	19.09
T6	62.83	42.66	37.32	20.06
T7	56.73	34.06	39.41	20.65
T8	53.10	28.71	37.09	20.58
T9	46.00	36.63	34.03	19.73
CD (0.05)	**9.965**	**3.812**	**9.179**	**1.304**

Table 5. Effect of CO_2 on growth parameters of *Amaranthus spinosus* Linn.

Treatments	Treatment means of			
	Plant Height (cm)	Leaf Area (cm^2)	Shoot weight (g)	Dry Matter Production (g)
OT1	52.86	28.23	43.16	10.91
OT2	39.83	23.69	25.27	7.29
OT3	44.03	30.04	28.89	7.05
OT4	48.55	31.68	35.52	9.47
OT5	34.75	13.94	19.96	5.60
OT6	34.28	24.44	23.48	4.68
CD (0.05)	**11.958**	**4.188**	**14.040**	**0.992**

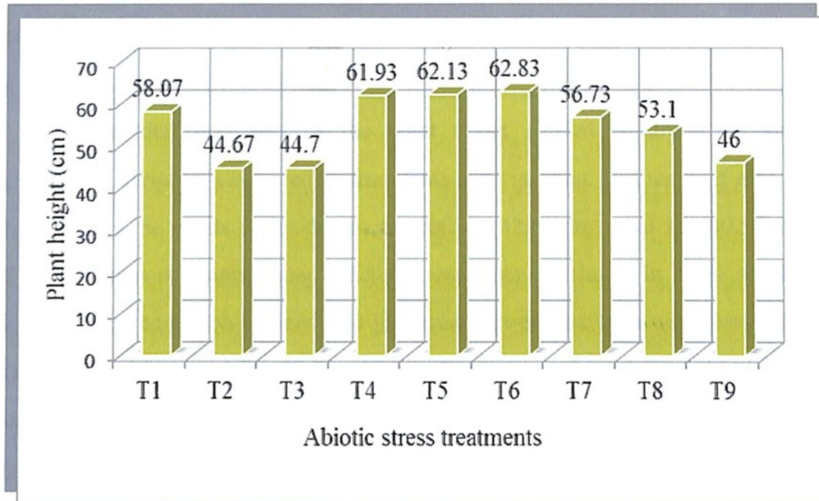


Fig.3. Effect of abiotic stress on plant height of *Amaranthus spinosus* Linn.

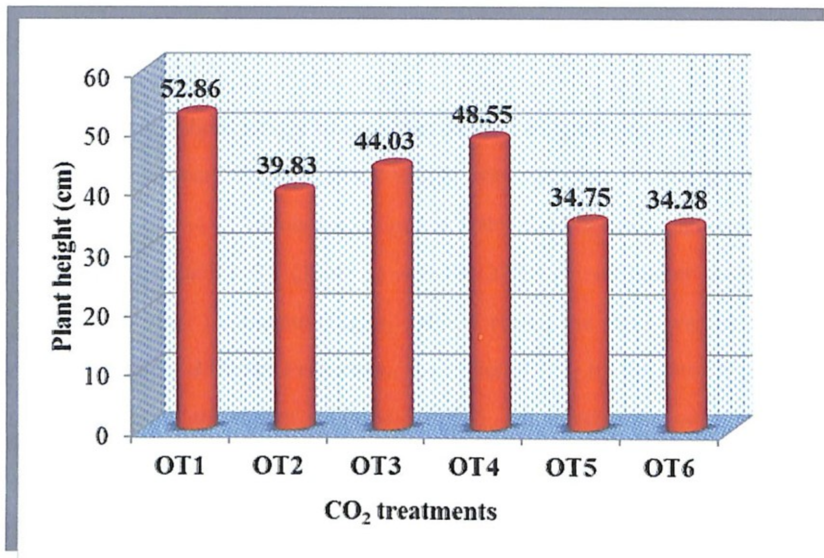


Fig.4. Effect of CO_2 on plant height of *Amaranthus spinosus* Linn.

The figures 3 and 4 show the graphical representation on effect of abiotic stress and CO_2 enrichment on plant height.

4.1.2 Leaf Area

The total leaf area of the plant under shaded condition showed a significant variation compared to the plants kept in open. Highest leaf area was recorded by the plants under the treatments T6 (42.63 cm^2) and T3 (40.57 cm^2). The leaf areas of plants under different treatments are shown in Table 4. Plants kept in open condition had reduced leaf area. Lowest leaf area was observed for the plants in treatment T8 (28.7 cm^2). Fig. 5 represents the effect of abiotic stress on leaf area.

Plants enriched with CO_2 did not show a significant increase in leaf area, when compared to the chamber control and open control. Leaf areas of plants under treatments OT1 and OT2 were 28.23cm and 23.69cm respectively. Table 5 shows the leaf area of plants under different treatments kept at Open Top Chamber.

4.1.3 Shoot weight

The total shoot weight of the plant showed a significant difference among the given treatments. Highest shoot weight was recorded for the plants under treatment T1 (48.34 g). Plants maintained at 100% field capacity had higher shoot weight. Treatments T1, T7 (39.41 g) and T4 (38.88 g) had higher shoot weight compared to other treatments (Fig.6). Table 4 shows the shoot weight of plants maintained under different treatments. The effect of CO_2 on the growth and shoot weight of plants are shown in Table 5(Fig.7). An increased shoot weight was observed for the plants enriched with CO_2, OT1 (43.16 cm).

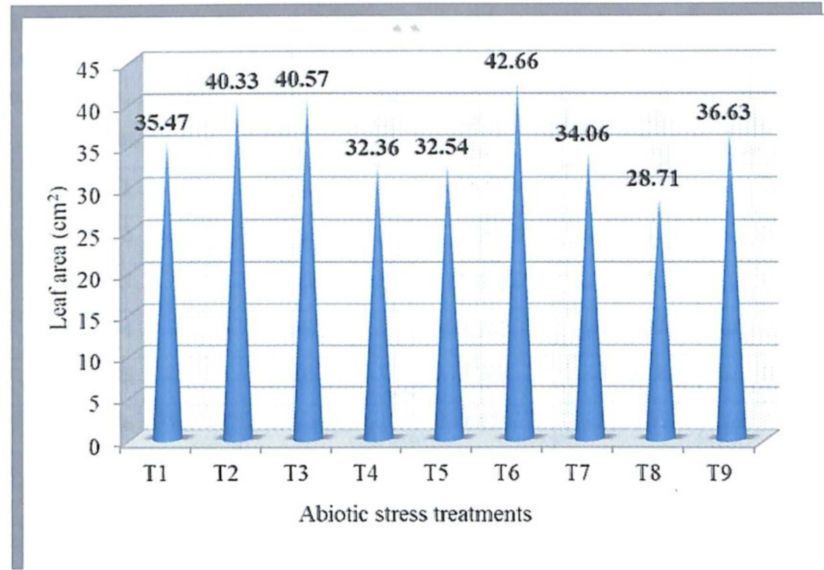


Fig. 5. Effect of abiotic stress on leaf area of *Amaranthus spinosus* Linn.

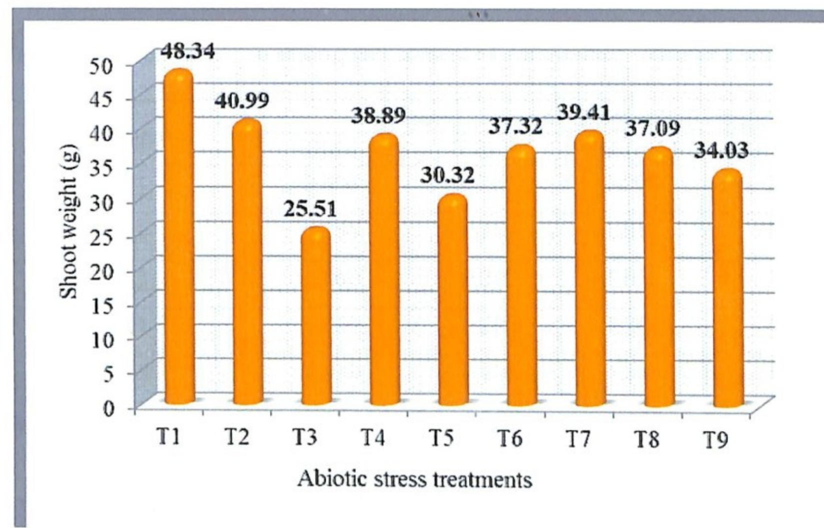


Fig.6. Effect of abiotic stress on shoot weight of *Amaranthus spinosus* Linn.

Table 6. Effect of abiotic stress factors on chlorophyll content of *Amaranthus spinosus* Linn.

Treatments	**Treatment means of Chlorophyll (mg/g)**			
	Chl a	Chl b	Chl (a+b)	Carotenoids
T1	1.323	0.400	1.727	0.718
T2	1.367	0.395	1.890	0.683
T3	1.273	0.442	1.607	0.611
T4	1.240	0.312	1.550	0.615
T5	1.280	0.303	1.587	0.602
T6	1.500	0.450	1.947	0.708
T7	1.013	0.175	1.183	0.568
T8	1.030	0.169	1.203	0.662
T9	1.043	0.107	1.151	0.650
CD (0.05)	**0.131**	**0.146**	**0.131**	**0.051**

Table 7. Effect of CO_2 on chloropyhll content of *Amaranthus spinosus* Linn.

Treatments	**Treatment means of Chlorophyll (mg/g)**			
	Chl a	Chl b	Chl (a+b)	Carotenoids
OT1	0.896	0.202	1.098	0.477
OT2	0.756	0.257	1.013	0.531
OT3	0.860	0.210	1.070	0.482
OT4	0.969	0.292	1.261	0.549
OT5	0.930	0.204	1.135	0.526
OT6	1.145	0.261	1.406	0.677
CD (0.05)	**0.151**	**0.056**	**0.147**	**0.052**

4.1.4 Dry Matter production

The total dry matter produced by plants in different treatments is shown in Table 4. Plants kept under open condition had higher dry matter production compared to shade treatments. Plants maintained in the treatments T7 (20.65 g) and T8 (20.58 g) showed highest dry matter production (Fig.8). An increase in total dry mater production is observed for the plants enriched with CO_2 as shown in Table 5 (Fig.9).

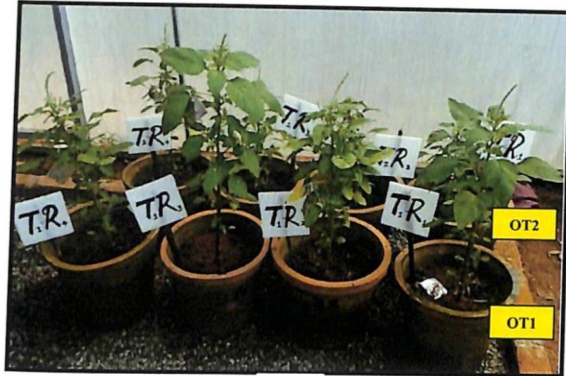
4.2 PHYSIOLOGICAL PARAMETERS

4.2.1 Chlorophyll content (mg per gram of fresh leaf tissue)

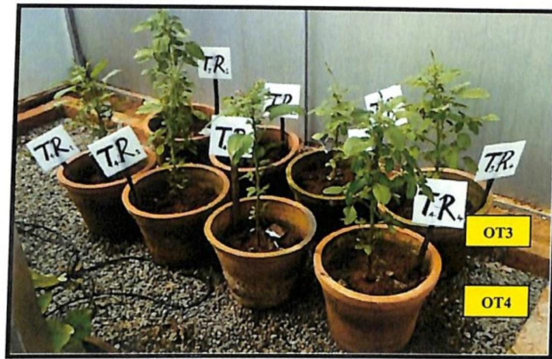
A general increase in total chlorophyll content was observed for the plants kept under artificial shades compared to the plants under open condition. Within a particular shade, the chlorophyll a content was observed to decrease with increase in water deficit stress. The plants under treatment T6 recorded the maximum chlorophyll a content (1.5 mg/g), chlorophyll b (0.450 mg/g) as well as total chlorophyll (1.947 mg/g) contents. The highest carotenoid content was observed in treatment T1 (0.718 mg/g). Lowest total chlorophyll content was observed in the treatment T9 (1.151 mg/g). Table 6 presents chlorophyll and carotenoid content of plants under various treatments imposed (Fig.10). CO_2 enrichment did not alter the chlorophyll and carotenoid content of the plants as given in Table 7 (Fig. 11).

4.2.2 Specific Leaf Area

Specific leaf area of plants observed for plants under various treatments are presented in Table 10. SLA was observed to be higher for plants kept under shade compared to the plants kept in open condition. SLA value was recorded maximum for treatment T3 (508.4 cm^2 /g). Lowest SLA value was observed in plants under the treatment T7 (265.9 cm^2 /g). Table 6 shows the SLA values for the corresponding treatments (Fig. 12). Plants which were given treatment OT2



(A)



(B)

(C)

Plate 5. CO_2 enrichment study (A) OT1, OT2; (B) OT3, OT4; (C) OT5, OT6

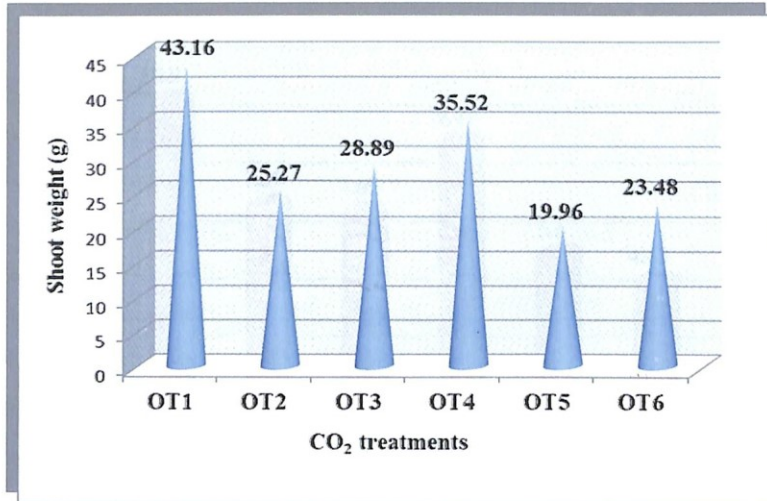


Fig.7. Effect of CO_2 on shoot weight of *Amaranthus spinosus* Linn.

