STUDIES ON DROUGHT AND HIGH TEMPERATURE STRESSES ON THE PHYSIOLOGY OF YOUNG PLANTS OF *HEVEA BRASILIENSIS*

Dissertation submitted to
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Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled **"Studies on drought and high temperature stresses on the physiology of young plants of *Hevea brasiliensis*"** is an authentic record of the project work done by **Ms. Veena Raj** at the Rubber Research Institute of India, Kottayam, under the guidance of Dr. K. Annamalainathan, Deputy Director, Crop Physiology Division, Rubber Research Institute of India, Kottayam, in partial fulfillment of the requirement for the award of Master of Science in Biochemistry at the School of Biosciences, Mahatma Gandhi university, Kottayam and this dissertation has not formed the basis for the award of any other degree or diploma earlier.

August 2012

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DECLARATION

I do hereby declare that the dissertation entitled "**Studies on drought and high temperature stresses on the physiology of young plants of *Hevea brasiliensis***" submitted to Mahatma Gandhi University in partial fulfillment for the award of degree of Master of Science in Biochemistry is a record of the original research work done by me under the guidance of Dr. K. Annamalainathan, Deputy Director, Crop Physiology Division, Rubber research Institute of India (RRII), Kottayam from April-June 2012 and no part of this dissertation had been presented earlier for any degree/diploma/fellowship or any other similar title of any university or institution.

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ABSTRACT

ABSTRACT

A study was conducted to understand the interactive effects of drought and high temperature stresses on photosynthesis and chloroplast profile of young plants of *Hevea.* A popular clone RRII 105 was raised in medium size poly bags and kept in open field. Around 8 months old young plants were subjected to drought stress by withholding irrigation under different temperature inside a plant growth chamber. Various physiological and biochemical parameters were analyzed after 5 days of withholding irrigation in poly bags. When compared to the irrigated plants there was a decline in leaf water potential in drought imposed plants under all temperature conditions. There was a significant reduction in photosynthetic pigments namely chlorophyll *a, b* and carotenoids contents in drought imposed plants when compared to the irrigated plants. The pigments reduction was minimum at 30^{0}C and very high at 35 and 40^{0}C. Similarly the effective quantum yield of PS II was drastically inhibited at 40^{0}C. A thylakoidal stress protein was found over expressed under drought condition. Further expression level of this protein was relatively higher at 35^{0}C. The present study obviously indicated that concomitant occurrence of high temperature stress and soil moisture deficit aggravated the damaging effects in the photosynthetic apparatus of young *Hevea* plants.

INTRODUCTION

Natural rubber (NR) is one of the most important polymers naturally produced by plants and it is a strategic raw material used in more than 4,000 products including around 400 medical devices (Mooibroek and Cornish, 2000). Owing to its special molecular structure and high molecular weight NR has better resilience, elasticity, abrasion resistance, efficient heat dispersion and impact resistance than artificially produced polymers. NR has been widely applied to many products, such as tires, gloves, balloons and balls for sports. Rubber tree has attracted attention as a substitute for the tropical rain forest as a wood resource. The wood is excellent for furniture and floor materials. Natural rubber also contributes to the global environment preservation due to its role as an efficient carbon sequesters (Rahman and Shivakumaran, 1998).

Natural rubber produced in the milky cytoplasm (latex) of specialized cells called laticifers of over 2000 plant species belonging to 311 genera of 79 families. The chemical nature of natural rubber is cis-1, 4 poly isoprene. *Hevea brasiliensis* (para rubber tree) is the major source of commercial natural rubber belonging to the family Euphorbiaceae. Its center of origin is the Amazon rain forests of Brazil. It was brought under domestication only in 1876. Now it is widely cultivated in the South and Southeast Asian countries including India, Thailand, Indonesia, Malaysia, Sri Lanka, Vietnam, Laos and China. It is a deciduous, sturdy perennial tree with orthotropic rhythmic growth and the mature tree attains a maximum height of 25 m. It grows on varying type of soils provided they are deep and well drained. A warm humid equable climate and fairly distributed annual rainfall of not less than 200 cm are necessary for the optimum growth. The temperature must be about 20-34°C and the atmospheric humidity (RH) might be of around 70-80%. Bright sunshine amounts about 2000 hours per year at the rate of 6 hours per day throughout the year may be required. The absences of strong winds are suitable for healthy growth (Rubber Grower's Companion 2010). The tree has a straight trunk with light gray bark and the branches are usually from an open leafy crown. The tree is monoecious with lateral inflorescence bearing both staminate and pistillate flowers.