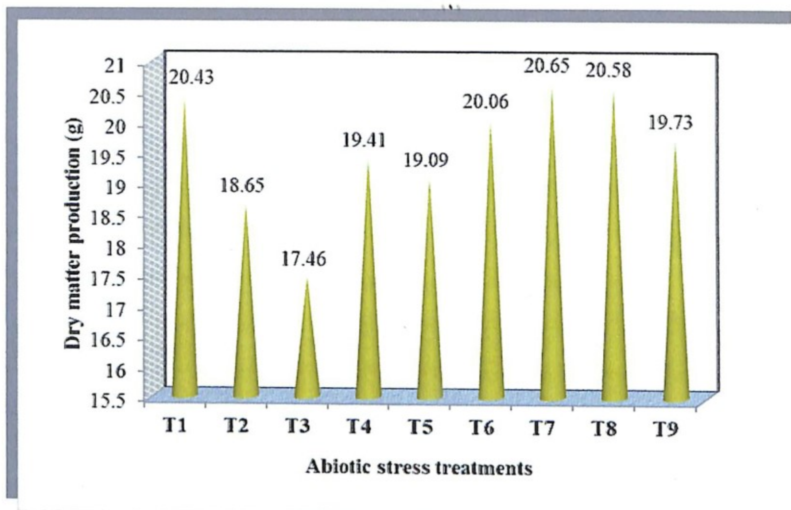


Fig. 8. Effect of abiotic stress on dry matter production of *Amaranthus spinosus* Linn.

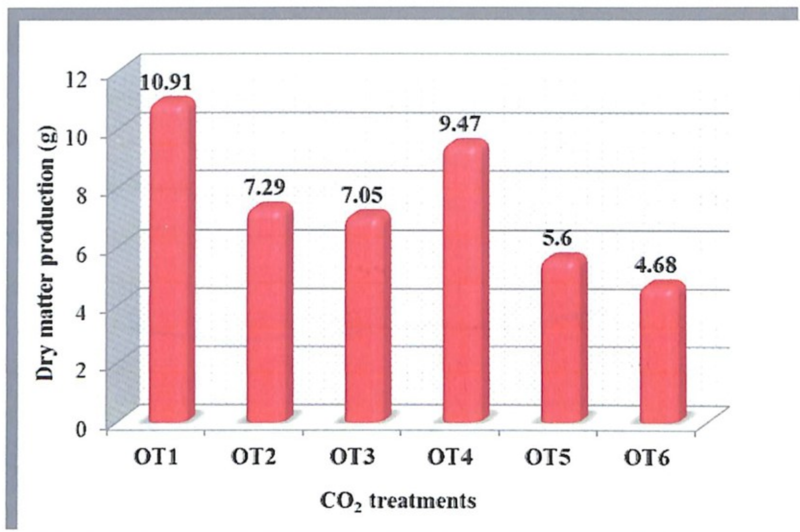


Fig. 9. Effect of CO_2 on dry matter production of *Amaranthus spinosus* Linn.

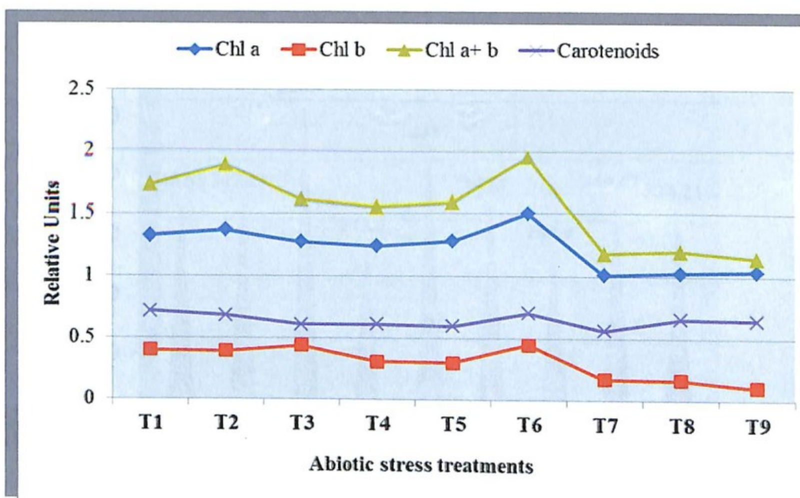


Fig. 10. Effect of abiotic stress factors on chlorophyll content of *Amaranthus spinosus* Linn.

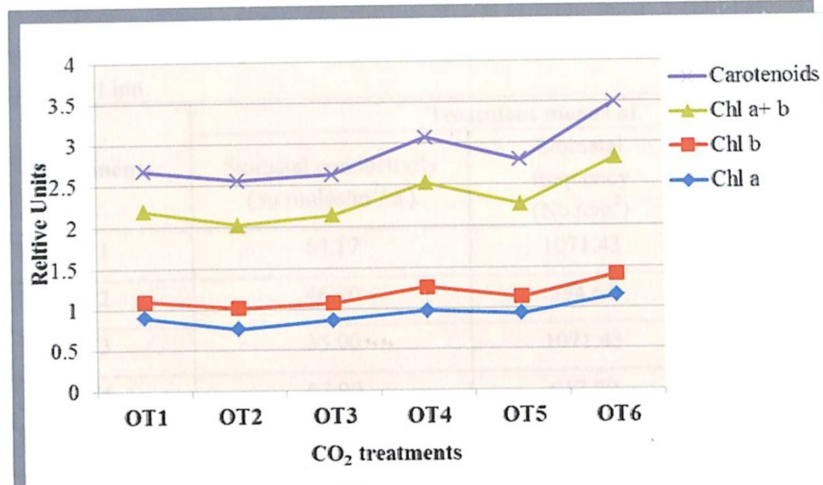


Fig.11. Effect of CO_2 on chlorophyll content of *Amaranthus spinosus* Linn.

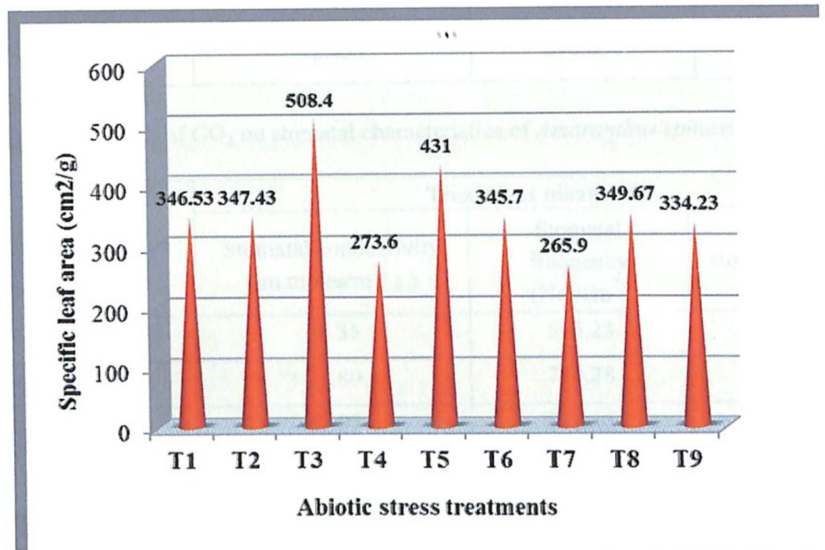


Fig. 12. Effect of abiotic stress on specific leaf area of *Amaranthus spinosus Linn.*

(394.79 cm^2) showed a higher specific leaf area as given in Table 11 (Fig.13).

Table 8. Effect of abiotic stress factors on stomatal characteristics of *Amaranthus spinosus* Linn.

Treatments	Treatment means of		
	Stomatal conductivity (m moles/m^2/ s)	Stomatal frequency (No./cm^2)	Stomatal index
T1	64.17	1071.43	22.123
T2	46.80	554.63	14.313
T3	35.00	1071.43	24.573
T4	67.90	912.70	19.743
T5	53.03	1190.47	26.927
T6	39.20	714.30	16.870
T7	44.43	873.00	22.073
T8	69.00	753.97	21.913
T9	57.50	992.07	24.827
CD (0.05)	**12.439**	**294.051**	**NS**

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Table 9. Effect of CO_2 on stomatal characteristics of *Amaranthus spinosus* Linn.

Treatments	Treatment means of		
	Stomatal conductivity (m moles/m^2/ s)	Stomatal frequency (No./cm^2)	Stomatal index
OT1	25.35	595.23	19.168
OT2	21.80	714.28	18.990
OT3	43.95	982.15	24.465
OT4	42.05	744.05	14.180
OT5	64.40	565.45	18.428
OT6	69.35	833.33	20.143
CD (0.05)	**3.134**	**205.278**	**NS**

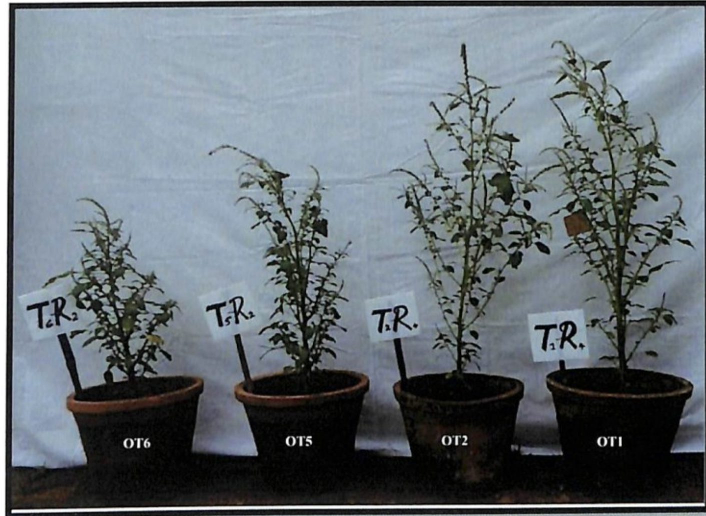
4.2.3 Stomatal Characteristics

Stomatal conductivity, stomatal frequency and stomatal index of plants under different treatments are presented in Table 8. Stomatal conductivity showed a decreasing trend within a particular shade with increase in water deficit stress. Maximum stomatal conductivity was recorded by the plants maintained under the treatment T8 (69.0 m moles/m^2/ s^{-1}). Lower stomatal conductivity was recorded by the plants under treatments 50% field capacities. Treatment T3 showed a lower stomatal conductivity of 35 m moles/m^2/ s^{-1} and treatment T6 recorded 39.2 m moles/m^2/s^{-1} of stomatal conductivity. Figure 14 shows the graphical representation of effect of stress treatments on stomatal conductivity. Total number of stomata was highest for the plants under treatment T5 (1190.5 per cm^2). No significant variation was observed in stomatal frequency among the different treatments given, although the treatments were significantly different. Stomatal index was observed to be non-significant among the various treatments given to the plants.

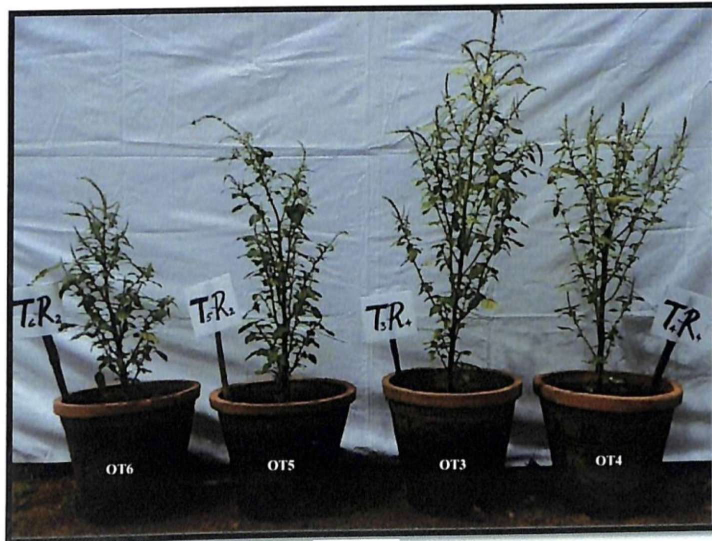
A significant reduction in stomatal conductivity was observed for the plants under treatments OT1 (25.35 m moles m^{-2} s-^{1}) and OT2 (21.8 m moles m^{-2}s-^{1}) when compared to plants that are kept in open control (Fig.15). No significant effect on stomatal frequency and stomatal index was observed for the plants under CO_2 enrichment. The Table 9 shows the effect of CO_2 enrichment on stomatal characters.

4.2.4 Relative Water Content

The relative water content of plants varied under different treatment combinations. A higher RWC was observed in plants under the treatment T5 (80.3 %). Treatments T3 (78.67%), T2 (78.47%) and T6 (78.03%) showed not much variation in RWC (Fig.16). RWC of plants under different treatments are given in Table 10. CO_2 influenced variation in RWC was not observed for the



(A)



(B)

Plate 6. Plants after CO_2 enrichment (A) Open Control *vs* CO_2 enriched plants; (B) Open Control *vs* Chamber control

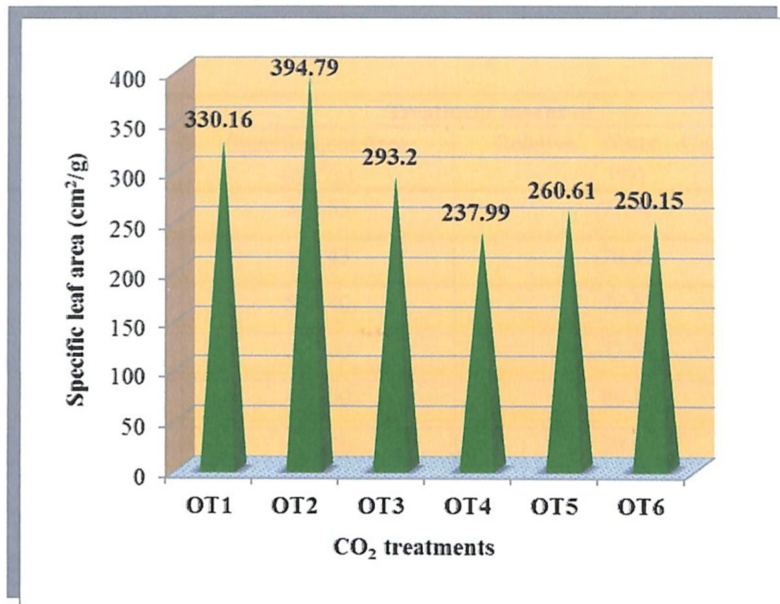


Fig. 13. Effect of CO_2 on specific leaf area of *Amaranthus spinosus* Linn.

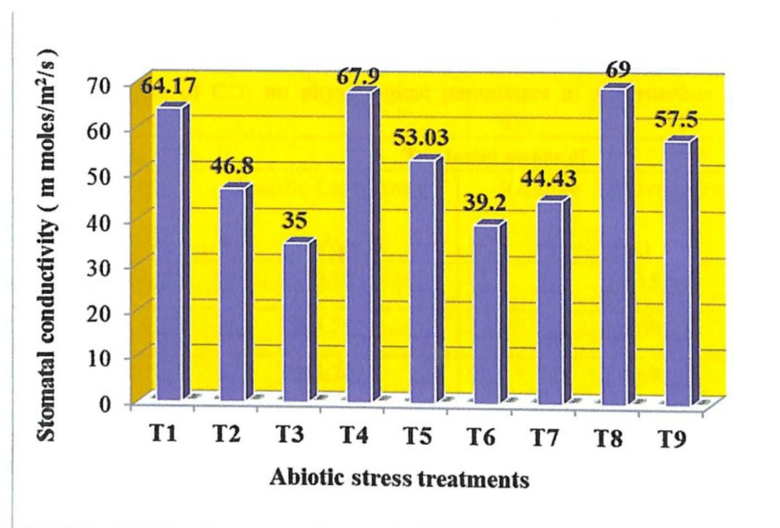


Fig.14. Effect of abiotic stress factors on stomatal conductivity of *Amaranthus spinosus* Linn.

Table 10. Effect of abiotic stress factors on physiological parameters of *Amaranthus spinosus* Linn.

Treatments	**Treatment means of**	
	Specific Leaf Area (cm^2/g)	Relative Water Content (%)
T1	346.53	77.40
T2	347.43	78.47
T3	508.40	78.67
T4	273.60	73.57
T5	431.00	80.30
T6	345.70	78.03
T7	265.90	69.17
T8	349.67	73.57
T9	334.23	72.03
CD (0.05)	**60.657**	**6.011**

Table 11. Effect of CO_2 on physiological parameters of *Amaranthus spinosus* Linn.

Treatments	**Treatment means of**	
	Specific Leaf Area (cm^2/g)	Relative Water Content (%)
OT1	330.16	72.35
OT2	394.79	80.08
OT3	293.20	61.68
OT4	237.99	72.48
OT5	260.61	89.98
OT6	250.15	68.40
CD (0.05)	**61.877**	**8.269**

plants kept in OTC as shown in Table 11 (Fig. 17).

4.3 BIOCHEMICAL PARAMETERS

4.3.1 Total Soluble Proteins

Total soluble proteins of the plants under different treatments were found to be significantly different. The total soluble protein content of plants maintained in different shade and water deficit tress are presented in Table 12. Maximum total soluble protein was observed in plants under the treatment T7 (4.63 mg/g). Within a shade level the total soluble protein content reduced considerably with increase in water deficit stress (Fig. 18). Under 50% shade treatment T1 recorded a total soluble protein content of (3.55 mg/g), which showed further reduction in treatments T2 (3.3 mg/g) and T3 (2.65 mg/g) with increase in water deficit stress in open condition treatment T9 was found to show the lowest total soluble protein content (3.79 mg/g). A significant effect of CO_2 on total soluble proteins was not observed as depicted in Table 13. However higher total soluble proteins was recorded for the plants under treatment OT4 (5.7 mg/g).

4.3.2 Total Starch

Total starch content was observed highest for plant which was given the treatment T4 (8.96 mg/g). Not much variation in starch content was found among treatments T1 (6.04 mg/g) T6 (6.09 mg/g) and T5 (6.47 mg/g). Total starch content was observed to be lower for plants in treatment T8 (3.99 mg/g). Reduction in starch content was observed for plants kept under shade with increased water deficit stress. Total starch content for T3 (4.04 mg/g) and T6 were reduced when compared to T1 and T4 respectively. Table 12 shows total starch content of plants under different treatment combinations. Total starch content was observed to be higher for treatments OT3 (9.44 mg/g) and OT1 (9.13 mg/g) as given in Table 13.

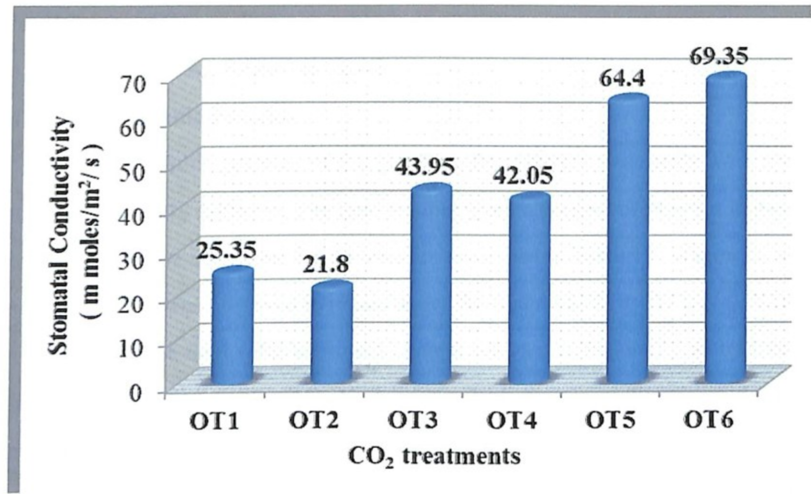


Fig.15. Effect of CO_2 on stomatal conductivity of *Amaranthus spinosus* Linn.

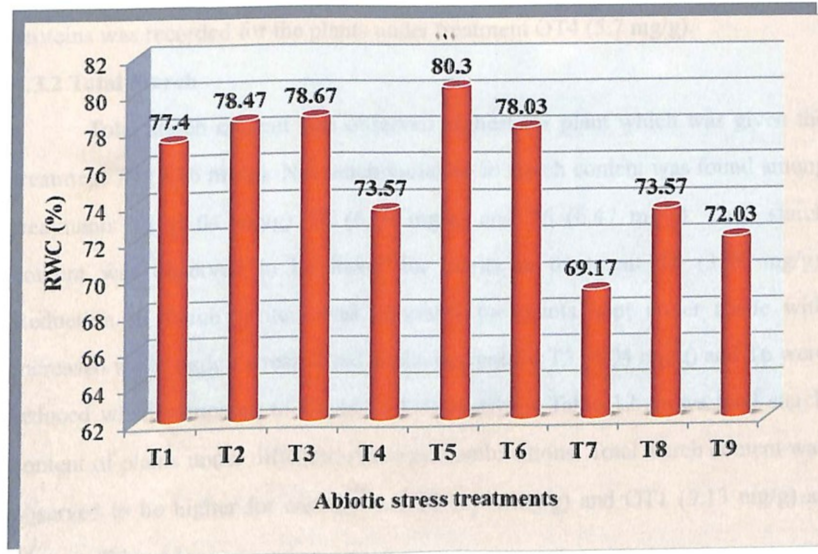


Fig.16. Effect of abiotic stress factors on Relative water content of *Amaranthus spinosus* Linn.

Table 12. Effect of abiotic stress factors on biochemical parameters of *Amaranthus spinosus* Linn.

Treatments	Treatment means of				
	Total soluble proteins (mg/g)	Total starch (mg/g)	Reducing sugars (mg/g)	Free amino acids (µg/g)	Proline (µg/g)
T1	3.55	6.04	10.02	16.82	0.044
T2	3.30	7.89	9.62	14.86	0.058
T3	2.65	4.04	9.82	13.59	0.026
T4	3.04	8.97	8.46	14.61	0.026
T5	3.87	6.48	8.09	17.43	0.048
T6	3.82	6.09	8.63	13.83	0.035
T7	4.63	4.34	7.26	18.09	0.036
T8	3.87	3.99	9.39	18.66	0.024
T9	3.79	8.49	8.30	12.01	0.052
CD (0.05)	**0.351**	**1.577**	**0.732**	**1.682**	**0.022**

Table 13. Effect of CO_2 enrichment on biochemical parameters of *Amaranthus spinosus* Linn.

Treatments	Treatment means of				
	Total soluble proteins (mg/g)	Total starch (mg/g)	Reducing sugars (mg/g)	Free amino acids (µg/g)	Proline (µg/g)
OT1	3.49	9.13	7.80	20.13	0.105
OT2	3.92	5.56	6.25	20.64	0.042
OT3	4.65	9.4	6.07	12.57	0.051
OT4	5.71	3.58	6.39	12.31	0.063
OT5	3.12	4.08	7.24	13.84	0.050
OT6	3.66	7.92	9.49	16.67	0.050
CD (0.05)	**0.286**	**0.324**	**0.440**	**1.679**	**0.009**

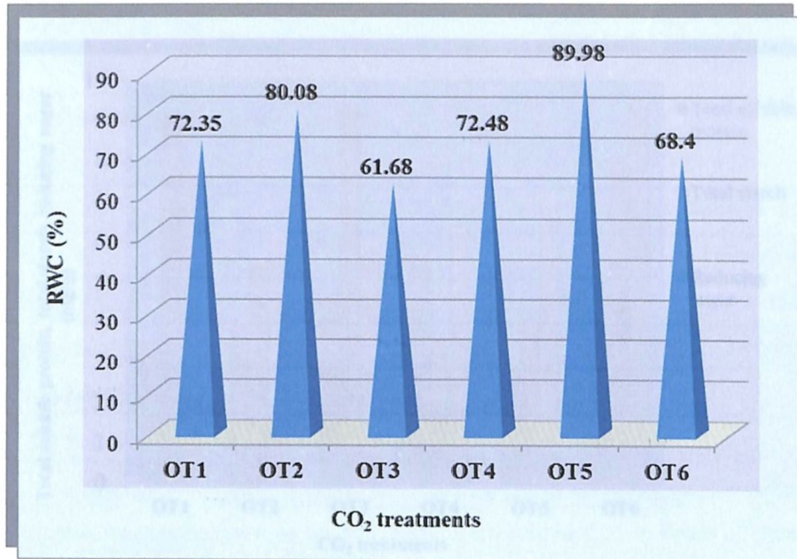


Fig.17. Effect of CO_2 on Realtive water content of *Amaranthus spinosus* Linn.

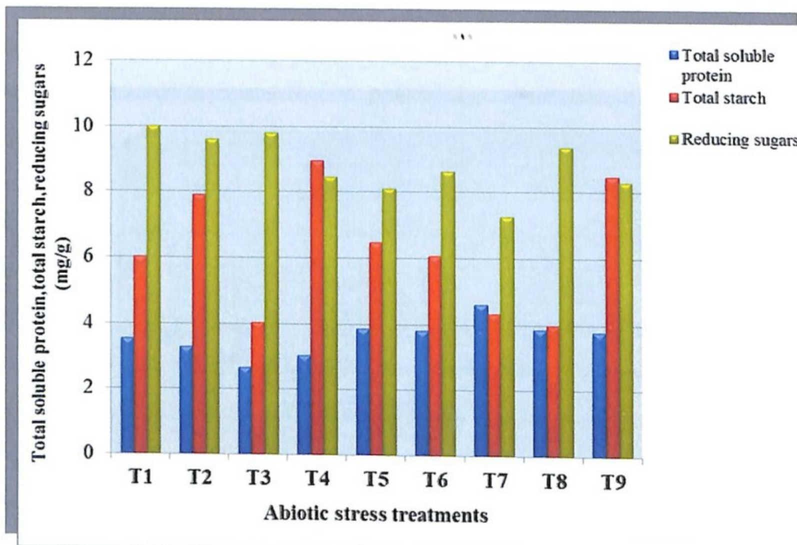


Fig. 18. Effect of abiotic stress on biochemical parameters of *Amaranthus spinosus* Linn.

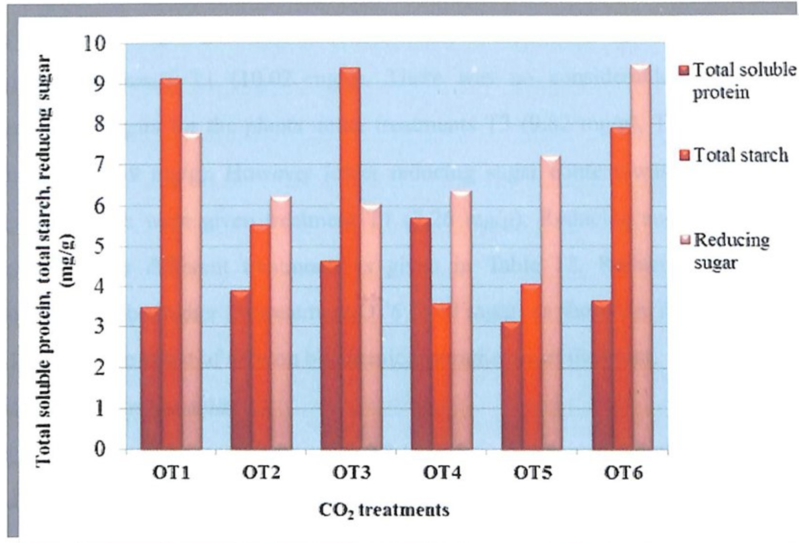


Fig. 19. Effect of CO_2 enrichment on biochemical parameters of *Amaranthus spinosus* Linn.