The world demand for natural rubber is steadily increasing; hence production has to be accelerated to meet the demand. The exclusive objective of rubber breading is to develop superior clones with improved dry rubber yield as well as wood. Other desirable secondary characteristics include high initial vigor, smooth and thick bark with good latex vessel system, good bark renewal, high growth rate after initiation of latex harvest, tolerance to major diseases, wind, abiotic stresses, low incidence of TPD, etc. (Varghese *et al.*,2000).

Adverse environmental conditions such as drought, high and low temperatures, high solar radiation, low atmospheric humidity, poor soils etc., limit the expansion of rubber cultivation to newer areas in several rubber producing countries including India. Stressful environment caused by the above conditions is a limiting factor in the traditional rubber growing areas too. Abiotic stresses affect every aspects of plant growth; modify plant anatomy, physiology, biochemistry and gene expression. Developing new clones with increased tolerance to environmental stress is highly essential, especially in the present scenario of global warming and related climatic changes. Abiotic stress is the primary cause of crop loss worldwide, reducing average yield for most major crop plants by more than 50% (Wang *et al.*, 2003). Drought is probably the largest factor which limits the agricultural productivity in general and is the most important factor that restricts the expansion of cultivation of *Hevea brasiliensis* to newer areas in habitat available to plants.

Drought stress is characterized by reduction of tissue water content, diminished leaf water potential and turgor loss, closure of stomata and decrease in cell enlargement and growth. Severe water stress may results in the arrest of photosynthesis, disturbance of metabolism and finally the death of plant (Jeleel *et al.*, 2008). Most of the field grown plants tolerate stress through many metabolic adaptations at cellular levels. Plants can tolerate certain level of environmental stress through modulating there metabolic activities and developing some defense mechanisms (Halliwell and Gutteridge., 1999). Almost all stresses induce the production of a group of proteins called heat-shock

proteins (Hsps) or stress induced proteins, a common phenomenon in all living things (Vierling, 1991).

NR is being extended to marginally suitable Konkan and east coastal areas of India where soil moisture deficit and very high temperature during summer months are the major environmental constraints for the establishment of young rubber plants. Drought and high temperature in central India and chilling winter in the North East are the two major limiting factors that restrict the growth and productivity of *Hevea.* Summer in the North Konkan can last for more than 6-7 months from mid-December onwards with practically no rain during this period, and characterized by fast depletion of soil moisture, high temperature and very low relative humidity. The atmospheric temperature may go up to 42°C in the Central India region. The fairly warm air and low atmospheric relative humidity (RH) lead to high evaporative demand causing atmospheric drought in North Konkan. Both in the North Konkan and North East the environmental stress is associated with light intensities of sunlight, much more than what is required to saturate photosynthesis of leaves. The interactive effects of water deficit and high temperature on the physiology of young rubber plants are largely unknown. No extensive studies have been done so far to understand the cumulative effects of various abiotic stresses in plantation crops including rubber.

In the present study a popular *Hevea* clone namely, RRII 105 was tested for its drought tolerance potential under varying temperature conditions for a short period inside a plant growth chamber.

OBJECTIVE

1. To study the interactive effects of drought and high temperature conditions on the physiology of young *Hevea brasiliensis.*
2. To study the level of chloroplast stress proteins and its role in water deficit stress tolerance of young plants of *Hevea* under three different temperature regimes.