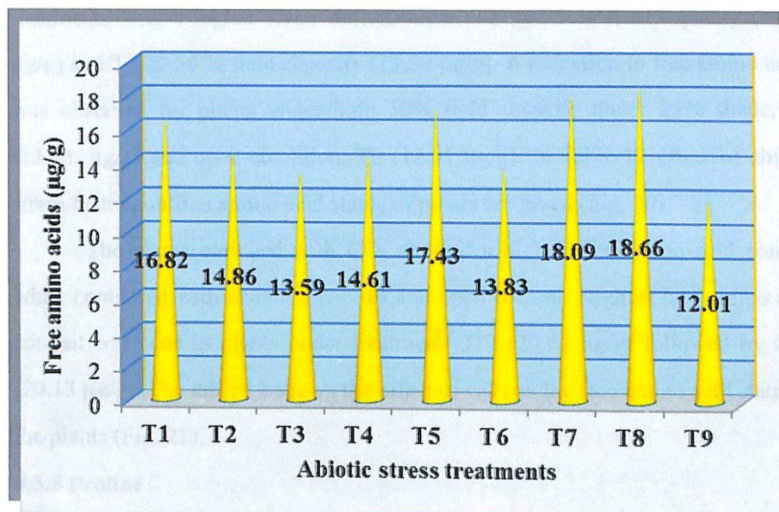


Fig. 20. Effect of abiotic stress factors on free amino acids of *Amaranthus spinosus* Linn.

4.3.3 Reducing Sugar

Reducing sugar content was maximum record for the plants which were given treatments T1 (10.02 mg/g). There was no considerable variation in reducing sugars for the plants under treatments T3 (9.82 mg/g), T2 (9.62 mg/g) and T8 (9.39 mg/g). However lower reducing sugar content was observed for plants which were given treatment T7 (7.26 mg/g). Reducing sugar content of plants under different treatments is given in Table 12. Reducing sugar was observed to be higher for treatment OT6 (9.49 mg/g) as shown in Table 13.Figure 19 shows the effect of CO_2 on biochemical parameters of the plant.

4.3.4 Free amino acids

Among the treatments given T8 (18.66 µg/g) showed a higher free amino acid status in plants, followed by T7 (18.97 µg/g). Within the two levels of shade given a reducing trend of free amino acids was observed with increase in water deficit stress. Under 50% shade, highest free amino acids was recorded for plants at 100% field capacity (T1 @ 16.82 µg/g) which was seen to reduce for the treatments with a higher water deficit stress, T2 @ 75 % field capacity (14.86 µg/g) and T3 @ 50 % field capacity (13.59 µg/g). A reduction in free amino acids was observed for plants under both 50% field capacity under 25% shade, T6 (13.83 µg/g) and open condition, T9 (12.01 µg/g). In Table 12 effect of abiotic stress factors on free amino acid status of plants are given (Fig. 20).

The plants enriched with CO_2 showed a higher free amino acid content when compared with chamber control and open control. Highest free amino acid content was seen in plants under treatment OT2 (20.64 µg/g) followed by OT1 (20.13 µg/g). The table 13 shows the effect of CO_2 on the free amino acid status of the plants (Fig. 21).

4.3.5 Proline

A considerable variation in proline status of the plants under different

treatments was not visible. However the treatments were significantly different for proline content. Highest proline content was reported for the plants which were imposed with treatment T2 (0.058 μg/g). Proline concentrations recorded by plants under different treatments are shown in Table 12. A higher proline content was observed for the plants under treatment OT1 (0.105 μg/g) when compared with open control as depicted in Table 13.

4.4 ANTIOXIDANTS

4.4.1 Enzymatic antioxidants

4.4.1.1 Superoxide dismutase (SOD)

The effect of abiotic stress factors on the activity of the enzyme SOD is presented in Table 14 (Fig. 22). The enzyme activity was recorded maximum for the plants maintained in the treatment T3 (0.032 EU/mg of protein). SOD activity was comparatively lower in plants maintained in 100% field capacities in shaded conditions, T1 (0.021 EU/mg of protein), T4 (0.029 EU/mg of protein) as well as in open field, T9 (0.019EU/mg of protein). CO_2 mediated increase in SOD activity was not observed among the plants kept in OTC, which is shown in Table 15.

4.4.1.2 Catalase (CAT)

The activity of CAT enzyme was observed maximum in the plants under the treatment T3 (0.086 EU/mg of protein). Increased activity of CAT was also observed in plants under 50% shade, T2 (0.070 EU/mg of protein) and T1 (0.041 EU/mg of protein) compared to other shade levels (Fig.22). Plants maintained at open conditioned showed lower CAT activity. The lowest CAT activity was shown by plants under treatment T7 (0.0270 EU/mg of protein). Table 14 shows CAT activity levels of plants given various treatments. A higher activity of CAT was seen for the plants enriched with CO_2 which were maintained a 50% field capacity. The effect of CO_2 on CAT activity is expressed in Table 15 (Fig 23).

Table 14. Effect of abiotic stress factors on enzymatic antioxidants in *Amaranthus spinosus* Linn.

Treatments	Treatment means (Enzyme Units/mg of protein)				
	SOD	CAT	GPx	POX	PPO
T1	0.021	0.041	0.715	0.003	0.003
T2	0.025	0.070	0.686	0.004	0.004
T3	0.032	0.086	0.731	0.008	0.006
T4	0.029	0.036	0.570	0.008	0.001
T5	0.021	0.029	0.686	0.006	0.001
T6	0.022	0.046	0.532	0.007	0.001
T7	0.019	0.027	0.518	0.006	0.001
T8	0.023	0.028	0.499	0.006	0.002
T9	0.023	0.035	0.544	0.005	0.001
CD (0.05)	**0.002**	**0.005**	**0.062**	**0.001**	**0.003**

Table 15. Effect of CO_2 enrichment on enzymatic antioxidants in *Amaranthus spinosus* Linn.

Treatments	Treatment means (Enzyme Units/mg of protein)				
	SOD	CAT	GPx	POX	PPO
OT1	0.007	0.085	0.699	0.007	0.001
OT2	0.008	0.169	0.671	0.006	0.002
OT3	0.011	0.082	0.663	0.005	0.001
OT4	0.011	0.115	0.711	0.005	0.001
OT5	0.022	0.040	0.616	0.008	0.002
OT6	0.017	0.052	0.600	0.007	0.002
CD (0.05)	**0.001**	**0.018**	**0.015**	**0.001**	**0.001**

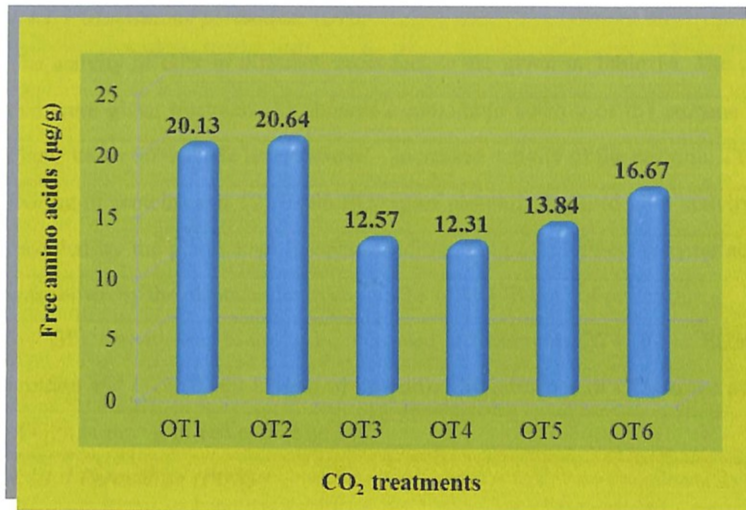


Fig. 21. Effect of CO_2 enrichment on free amino acids of *Amaranthus spinosus* Linn.

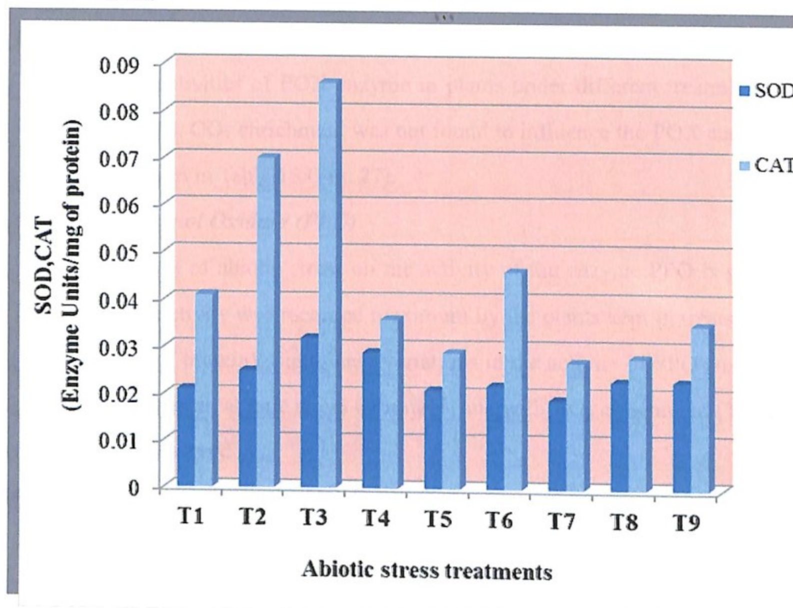


Fig. 22. Effect of abiotic stress factors on enzymes SOD and CAT in *Amaranthus spinosus* Linn.

4.4.1.3 Glutathione peroxidase (GPx)

The activity of GPx to different stress factors are given in Table 14. The plants that were given treatment T3 showed a maximum activity of the enzyme GPx. Plants under 50% shade level showed increased activity of the enzyme, T1 (715 EU/mg of protein) and T2 (0.686 EU/mg of protein). Reduced GPx activity was recorded by the plants kept in open condition (Fig.24). Lowest enzyme activity was shown by the plants under treatment T8 (0.499 EU/mg of protein).

GPx activity was found to be increased in treatments OT4 (0.711 EU/mg of protein) and OT1 (0.699 EU/mg of protein). The influence of CO_2 in the activity of GPx is shown in Table 15 (Fig. 25).

4.4.1.4 Peroxidase (POX)

The activity of POX enzyme did not have a significant variation with the treatments given, however the treatments were significantly different. The plants under treatment T3 (0.008 EU/mg of protein) showed the highest POX activity (Fig. 26). The activities of POX enzyme in plants under different treatments are given in Table 14. CO_2 enrichment was not found to influence the POX activity of the plants as given in Table 15 (Fig. 27).

4.4.1.5 Poly Phenol Oxidase (PPO)

The effect of abiotic stress on the activity of the enzyme PPO is given in Table 14. PPO activity was recorded maximum by the plants kept in treatment T3 (0.006 EU/mg of protein). Significant variations in the activity of PPO among the plants under different abiotic stress treatments and with CO_2 enrichment (Table 15) were also not observed.

4.4.2 Non – enzymatic antioxidants

4.4.2.1 Vitamin A

Plants maintained in treatment T3 (42.01 μg β carotene/g) showed a higher vitamin A content. Within a shade level an increasing trend of vitamin A content

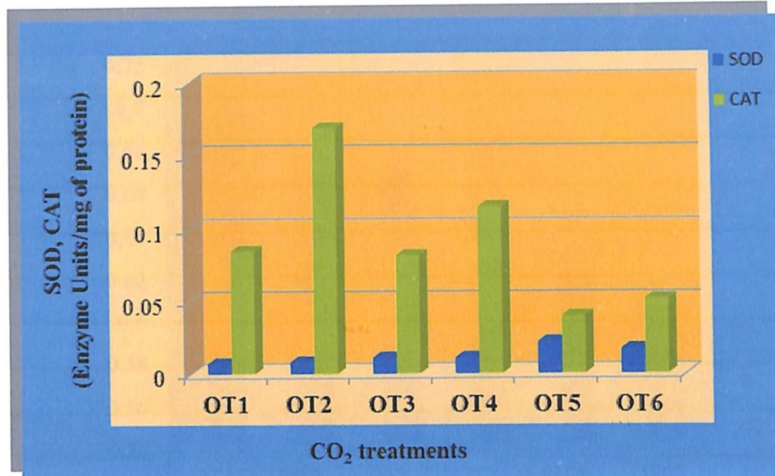


Fig. 23. Effect of CO_2 enrichment on enzymatic antioxidants SOD and CAT in *Amaranthus spinosus* Linn.

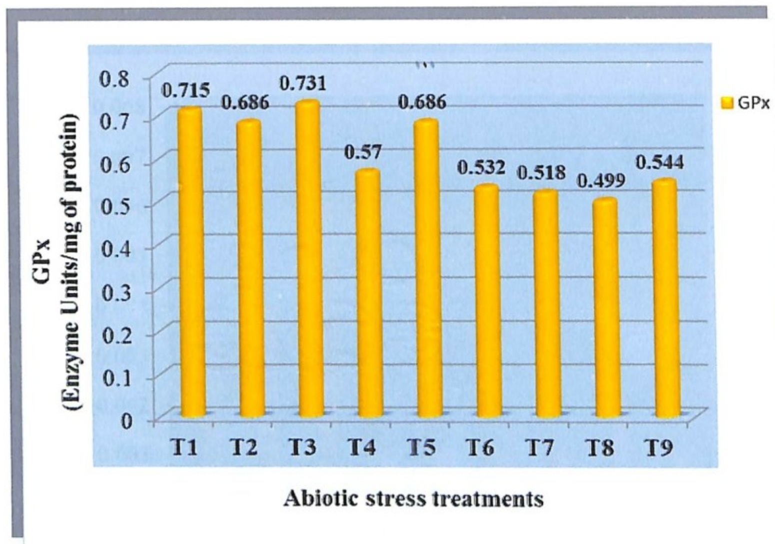


Fig.24. Effect of abiotic stress factors on the enzyme GPx in *Amaranthus spinosus* Linn.

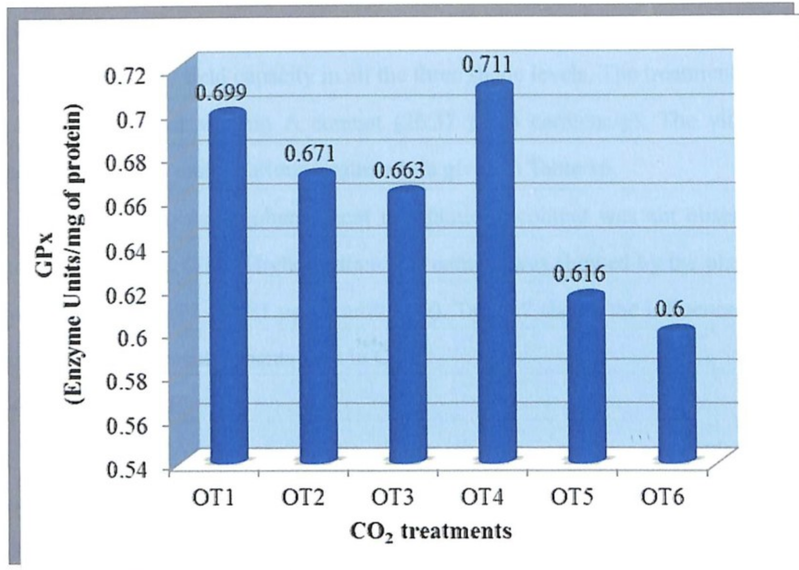


Fig. 25. Effect of CO_2 enrichment on GPx in *Amaranthus spinosus* Linn.

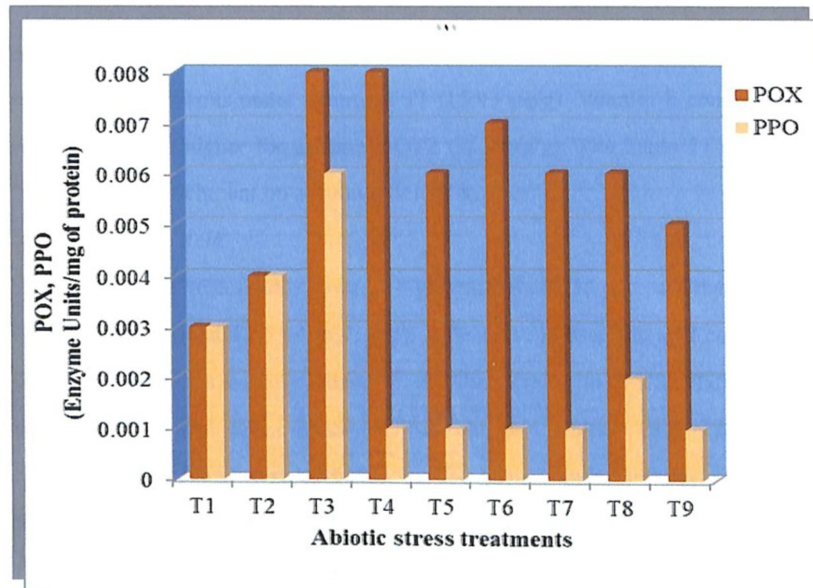


Fig. 26. Effect of abiotic stress factors on the enzymes POX and PPO in *Amaranthus spinosus* Linn.

was observable with increase in WDS. Lower vitamin A content was observed for plants under 50% field capacity in all the three shade levels. The treatment T4 has shown the lowest vitamin A content (26.37 µg β carotene/g). The vitamin A content of plants under various treatments is given in Table 16.

CO_2 influenced enhancement in vitamin A content was not observed for the plants kept in OTC. Highest vitamin A content was showed by the plants kept with treatment OT3 (36.81 µg β carotene/g). Table17 shows the influence of CO_2 on vitamin A content of pants kept in OTC.

4.4.2.2 Vitamin E

The influence of abiotic stress factors on the accumulation of α-tocopherol / Vitamin E is presented in Table 16. The level of Vitamin E was higher for plants kept under open condition. Maximum Vitamin E concentration was recorded in the plants maintained under treatment T9 (20.7 µg/g), followed by T7 (20.4 µg/g). Plants kept under 50% shade were found to accumulate lower concentrations of Vitamin E (Fig. 28). Lowest vitamin E concentration was recorded by the plants under treatment T1 (15.93 µg/g). Vitamin E concentration was found to be higher for treatment OT2 (20.7 µg/g). The Table 17 shows the effect of CO_2 enrichment on accumulation of Vitamin E.

4.4.2.3 Ascorbic Acid

Plants under 50% shade showed a higher ascorbic acid content, treatments T1 (3.643 mg/100g) and T2 3.64 mg/100g). A decrease in ascorbic acid content was found among plants within a shade level with increase in WDS. Plants kept in open condition had lower levels of ascorbic acid content (Fig. 29). Lowest ascorbic acid content was recorded by the plants under treatment T9 (2.28 mg/100g). Table 16 shows the ascorbic acid content of plants under various treatments. Ascorbic acid content was found to have not much variation with CO_2 enrichment when compared with open control. The ascorbic acid content for

Table 16.Effect of abiotic stress factors on non-enzymatic antioxidants in *Amaranthus spinosus* Linn.

Treatments	Treatment means				
	Vitamin A (µg/g)	Vitamin E (µg/g)	Ascorbic Acid (mg/100g)	Phenols (mg CE/ml)	Flavanoids (mg CE/ml)
T1	31.93	15.93	3.64	3.20	0.814
T2	26.71	16.42	3.64	2.20	0.841
T3	42.01	17.48	3.15	1.91	0.787
T4	26.37	18.44	3.26	2.52	0.796
T5	29.16	19.18	2.63	2.13	0.804
T6	32.85	19.67	2.10	1.95	0.764
T7	30.36	20.40	2.47	3.42	0.783
T8	32.52	20.25	2.37	3.31	0.769
T9	33.94	20.78	2.28	3.06	0.748
CD (0.05)	**0.625**	**0.674**	**1.018**	**0.352**	**0.021**

Table 17. Effect of CO_2 enrichment on non-enzymatic antioxidants in *Amaranthus spinosus* Linn.

Treatments	Treatment means				
	Vitamin A (µg/g)	Vitamin E (µg/g)	Ascorbic Acid (mg/100g)	Phenols (mg CE/ml)	Flavanoids (mg CE/ml)
OT1	26.40	19.55	1.06	3.28	0.719
OT2	27.07	20.87	1.50	1.32	0.725
OT3	36.81	16.51	1.38	1.17	0.788
OT4	27.95	17.88	1.44	1.12	0.779
OT5	33.34	17.70	1.56	4.55	0.717
OT6	27.19	19.89	1.75	2.78	0.752
CD (0.05)	**1.303**	**0.392**	**0.404**	**0.156**	**0.005**

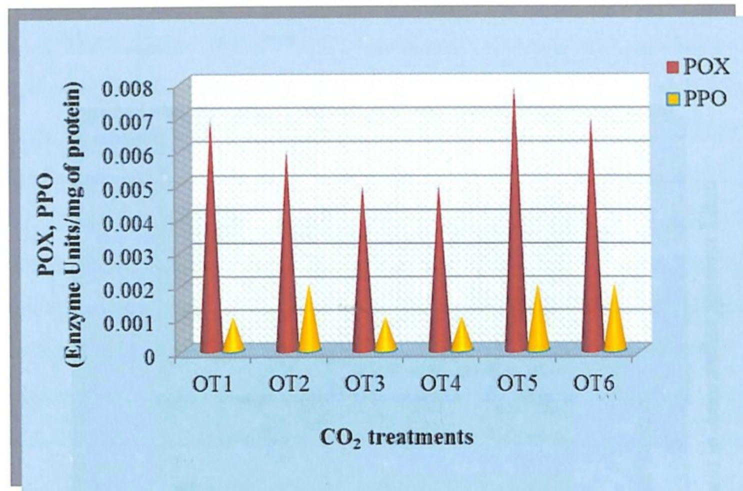


Fig. 27. Effect of CO_2 enrichment on POX and PPO in *Amaranthus spinosus* Linn.

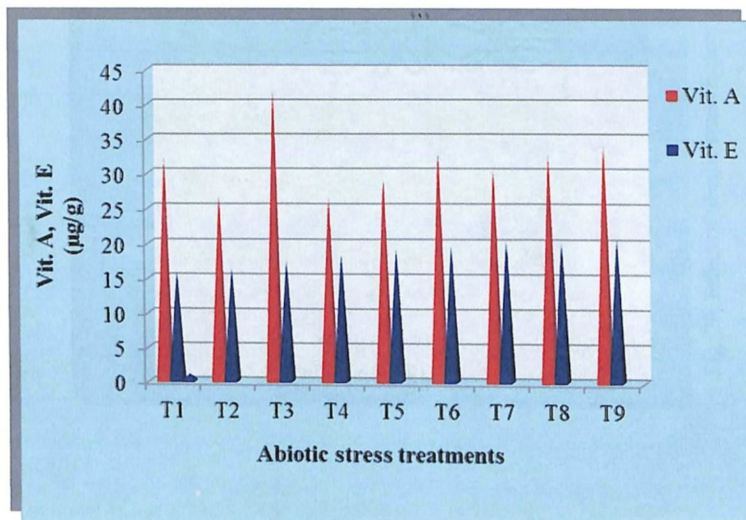


Fig. 28. Effect of abiotic stress on Vitamin A and Vitmin E in *Amaranthus spinosus* Linn.

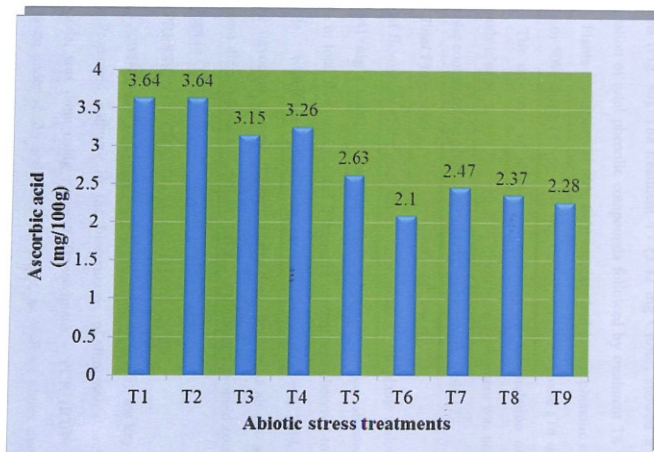


Fig. 29. Effect of abiotic stress factors on Ascorbic acid in *Amaranthus spinosus* Linn.

plants under CO_2 enrichment is shown in Table 17.

4.4.2.4 Total Phenolic compounds

Total phenolic compounds were found to be higher for plants kept under open conditions (Fig. 30). The treatment T7 (3.42 mg CE/ml) showed a maximum concentration of total phenolic compounds followed by treatment T8 (3.307 mg CE/ml). Plants maintained at 50% field capacity had lowest phenolic compounds under both 50% shade (T3 @ 1.91 mg CE/ml) and 25% shade (T6 @ 1.95 mg CE/ml). The influence of abiotic stress factors on accumulation of phenolic compounds is expressed in Table16. CO_2 mediated enhancement was not observed in phenolic content of the plants kept in OTC as shown in Table 17.

4.4.2.5 Total Flavanoids

Total flavonoids of the plant showed maximum for the plants under treatment T2 (0.841 mg CE/ml). Plants kept under shaded condition showed a slight increase in total flavonoid content. Flavonoid content was observed to be lowest for plants which were given treatment T9 (0.748 mg CE/ml). Table 16 shows the total flavonoid content of plants under the influence of abiotic stress. CO_2 enrichment did not have a significant effect on the total flavonoid content of the plants kept in OTC as depicted in Table 17 (Fig.31).

4.5 EXPRESSION OF CHALCONE SYNTHASE GENE

Expression study of the gene chalcone synthase (CHS), the key enzyme in Phenyl Propanoid pathway in the biosynthesis of flavonoids and phenolic compounds, was done using Reverse Transcriptase – PCR (RT-PCR). Plants which were under shade and WDS treatment, as well as plants maintained at 100% field capacity and CO_2 enrichment were considered for expression study. The gene showed overexpression under shade stress. The PCR product showed an amplicon of approximately 560bp in 2% agarose gel (Plate.7). Plants grown under mild shade stress along with WDS showed a prominent luminescence. Slight

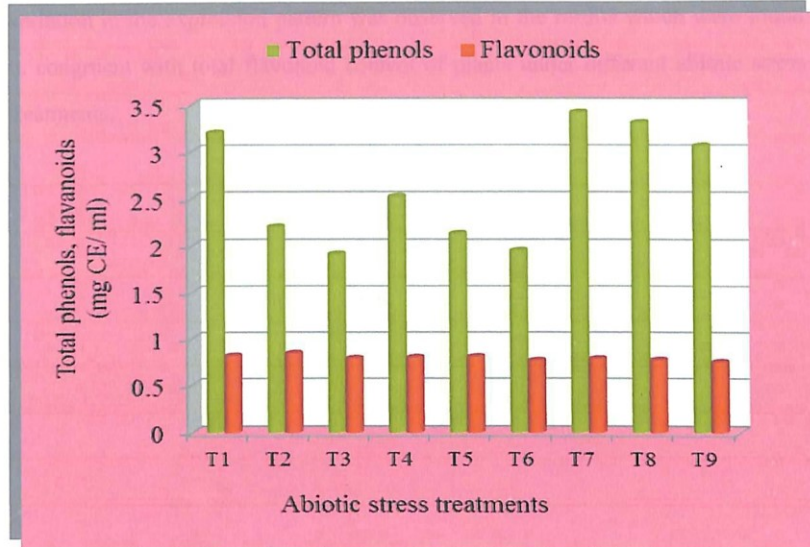


Fig. 30. Effect of abiotic stress on phenols and flvanoids in *Amaranthus spinosus* Linn.