REVIEW OF LITERATURE

Rubber plants

Hevea brasiliensis is the most important commercial source of natural rubber (NR). About 90% of the total world production of natural rubber is obtained from *H. brasiliensis.* Owing to the increased global demand for NR and its limited scope for expansion in the traditional belts, attempts are being made to extend the cultivation to marginally suitable areas in most rubber growing countries with varied climatic constraints like moisture stress and high and low temperature conditions. In India, the cultivation of rubber is being extended to North East and Central India. The tree requires deep soils, relatively stable warm temperature and continuous moisture throughout the year; soil fertility is less important than physical soil properties. Dry periods just for 2-3 months do not specifically damage vegetative growth but seriously affect rubber production and quality of latex. However, prolonged drought coupled with high temperature in central India and very low temperatures during winter in NE India are the major constraints for young plants establishment in this regions (Jacob *et al.,* 1999).

Climatic constraints and Young plants establishment

Climatic factors play a pivotal role in the establishment and development of any crop. Ambient temperature, rainfall, wind speed, vapor pressure deficit and the number of hours of sun shine are some of the factors that govern these. Drought combined with high solar light intensity has been reported as a major environmental constraint for establishing rubber cultivations in areas such as the North Konkan region of India (Jacob *et al.*, 1999; Alam *et al.*, 2005). Different genotypes need to be evaluated under various environments in order to give an idea of which genotype(s) would be suitable for planting in a particular environment.

High vapour pressure during the summer months can be a problem for growing rubber trees in parts of India, for example in the Konkan region. Wind is yet another abiotic stress influencing the establishment and growth of rubber. One impact is a contribution to the drying effect of drought conditions, especially

with regimes of long lasting- steady winds such as occurs during the dry season in the highlands of Vietnam. High solar radiation coupled with high temperature and low relative humidity (RH) result in the high vapour pressure deficit between the leaf and the surrounding atmosphere, and this subsequently increases the evapotranspirative demand of the atmosphere. Thus rubber trees in this region are subjected to prolonged periods of both soil and atmospheric drought stress. Agro-climatic constraints cause direct or indirect losses in the yield and quality of produce.

Polybag plants of 2-3 whorls (8-10 months old) raised from budded stumps are used as planting material in the field. Planting may be carried out during favorable climate with sufficient soil moisture. It should be either during pre- monsoon period or immediately after the intensive rainy season. If polybag plants are used the top storey of the leaves should be mature at the time of planting. When the polybag plants are taken out of the trench dressing of the roots is necessary. While planting the scion of the polybag plants should be directed towards the North East to minimize the adverse effect of direct sunlight on the bud patch (Rubber Growers companion 2012).

Recent studies showed that there are indications of climate change in the NR growing regions in India and increasing temperature is the most prominent change indicated (Shammi *et al.,* 2010). Under the changing climate, survival/establishing young plants during summer is a major problem to be addressed seriously. The following management practices which have been tested in various experiments at Rubber Research Institutes of India can be practiced for the protection of immature and mature plants in the field. Partial shade (30 % shade) is advisable for the establishment of young nursery plants (Nair *et al.*, 2002, Annamalainathan *et al.,* 2005). During the year of planting in main field the plants may be provided with shade before the onset of summer. Plaited coconut leaf or used gunny bags can be used as shade providing materials. Mulching the base with cut grasses, dried plant materials or plastic with punched holes can be practiced in young immature plants (Rubber Growers Companion 2012). From the second year onwards till the canopy development

the young brown stems are generally white washed with lime during summer in almost all rubber growing regions of India.

Experiments conducted with tilling the plant base in young plants resulted in significant level of soil moisture retention and the growth was significantly superior to that of untilled plants. Both tillage and life saving irrigation were found effective overcoming the transient drought condition. Under short dry spell, application of potassium and silicon were found effective in reducing the adverse effect of water stress in young plants. Thus to enhance the ability of plants to tide over drought stress in addition to irrigation proper nutritional management is also required (Jessy *et al.*, 2010). Adopting both these management practices together may give adequate protection to plants to withstand drought stress.