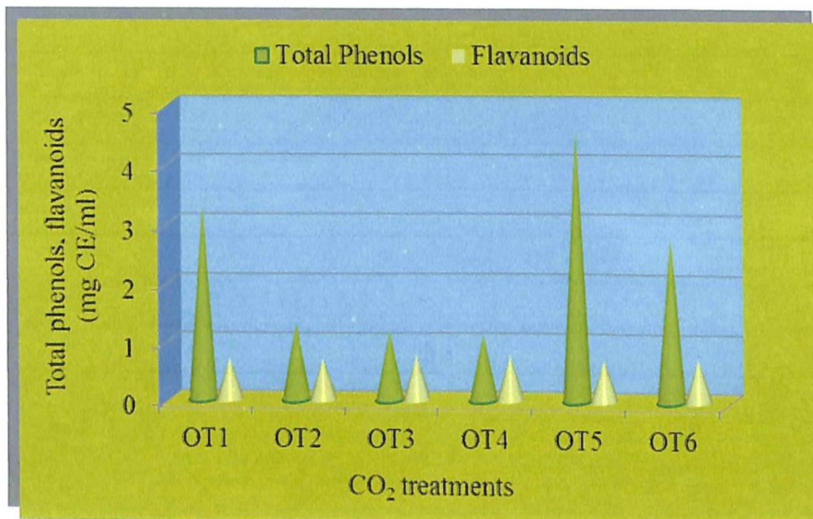


Fig. 31.Effect of CO_2 enrichment on phenols and flavanoids in *Amaranthus spinosus* Linn.

variation in the expression pattern was observed in the results which were found in congruent with total flavonoid content of plants under different abiotic stress treatments.

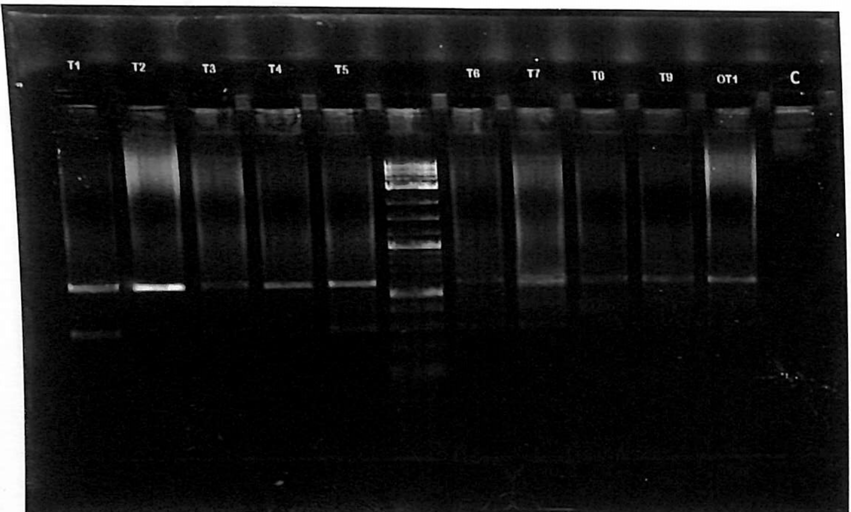


Plate 7. Expression pattern of CHS gene in *Amaranthus spinosus* Linn. under various treatments.

DISCUSSION

5. DISCUSSION

Plant response studies to abiotic factors provide information on the physiological basis of mechanism by which they adapt to the ever changing environment. Plants have evolved to secondary biochemical pathways in response to specific environmental stimuli which do not play any role in the plant's primary metabolic requirements, but necessary to survive and overcome local challenges by allowing them to interact with their environment. Coupled action of secondary metabolites as well as inherently present enzymatic antioxidants in plants help them to circum the varying degrees of oxidative stresses produced as a result of abiotic and biotic stress factors.

Plants are the richest source of natural antioxidants. The antioxidant properties of plants make them therapeutically valuable. Enhanced production of these secondary metabolites and anti-oxidative capacity of plants under abiotic stress improves the medicinal property of plants and this can be exploited in the pharmaceutical manufacturing of drugs.

To utilize plants for the benefit of humankind it is necessary to study the mechanism behind the influence of environmental factors in modulating the medicinally active compounds in plants. The extent to which the production of phytochemical compounds are controlled by genetic as well as environmental cues might be helpful in establishing a strategy to enhance the antioxidant content of plants which in turn pave way for formulating a cure for deleterious and degenerative diseases. Thus the present study was done to analyse the influence of stress factors like water deficit stress and shade and also CO_2 enrichment on growth and the accumulation of antioxidants in *Amaranthus spinosus* Linn.

5.1 EFFECT OF ABIOTIC STRESS FACTORS ON GROWTH PARAMETERS

Abiotic stress *viz*. water deficit stress and shade stress were imposed on

Amaranthus spinosus Linn. for a period of 30 days. The treatments were given at three levels; WDS at 100%, 75% and 50% field capacity and shade stress at 50%, 25% and in open condition as a combination. Observations on the growth parameters like plant height, leaf area, shoot weight and dry matter production were made. The influence of water deficit stress and shade stress on these parameters is discussed in following session.

Plant height was differentially affected by shade stress. However an increasing trend in plant height was observed under shaded condition as compared to open condition. The finding was in agreement with the results in *Amaranthus viridis* reported by Farrukh *et al*. (2003). In the present study the plant height decreased with decreasing field capacities. These results were in congruent with the findings by Chauhan and Abugho (2013). Similarly plant height decreased in *Amaranthus retroflexus* at early vegetative phase, under WDS (Quasem and Biftu, 2010).

According to Jensen *et al.* (1998) auxin transport plays an important role in stem elongation and the rate of transport is dependent on light intensity. Shading resulted in a change of gibberellin concentration in plants, which in turn resulted in increase plant height under shade condition. Increase in plant height is the result of phytochrome mediated shade avoidance mechanism of the plants as reported by Lambers *et al*. (1998). The primary plant process affected by WDS is the cell elongation which is the basic cause for reduced plant height under WDS (Taiz and Zeiger, 2010).

Leaf area was found to be increased under shaded condition when compared with open condition. The result was in accordance with earlier reports in cucumber plants (Yang *et al*., 2000) and in *Ageratum conyzoides* (Sun *et al*., 2012). The leaves under open condition are thicker due to the formation taller palisade cells and increased number of layers of palisade cells and hence

have lesser area since light is not limiting. The increased leaf area under shade was due to the plant adaptation to expose larger photosynthetic surface under limited illumination (Parthasarathy, 2004). The shoot weights of plants were found to have an increasing trend with increase in shade intensity. A similar result was reported in *Amaranthus spinosus* Linn. by Chauhan and Abhugo (2013). Since the plants under shade had higher plant height and stem elongation a complimentary increase in vegetative growth has favoured resulting in increased shoot weight of plants.

Dry matter production of the plants was enhanced under open condition in general. The results were found to be similar as in *Amaranthus cruentus* (Ejieji and Adeniran, 2010), under water available situations. The result was in accordance with that of Paez *et al*. (2000) in *Aloe vera*. This suggests that the dry matter production is favoured in the natural habitat of the plant than under shaded condition. The medicinal value of the roots can also be utilized under elevated dry matter production in natural conditions. However a decrease in dry mater production was noticed with increase in WDS. A similar trend was also noticed in sugarcane (Ramesh, 2000). Dry matter production was found to be lowest in 50% field capacity under all the levels of shade.

The increased oxidative stress might have reduced the assimilatory activities in plants resulting in reduced dry matter production, under water deficit stress. When plants were exposed to WDS the first line of defense is the stomatal closure because of chemical signal ABA produced by roots in contact with the dry soil is transported to the leaves, thus reducing the transpiratory water loss (Taiz and Zeiger, 2010). This causes a proportionate decrease in the assimilation rate and thus the response of water to plants will be reduction in leaf area and total biomass accumulated.

5.2 EFFECT OF ABIOTIC STRESS FACTORS ON PHYSIOLOGICAL PARAMETERS

The effect of abiotic stress factors on various physiological parameters like chlorophyll content, specific leaf area, stomatal characteristics and relative water content were analyzed. The results of these parameters are discussed below. A general increase in chlorophyll content of plants was noticed in those kept under shade than plants in open condition. The results were in congruent with the findings of Pratima (1999) and Parthsarathy (2004). According to Lambers *et al.,* (1998) chloroplasts produce larger grana under shade, which contain major part of the chlorophyll. This might contribute for higher chlorophyll content under shaded condition, which is an adaptation of these plants to harvest more radiation. However, due to reduced Calvin cycle enzymes there is a chance of reduced photosynthetic accumulation which might be the reason for the observed reduction in the dry matter production in shade.

Among the given treatments with increase in water deficit stress the chlorophyll content is found to be reduced. Plants kept under 50% field capacity were observed to contain lower chlorophyll content in 50% and 25% shade as well as in open condition. Lini (2006) has also reported a similar result. According to Walter (1995), the reduction of the chlorophyll might have contributed by the disruption of thylakoids and the chloroplast swelling and breaking down under drought condition. The generation of ROS induced by WDS also have resulted in damage of the membrane and caused the photoxidation. This can be the reason for reduced chlorophyll contents under water stress (Lambers *et al.,* 1998).

Specific leaf area (SLA) followed an increasing trend in plants under shade compared to the plants in open condition. Highest SLA was observed for the plants under 50% shade and lowest SLA was observed for the plants in open

condition. According to Parthasarathy (2004), the lower value of the specific leaf area in open condition was due to lesser leaf area and higher leaf weight. The plants in shade have thin and a broad leaf to harvest maximum light since light is the limiting factor under shade. Such leaves will have higher stromal thylakoids than thylakoids in granum. The palisade parenchyma thickness will be low in shade leaves whereas under open condition the leaf thickness is more and they will have more cell layers in the leaf to have more enzymes for utilizing the assimilatory power generated due to high light intensity (Lambers *et al.,* 1998).

Stomatal conductance and stomatal frequency varied significantly due to different shade levels. The highest stomatal conductance was recorded under open condition and the lowest stomatal conductance was reported in plants under 50% shade and 50% field capacity. According to Parthasarathy (2004), since stomatal opening is regulating by light which is in turn regulated by the blue light receptor cryptochrome, the stomatal conductance was higher under open condition compared to shaded plants. A decreasing trend in stomatal conductance within a particular shade with increase in WDS was also noticed. This reduction was due to chemical signal- ABA arriving from the roots as response to WDS that cause stomatal closure and in turn the decrease in stomatal conductance (Else *et al.,* 1996). Hence the plants' survival strategy of ABA mediated stomatal closure resulted in the decrease in stomatal conductance under increased WDS. WDS also causes stomatal closure reduced transpiration rate, a decreasing in the water potential of plant tissue, decrease in photosynthesis and growth inhibition and accumulation of oxygen radical scavenging compounds (Yordhan *et al.*, 2003).

No significant variation was observed in stomatal frequency and stomatal index with the different treatments given.

The Relative Water Content (RWC) of plants varied under different treatment

combinations. A higher RWC was observed in plants under 25% shade. *Amaranthus spinosus* Linn. being a C4 plant, was found to have efficient strategies for water management under stressed situation. The plant might be able to tolerate well with the available moisture, with a deeper root system and increased dry matter production. The plants might not be experiencing a high demand for increased transpiration rate, which can be a reason for the plants not to show a significant increase in RWC. As reported by Rahimi *et al*. (2010), RWC is more sensitive to drought stress, which may result in decrease in RWC under mild stress. According to Cornic *et al*. (1992), under lower water availabilities the primary activities may stay unaffected even under low RWC. The plant is hence competent enough to survive under low water regimes.

5.3 EFFECT OF ABIOTIC STRESS FACTORS ON BIOCHEMICAL PARAMETERS

The influence of abiotic stress factors - WDS and shade on the biochemical characteristics like total soluble proteins, total starch, reducing sugars, free amino acids and proline were studied. In general total soluble proteins were found to be higher in plants kept in open condition. This supports that the ideal condition for the plant to undergo primary metabolism is in its natural habitat. The plant prefers high light intensities and is capable to survive in high irradiance. The plants were observed to contain reduced amounts of total soluble proteins under increased WDS. This is in favour of earlier reports by Parthasarathy (2004) and Agami (2013). The decrease of protein and amino acid content was due to impaired protein metabolism and synthesis. The protein fractions were converted into compatible cytoplasmic osmotic agents and other stress proteins (Walter, 1995).

Variation in starch content was not much prominent among shade and open conditions. However a decreasing trend in total starch content was noticed among

the plants with increase in WDS. Similar results were also observed in *Vinca major* by Logan *et al*. (1998) and in chickpea by Yadav *et al*. (1996). Considerable variation in reducing sugar was not observed among plants under different shade levels. The ability of the plant to withstand high irradiance may be the reason for this. But a reduction in reducing sugar was observable with increased WDS. This was in accordance with the findings of Lini (2006). The decrease of carbohydrate fractions might be due to the reversible decrease in sucrose phosphate synthase activities (Vassey *et al.,* 1991).

The free amino acids in plants kept in open condition were comparatively high with those plants kept under shade. WDS sowed a reduction in the amino acid content of plants. A similar result was also reported by El-Tayeb (2005) in barley. Lower amino acid status might be due to reduced metabolism. A reduction in stomatal conductance under WDS might have also contributed to an associated reduction in nitrogen assimilation, following decreased amino acid content in stressed plants.

Proline status of plants under different treatment combination has not followed increasing trend suggesting that proline shall not be considered as an indicator of stress for this plant. This was in contradictory to the earlier findings by Agami (2013) and Slabbert and Kruger (2014). However, in another study by Brandt *et al*. (2011), only lower concentration of proline accumulation in C4 plant, *Amaranthus cruentus* and sorghum when imposed with oxidative stress due to WDS was observed. Apart from this, with the available results of RWC and the ability of the plant to tolerate WDS, the plant might not been experiencing a severe WDS, can also be a reason for reduced proline content in the plant.

5.4 EFFECT OF ABIOTIC STRESS FACTORS ON ANTIOXIDANTS

The activity and abundance in response to shade stress and WDS of five different enzymes like Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Peroxidase (POX) and Polyphenol oxidase (PPO) were studied. SOD showed more or less similar activity in plants under shade and open condition. This was found in contradiction to earlier reports by Yang *et al*. (2008). However a fluctuating range of activities of enzymatic antioxidants under oxidative stress were also reported (Yong *et al*., 2006). In the present study a slight increase in SOD activity was observed in plants which were imposed with a WDS of 50% field capacity. The plants kept under 100% and 75% field capacity did not show much difference in SOD activity. Similar results were also reported in Sorghum (Zhang and Kirkham, 1996) and in wheat (Bartoli *et al*., 1999).

Catalase activity showed significant variation among the different shade levels. CAT activity was observed to be higher for the plants kept under 50% shade than in 25% shade and open condition. Reduced activity of the catalase under open condition can be due to the photo-philic character of the plant. Enhanced production of ROS might not have been occurred in the plant to induce an intensified CAT activity. The catalase activity was increased under WDS. This was found to be in accordance with the report of Hertwig *et al*. (1992). In general an increase in catalase activity was observed with a corresponding increase in stress levels with the purpose of scavenging the free radicals produced during stress.

Glutathione peroxidase activity was observed to increase under 50% and 25% shaded condition, when compared to open condition. The plant tries to maintain the equilibrium of ROS generation to defend the oxidative damage during stress

condition experienced by the plant. Similar results were also reviewed by Milla *et al*. (2003). Increased activity of GPx was also observed in treatments with limited water availability especially under 50% field capacity. This is in congruent with earlier reports (Holland *et al.,* 1993; Avsian-Kretchmer *et al*., 2004; Ramos *et al*., 2009; Chang *et al*., 2009; Faltin *et al*., 2010; Miao *et al*., 2006; Fu, 2014).

Peroxidase and polyphenol oxidase activities were not found to have significant variation with the given treatments. However, comparatively increased activity of both the enzymes was observable in the treatment with highest shade level (50%) and highest WDS (50%). Plants under this treatment might have experienced a considerable level of oxidative stress. The level of activation strategy of oxidation enzymes under various stress conditions might differ among species in plants. This can be a reason for the lower activity of POX and PPO in *Amaranthus spinosus* Linn. compared to the activity of other enzymes like SOD, CAT and GPx.

The contribution of non-enzymatic antioxidants like vitamin A, vitamin E, ascorbic acid, total phenols and total flavonoids were also studied. Vitamin A content was found to be highest for the plants which were imposed with 50% shade stress and 50% field capacity. Increase in vitamin A was also noticed with an increase in WDS under each level of shade. These results were in accordance with earlier findings of Keles and Oncel (2002) and Ramel *et al*. (2012).

Vitamin E content was higher for plants kept in open condition. This was in accordance with the report revealing induction of tocopherol biosynthesis by light stimulus (Collakova and Penna, 2003). A general increase in vitamin E is observed with increased WDS among the different shade levels. Vitamin E has protective role against the stress induced oxidative damage (Espinoza *et al*.,

2013).

Ascorbic acid content was found to be comparatively higher for plants kept under 50% shade. Increased ascorbate content is one of the effective defense mechanisms against stresses in plants (Michael and Krishnaswamy, 2011). Ascorbic acid content in the plants under 50% shade might have helped the plants to withstand the shade stress to a certain extend. With the increase in WDS, ascorbic acid content was observed to be slightly reduced. Similar results were also reported in cucumber seedlings when exposed to short term water deficit (Fan *et al*., 2014).

Total phenolic content of the plants increased with decreased shade levels. The increased content of phenol under open condition was due to its protective role against high light intensity in open condition (Walter, 1995). Drought stress is found to decrease the total phenolic content of the plants. Plants maintained at 50% field capacity had lowest phenolic compounds throughout at all the shade levels. This result was in congruent with former reports by Tattini *et al.* (2004) and Robert *et al*. (2010). Plants maintained in shaded condition were found to have slightly increased flavonoid content. It was reported that accumulation of flavonoid compounds were promoted under low light intensities (Ghasemzadeh and Ghasemzadeh, 2011). Shade stress is found to have more role in flavonoid accumulation than drought stress.

5.5 CO_2 RESPONSE STUDY IN REALTON WITH ANTIOXIDANTS

In the present study, Amaranthus plants were exposed to CO_2 enrichment @ 500 ppm for a period of 30 days. The influence of different growth parameters, physiological and biochemical parameters and antioxidants in response to CO_2 enrichment were studied. Among the growth parameters, plant height, shoot weight and dry matter production were found to have a promotional response with

CO_2 enrichment. Total biomass accumulation in *Hevea brasiliensis* increased under conditions of elevated CO_2 (Devakumar *et al*., 1998). The results are in total agreement with the findings of Chaturvedi *et al*. (2009).

The major physiological parameter influenced by CO_2 enrichment was stomatal conductance. Around 40% reduction in stomatal conductance was observed under increased CO_2 concentration. Similar result was also reported by Chaturvedi *et al*. (2009), which follow the theory of CO_2 mediated stomatal closure. A decrease in chlorophyll content of plants was noted in the plants which were exposed to CO_2 enrichment. This result was in accordance with the earlier report by Zhu *et al*. (1997). Specific leaf area was found to be higher for the plants exposed with CO_2, which were maintained at 50% field capacity. This was found to be in contradiction to the report by Garbut *et al.* (1990). However oxidative stress due to WDS can also result in increased specific leaf area as formerly discussed in his chapter. There was no significant difference in RWC of the plant with the exposure to elevated CO_2 level. Aranjuelo *et al*. (2005) also reported similar result.

In the present study, total starch content of Amaranthus plants was found to have enhanced whereas total soluble proteins were slightly reduced under increased CO_2 concentration. Reducing sugars were also observed to have reduced. These results were found in accordance with earlier findings of Garg *et al*. (2001). Free amino acids were found to be higher for the plants enriched with CO_2 which directly indicates the health status of the plant. Antioxidants were not significantly affected with CO_2 enrichment. Amaranthus, being a C4 plant is equipped inherently with CO_2 concentrating mechanism. This can be one of the reasons for not observing remarkable highlights with regard to biochemical parameters in the plants with CO_2 enrichment. Moreover, the enrichment with CO_2 is only a slight increase in concentration. Hence the plants might not have

experienced a severe modification in its environment. C4 plants are acclimatized to perform under elevated CO_2 levels. Similar reports were also given by the studies conducted on Maize; a C-4 plant (Kim *et al.*, 2007). Hence a lower increase in CO_2 was not influential to alter the physiology of the plants.

5.6 EFFECT OF ABIOTIC STRESS AND CO_2 ENRICHMENT ON THE EXPRESSION OF CHALCONE SYNTHASE GENE

Reverse transcriptase PCR (RT-PCR) has been done to study the effect of abiotic stress and CO_2 enrichment on the level of expression of the chalcone synthase gene (CHS). The amazing diversity of polyketide derivatives in plants are generated by a group of structurally related enzymes called as the type III polyketide synthases (PKSs). Enzymes of the type III polyketide synthase super family play an important role in the biosynthesis of medicinal natural products in plants (Radhakrishnan *et al.*, 2009).

The most well-known and widely distributed member of the PKS superfamily is the chalcone synthase (Winkel-Shirley, 2001). CHS is the key enzyme of the flavonoid biosynthesis pathway. A preliminary qualitative study on the expression level of CHS gene by the plants under abiotic stress treatments and CO_2 enrichment was done using RT-PCR. The results have shown slight variation in the expression level of CHS gene in the plants under shade and WDS. This was in accordance with an earlier report where an upregulation of CHS gene under drought stress was observed (Bray, 2002).

An overexpression of CHS gene under elevated CO_2 was also observed. However, a significant overexpression when compared to abiotic stress was not noticed in CO_2 enrichment. A slight increase of CO_2 did not induce the gene expression of CHS. But, higher levels of CO_2 to the tune of 10,000 ppm to 50,000 ppm were found to have an effect on overexpression of flavonoid biosynthesis

genes and an associated increase in flavanoids (Ali *et al*., 2005). *Amaranthus spinosus* Linn. being a C4 plant, has an inherent acclimatized CO_2 concentration mechanism, which can be a reason for the observed results.

SUMMARY

6. SUMMARY

Plants continue to serve mankind as food as well as medicine since the time immemorial. The medicinal effects in plants are due to the presence of various active principles which are the products of secondary metabolism in plants. Plants have evolved a secondary metabolic pathway to survive it from numerous biotic and abiotic stresses. These stresses contribute to the increased production of reactive oxygen species (ROS), apart from the normal metabolism, which finally creates oxidative stress to plants. Antioxidants are capable of maintaining the equilibrium of generation of ROS. There are enzymatic and non-enzymatic antioxidants in plant system in order to maintain the balance between ROS metabolism and ROS scavenging.

It has been found that abiotic stress acts as an environmental cue for the enhanced production of antioxidants. Increase in antioxidant capability of plants increases its therapeutic value. Apart from the abiotic stress modification in climate also contributes to certain changes in plant system. The CO_2 level of the atmosphere is reported to be increasing. Thus the current investigation attempts to study the influence of abiotic stress factors on the growth characteristics, physiological and biochemical parameters as well as on the various enzymatic and non-enzymatic antioxidants in *Amaranthus spinosus* Linn. The programme also investigates influence of CO_2 on the above mentioned parameters on the plant.

The study was conducted in two separate experiments. In one experiment, abiotic stress factors like WDS and shade stress were imposed at three levels in combinations. WDS @ 100%, 75% and 50% field capacities and shade stress @ 50% and 25 % and open condition were imposed. CO_2 enrichment @ 500 ppm and WDS at 100% and 50% field capacities in combination were given as treatments in the second experiment. All the treatments were given for a period of

30 days and observations on various growth parameters like plant height, leaf area, shoot weight and dry matter production were taken. Physiological parameters like chlorophyll content, specific leaf area, stomatal characteristics and relative water content were studied. Influence of the different treatments on the biochemical parameters like total soluble proteins, total starch, reducing sugars, free amino acids and proline content were analysed. The accumulation of various enzymatic and non- enzymatic antioxidants in response to the abiotic stresses and CO_2 enrichment given were studied. The expression level of CHS gene, the key enzyme in flavonoid biosynthesis was also studied. The salient features of the investigation are summarized below.

6.1 EFFECT OF ABIOTIC STRESS FACTORS ON GROWTH PARAMETERS

The height of the plant was found to have an increasing trend in shaded condition when compared to open condition. The maximum plant height of 62.83 cm was recorded for the plants kept under 50% shade. Within a shade level the plant height was decreased as the WDS increased. The lowest plant height (46 cm) was recorded by those plants under open condition with highest WDS i.e. 50% field capacity. Leaf area was also higher for plants kept in shade Shoot weight also followed the same trend. Plants maintained at 100% field capacity and 50% shade showed the highest shoot weight. Dry matter production was maximum for plants kept in open condition, indication more contribution of dry matter from root biomass. Since the plant had positive response to growth parameters, it can be cultivated as an intercrop in plantations, under proper irrigated condition, which will help in initial establishment of the plant.

6.2 EFFECT OF ABIOTIC STRESS FACTORS ON PHYSIOLOGICAL PARAMETERS

Among the physiological parameters, chlorophyll content was maximum for the plants kept in shade. Plants maintained at 50% field capacity were found to

contain the highest chlorophyll content of 1.947 mg/g. In general, WDS decreased chlorophyll content. Plants kept at 50% field capacity observed to have decreased chlorophyll content in each shade level. Specific leaf area was also found to be increased under shade. Plants at 50% shade recorded a maximum specific leaf area of 508.4 cm^2 /g. Among the stomatal characteristics studies, stomatal conductivity was found to be significantly influenced by WDS. A decreasing trend of stomatal conductivity was followed with an increase in WDS. Stomatal conductivity was highest for the plant kept in open condition (69.0 m moles/m^2/ s^{-1}) and lowest for plants maintained at 50% field capacity (35 m moles/m^2/ s^{-1}). Stomatal frequency and stomatal index was not found to be profoundly varying under different abiotic stresses imposed. Relative water content of plants was found to have varied effects on abiotic stress treatments. Even though the plant has its original habitat under open condition, it could withstand varying levels of shade stress and WDS with respect to relative water content.

6.3 EFFECT OF ABIOTIC STRESS FACTORS ON BIOCHEMICAL PARAMETERS

The total soluble proteins were found to be higher in plants kept in open condition in general. A maximum of 4.63 mg/g of soluble proteins were recorded for the plants kept in open condition. WDS is found to decrease the total soluble protein content in plants. Under 50% shade treatment the lowest total soluble proteins were reported by those plants which were given a WDS of 50% field capacity. Total starch content is plants were also found to have decreasing effect by increased WDS. Under 25% and 50% shade lowest starch content was reported for the plants maintained at 50% field capacities. Reducing sugar content was also found to decrease as the WDS imposed was increased under shaded condition. Highest reducing sugar was recorded for plants kept at 50% shade and 100% field capacity. Free amino acid status of the plants also followed similar trend. Under

50% shade a higher value for free amino acids was recorded for plants at 100% field capacity and lowest value for the plants at 50% field capacity (13.59 µg/g). Proline status of plants was not observed to be significantly varying with the given stresses. However the treatments were significantly different. The data on biochemical parameters of the plant shows that, the plant favours its native habitat for an enhanced production of biochemicals. But the plants were able to tolerate moderate levels of WDS as well as shade stress. Upto 50% reduction in irrigation was not lethal to the plant. This ability of the plant can be utilized in the cultivating the plant in regions of reduced water availability.