Abiotic stress and photosynthesis

Stress is defined as "any environmental variable, which can induce a potentially injurious strain in plants". In a persistently changing environment, plants are constantly challenged by various abiotic stresses such as salinity, drought, temperature extremes, heavy metal toxicity, high-light intensity, nutrient deficiency, UV-B radiation, ozone, etc. In general, abiotic stress often causes a series of morphological, physiological, biochemical and molecular changes that unfavorably affect plant growth, development and productivity. Drought, salinity, extreme temperatures (cold and heat) and oxidative stress are often interrelated; these conditions singularly or in combination induce cellular damage. A key sign of such stresses at the molecular level is the accelerated production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) (Halliwell and Gutteridge, 1999).

Abiotic stresses alter gene networks and signaling cascades in an effort to restore cellular homeostasis. Recent studies in plants have shown that relatively low levels of ROS act as signaling molecules that induce abiotic stress tolerance by regulating the expression of defense genes (Apel and Hirt, 2004). Additionally,

numerous results have shown that plants with higher levels of antioxidants, whether constitutive or induced, showed greater resistance to different types of environmental stresses.

Drought remains one of the most biologically damaging and ecologically limiting factors among all environmental stresses. Drought stress can occur at any stage of the growing process, and can cause complete loss of crop or serious damage to yield. Heat is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause irreversible damage to plant growth and development. Heat stress due to high ambient temperatures is a serious threat to crop production worldwide (Hall, 2001). High temperature during day time can have direct damaging effects associated with hot tissue temperatures or indirect effects associated with the plant-water-deficits that can arise due to high evaporative demands. Evaporative demand exhibits near exponential increases with increases in day-time temperatures and can result in high transpiration rates and low plant water potentials (Hall, 2001).

During the vegetative stage, high day temperature can cause damage to components of leaf photosynthesis, reducing carbon dioxide assimilation rates compared with environments having more optimal temperatures. Sensitivity of photosynthesis to heat mainly may be due to damage to components of photosystem II located in the thylakoid membranes of the chloroplast and membrane properties (Al-Khatib and Paulsen, 1999). Membrane thermo stability has been evaluated by measuring electrolyte leakage from leaf discs subjected to extreme temperatures (Blum, 1988). More stable membranes exhibit slower electrolyte leakage.

The process of photosynthesis takes place in the chloroplast, specifically using the green pigment chlorophyll . In addition to chlorophyll, carotenoids and xanthophylls are also present. These are embedded in a special antenna protein called light harvesting complex (Anderson and Barber, 1996). The function of the vast majority of chlorophyll (up to several hundred molecules per photosystem) is to absorb light and transfer that light energy to a specific chlorophyll pair in the reaction centre of the photosystems. Photosynthesis the light driven carbon

dioxide assimilation process and the primary means of energy production in plants, is extremely sensitive to elevated temperatures. Photosynthesis converts light energy into the chemical energy of sugars and other organic compounds. This process consists of a series of chemical reactions that require carbon dioxide (CO_2) and water (H_2O) and store chemical energy in the form of sugar. Oxygen (O_2) is a byproduct of photosynthesis and is released into the atmosphere. (Campbell *et al.*, 1999).

The light reaction happens in the thylakoid membrane and converts light energy in to chemical energy. This chemical reaction must, therefore, take place in the light. Chlorophyll and several other pigments such as beta-carotene are organized in clusters in the thylakoid membrane and are involved in the light reaction. The energy harvested via the light reaction is stored by forming a chemical called ATP (adenosine triphosphate) a compound used by cells for energy storage. The dark reaction takes place in the stroma within the chloroplast and converts CO_2 to sugar. This reaction doesn't directly need light in order to occur, but it does need the products of the light reaction (ATP and another chemical called NADPH). The dark reaction involves a cycle called the Calvin cycle in which CO_2 and energy from ATP are used to form sugar (Campbell *et al.*, 1999).