6.4 EFFECT OF ABIOTIC STRESS FACTORS ON ANTIOXIDANTS

Enzyme activity of Superoxide dismutase (SOD) was found to be maximum for plants kept at 50% field capacity, under 50% shade. At 100% field capacity, the enzyme activity was lower in plants at both 50% shade and 25% shade. Catalase (CAT) activity showed an increased activity at increased WDS. Maximum CAT activity was recorded for the plants maintained at 50% field capacity under 50% shaded condition (0.086 EU/mg of protein). Lowest activity of the CAT enzyme was shown by the plant kept at 100% field capacity in open condition. Similar trend was noticed for the enzyme Glutathione peroxidase (GPx). Plants at 50% field capacity and 50% shade showed maximum GPx activity. Peroxidase (POX) and polyphenol oxidase (PPO) activity did not show a significant variation with the abiotic stress treatments. A comparative increase in the activity was shown in the plants kept at 50% field capacity and 50% shade for both POX and PPO.

Among the non-enzymatic antioxidants, an increase in vitamin A content was noticed with an increase in WDS. Maximum vitamin A content was seen in the plants maintained at 50% field capacity and 50% shade. Plants maintained at open condition were found to have maximum vitamin E content (20.7µg/g).

Lower amounts of vitamin E was seen in plants kept under 50% shade. Highest ascorbic acid was observed in plants maintained at 50% shaded condition. WDS was found to decrease the accumulation of ascorbic acid content. Lowest ascorbate content was observed for plants in open condition and 50% field capacity (2.28 mg/100g). Plants kept in open condition had increased levels of phenolic acid content, which was also found to decrease with WDS. Plants kept at 100% field capacity in open condition showed maximum phenolic compounds (3.42 mg CE/ml). A slight increase in flavonoid content was noticed for plants kept under shade. In open condition plants at 50% field capacity lowered the flavonoid content in plants.

There is a general increase of antioxidants under WDS. This favours plant to survive under stress. Moreover, the non-enzymatic antioxidants present in the plant like β- carotene and ascorbate increases the nutritive value of the plant. Hence apart from the medicinal value of the plant, it is concluded that the plant can be cultivated with minimum maintenance and can be consumed.

6.5 CO_2 RESPONSE STUDY IN RELATION WITH ANTIOXIDANTS

The response study of plants to enriched CO_2 content showed variation with respect to different parameters. Among growth parameters, a general increase of plant height, shoot weight and dry matter production showed a positive response to CO_2 enrichment, whereas leaf area did not differ significantly. Among the physiological factors studied, CO_2 significantly reduced stomatal conductance. Specific leaf area, relative water content and stomatal frequency and index were not found to vary enormously with the enrichment given. Among the biochemical parameters investigated, total starch was found to be enhanced under elevated CO_2 level, whereas reducing sugars and total soluble protein content were found to be comparatively reduced. Free amino acid status of enriched plants was higher compared to the open control. Enzymatic and non-enzymatic antioxidants were

not found to have significant changes with the CO_2 enrichment given. Hence it can be concluded that the innate character of C4 plants was responsible for the mild response of the plant to CO_2 enrichment.

6.6 EFFECT OF ABIOTIC STRESS AND CO_2 ENRICHMENT ON CHALCONE SYNTHASE GENE EXPRESSION

CHS gene is the key enzyme in the Phenyl Propanoid pathway in the biosynthesis of flavonoids and phenolic compounds. A preliminary qualitative study on the expression level of the gene by reverse transcriptase PCR revealed that abiotic stress and CO_2 enrichment resulted in overexpression of the gene. It was observed with mild increase in shade and WDS. Healthy shoot establishment supported with associated carbohydrate and amino acid status of the plants might have enabled the overexpression and enhanced secondary metabolism in plants under moderate stress condition.

Thus, the plant can be exploited for its therapeutic as well as nutritive characters. The information from the response of plants to various stresses can be utilized in cultivation practices of the plant. The plant has been under exploited and neglected from the main stream of crops now days. The plant served to be a famine food in many places around the globe among aborigines. The medicinal as well as nutritive potency of this traditionally known plant needs to be acknowledged.

6.7 CONCLUSION

The present investigation studied on the influence of Water Deficit Stress (WDS), shade stress and CO_2 enrichment on the physiological, phytochemical and molecular parameters involved in the accumulation if antioxidants in *Amaranthus spinosus* Linn. The natural habitat of the plant is open and arid conditions. However, the plant was tolerant upto 50% shade condition with optimum dry

biomass production as evident from the present study. CO_2 enrichment also had a positive relation with dry biomass production. In general the dry biomass production, total soluble proteins, starch, reducing sugars and free amino acids were reduced at 50% WDS. The highest accumulation of enzymatic and non-enzymatic antioxidants at 50% field capacity shows that the plant experienced stress at 50% field capacity. Thus the plant can serve as an intercrop in plantations with lower light intensity and moderate WDS.

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7. REFERENCES

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PHYSIOLOGICAL, PHYTOCHEMICAL AND MOLECULAR STUDIES ON ABIOTIC STRESS MEDIATED ANTIOXIDANT ACCUMULATION IN *Amaranthus spinosus* Linn.

by

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ABSTRACT

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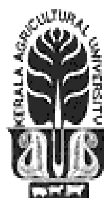

DEPARTMENT OF PLANT PHYSIOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM – 695 522
KERALA, INDIA

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ABSTRACT

The study entitled "Physiological, phytochemical and molecular studies on abiotic stress mediated antioxidant accumulation in *Amaranthus spinosus* Linn." was conducted during the period 2012-14 in the Department of Plant Physiology, College of Agriculture, Vellayani, Thiruvananthapuram. The objective of the study was to elicit information on the physiological, biochemical and molecular attributes associated to the antioxidant accumulation due to abiotic stress factors *viz*. light stress and water deficit stress and CO_2 enrichment in *Amaranthus spinosus* Linn. The study was conducted as two separate experiments. One experiment was laid out in pot culture at Instructional Farm Vellayani, in Completely Random Design (CRD) with nine treatments which included combinations of three levels of water deficit stress and three levels of shade stress in three replications. The second experiment was laid out in Open Top Chamber (OTC) in CRD with six treatments which included combinations of CO_2 enrichment and two levels of water deficit stress in four replications.

The observations on growth parameters revealed a general increase in plant height under shaded condition. Leaf area was maximum for plants under 50% FC and 25% shade, T6 (42.663 cm^2) and plants under 50% FC and 50% shade, T3 (40.567 cm^2). The results of physiological observation showed maximum concentration for total chlorophyll for plants under 50% FC and 25% shade, T6 (1.947 mg/g of leaf tissue). The analysis of biochemical parameters showed the plants under 25% shade + 100% FC (T4) and plants under open condition + 50% FC (T9) had maximum starch content of 8.963 mg/g and 8.49 mg/g respectively. The results of enzymatic antioxidant assays showed Superoxide dismutase activity was higher for plants under treatments T9 (Open + 50% FC) and T3 (50% shade + 50% FC); 0.032 Units. The non-enzymatic antioxidant assays showed the

maximum Ascorbic acid content of 3.6mg/g for treatment T1 (50% shade +100% FC) and Vitamin A content was maximum for T3 (50% shade +50% FC). Total flavonoids recorded highest in plant under treatment T2 (50% shade+75 % FC) whereas total phenol was maximum for the treatment T7 (Open + 100% FC).

CO_2 enrichment has no significant effect on plant height. Stomatal conductivity was significantly low for CO_2 enriched plant under 100% FC (25.35 m moles s^{-1} for OT1) and 50% FC (21.8 m moles s^{-1} for OT2). Plants under CO_2 enrichment and 50% FC showed maximum Catalase activity (0.169 Units).CO2 treatment did not show a significant variation with respect to antioxidants. Expression level study of the gene CHS (Chalcone synthase) revealed overexpression of the gene under abiotic stress, especially under moderate shade stress. Hence the plant can be exploited for its therapeutic value and can be cultivated as an intercrop in plantations.

APPENDICES

Appendix- I

Weather data during the treatment period (June1st – July10th , 2014)

Date	Temp. (°C)		RH (%)		Wind Velocity (km/h)	Sunshine hours	Rain (mm)	Evpn. (mm)
	Max.	Min.	I	II				
1-Jun	31.8	26.8	93	71	9.36	9.6		
2-Jun	31.7	26.6	92	81	9	9.3		
3-Jun	31.2	24.8	93	73	7.6	8.1	2.6	1.4
4-Jun	31.9	23.9	98	82	12.6	9.5	9.0	1.9
5-Jun	29.6	25.1	96	73	6.5	9.1	1.0	1.8
6-Jun	30.1	24.6	93	81	8.64	8.1	1.6	1.9
7-Jun	30.0	24.4	92	91	9.72	8.3	2.1	
8-Jun	30.6	24.7	96	98	5.4	4.7	3.5	1.5
9-Jun	28.2	24.3	92	84	8.28	9.3	13.5	1.5
10-Jun	30.6	24.4	93	74	6.48	8.5	3.6	2.6
11-Jun	31.2	23.4	93	86	5.76	8.3	19.0	3.0
12-Jun	29.5	24.4	97	80	9.72	7.52	3.0	3.1
13-Jun	29.5	25.0	88	72	9.36	8.6	1.0	3.1
14-Jun	30.8	25.6	88	70	8.64	9.37	0.0	3.1
15-Jun	31.1	25.5	88	68	12.60	10.16	0.0	3.1
16-Jun	31.3	25.7	91	83	9.00	9.57	0.0	3.1
17-Jun	31.5	25.9	91	83	12.24	9.5	0.0	4.0
18-Jun	30.7	26.3	85	69	17.64	10.15	0.0	2.3
19-Jun	31.3	26.1	95	77	8.28	7.18	0.0	2.3
20-Jun	31.7	26.1	99	78	12.96	8.27	15.0	2.3
21-Jun	31.5	24.5	92	83	9.72	9.13	3.4	7.5
22-Jun	30.6	25.5	91	74	6.12	10.52	1.0	5.0
23-Jun	30.8	25.7	92	80	7.92	10.2	0.0	2.5
24-Jun	31.3	25.4	92	83	14.04	9.5	0.0	4.4
25-Jun	30.7	25.3	93	77	10.80	9.3	0.0	3.3
26-Jun	30.8	25.8	91	73	7.92	10.3	0.0	3.3
27-Jun	30.7	25.5	89	80	8.64	9.4	0.0	3.4
28-Jun	31.6	25.7	90	84	4.68	8.4	0.0	6.8
29-Jun	30.1	24.9	96	76	7.20	10.4	0.0	4.4
30-Jun	30.5	24.5	95	91	6.12	9.35	8.7	4.3

Appendix- II

Weather data from OTC during the treatment period

(June1st – July5th, 2014)

Date	CO_2 (ppm)	Air Temp A (°C)	Air tem B (°C)	Rh A (%)	Rh B (%)	Sun shine duration (min.)	Solar Incidence (micro Enst.)
1/06/2014	541	47.96	47.9	33.36	33.09	159.3	1408.87
2/06/2014	537	46.86	47	36.5	35.86	467.4	1349.2
3/06/2014	540.2	42.59	42.6	45.4	44.7	764.06	1155.9
4/06/2014	543.9	45.9	44.2	38.59	40.8	1053.65	1433.6
5/06/2014	548	47.2	46.4	36.8	37.9	1352.11	1616.2
13/06/2014	537.7	41.3	42	49.2	47.8	1622.28	1119.7
14/06/2014	539.7	42.2	42.3	47.4	47.0	1070.12	1943.4
16/06/2014	542.7	47.9	47.7	33.2	33.1	2325.7	1662.0
17/06/2014	539.6	44.7	45.3	39.7	38.05	2654.5	1401.5
18/06/2014	544.9	46.01	46.1	36.7	35.5	3007.8	1431.9
19/06/2014	542.3	42.9	42.9	46.05	45.9	3341.8	1223.4
20/06/2014	543.2	42.6	42.6	47.6	46.2	3664.7	1324.6
21/06/2014	549.9	44.4	44.3	42.8	42.6	3978.9	1588.8
22/06/2014	535.4	45.02	44.5	41.2	41.4	4343.6	1425.2
23/06/2014	541.2	45.1	45.4	41.18	39.8	4722.08	1556.3
24/06/2014	542.6	45.6	46.2	39.1	37.3	5044.3	1593.5
25/06/2014	553.4	43.6	44.4	42.5	40.2	5383	1283.5
26/06/2014	540.4	44.5	44.7	41.9	40.6	5706.8	1400.6
27/06/2014	544.2	46.9	47.1	37.9	36.2	6048.9	1560.3
28/06/2014	548	46.9	47.2	39.8	37.6	6371.9	1531.4
29/06/2014	537.7	47.5	47	38.3	38.6	6709.2	1486.3
30/06/2014	550.6	40.2	40.5	55.0	53.8	7057.5	1069.0
1/07/2014	541.3	42.8	42.9	51.2	49.9	148.2	1338.5
2/07/2014	550.3	29.3	29.3	91.5	97.2	449.3	444.7
3/07/2014	544.6	41.5	41.3	54.8	56.2	766.3	1131.8
4/07/2014	546.4	37.2	36.6	64.0	68.0	998.4	911.7
5/07/2014	528.8	39.7	39.8	59.5	57.5	1110.8	1258.8