Drought stress induces several changes in various physiological, biochemical, and molecular components of photosynthesis. Drought can influence photosynthesis either through pathway regulation by stomatal closure and decreasing flow of CO2 into mesophyll tissue (Chaves, 1991; Chaves *et al.*, 2003; Ort *et al.*, 1994; Flexas *et al.*, 2004) or by directly impairing metabolic activities (Farquhar *et al.*, 1989). The main metabolic changes are declines in regeneration of ribulose bisphosphate (RuBP) and ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) protein content (Bota *et al.*, 2004), decreased Rubisco activity (Parry *et al.*, 2002), impairment of ATP synthesis, and photophosphorylation or decreased inorganic phosphorus. In general, during the initial onset of drought stress, decreased conductance through stomata is the primary cause of decline in photosynthesis (Cornic, 2000). At later stages with

increasing severity, drought stress causes tissue dehydration, leading to metabolic impairment. In contrast, there is evidence in some species that nonstomatal inhibition (metabolic activities) may occur first, causing a temporary increase in internal CO_2 concentration (Ci), which causes stomata to close (Briggs *et al.,* 1986). Drought stress has been shown to cause increases in Ci (Siddique *et al.,* 2001; Kicheva *et al.,* 1994). Recent studies suggest that both diffusive limitation through stomatal closure and nonstomatal limitation (such as oxidative damage to chloroplast) are responsible for decline in photosynthesis under drought stress (Zhou *et al.*, 2007).

The processes involved in photosynthesis are much more tolerant to heat stress and are mostly stable in the temperature range of up to 30 to 35°C, depending on crop species. However, very high temperatures (>40°C) can negatively affect photosynthesis. The response of photosynthesis to heat stress is related to temperature dependence of Rubisco to the two substrates, carbon dioxide and oxygen. At high temperatures, the solubility of oxygen is decreased to a lesser extent than CO2, resulting in increased photorespiration and lower photosynthesis (Lea and Leegood, 1999). In addition, the activation and activity of Rubisco are also decreased at high temperatures (Prasad *et al.,* 2004). Heat stress primarily deactivates Rubisco by inhibiting the enzyme Rubisco activase (Crafts-Brandner and Salvucci, 2000). The mechanism responsible for inactivation of Rubisco under heat stress is related to inability of activase to overcome the inherently faster rates of Rubisco inactivation (Salvucci and Crafts-Brandner, 2004).

The photosynthesis apparatus, photosystem II (PSII), plays a key role in the response of leaf photosynthesis to environmental stresses. Photosystem II is relatively more tolerant to drought stress than heat stress (Havaux, 1992). Drought stress resulting in relative water content (RWC) and leaf water potential of 40% and −4 MPa, respectively, did not affect PSII functioning in dark- and light adapted leaves (Havaux, 1992). In contrast, PSII is most sensitive to heat stress. There are two main factors which make the PSII electron transport most sensitive to heat stress. First, the fluidity of thylakoid membranes increases at high temperatures; this leads to dislodging of PSII light harvesting complexes

from thylakoid membrane. Second, the PSII integrity is dependent on electron dynamics. Therefore, if heat stress disrupts metabolic processes that either deliver or accept electrons from PSII, then the PSII is likely to dislodge from the thylakoid membrane.

Havaux (1992) investigated the impact of drought, heat, and strong light applied separately and in combination on PSII activity and found that drought stress enhances the resistance of PSII to heat and light stress. Although Rubisco activation was more closely correlated with photosynthesis than the maximum quantum yield of photochemistry of PSII, both processes could be acclimated to heat stress by gradually increasing the leaf temperatures (Law and Crafts-Brandner, 1999). The inhibition of PSII electron transport under heat stress is often indicated by sharp increase in basal level of chlorophyll fluorescence that corresponds to photosynthetic inhibition. Use of chlorophyll fluorescence measurements have been shown to be useful in quantifying the impact of drought and heat stress on plants (Oukarroum *et al.,* 2007; Ristic *et al.,* 2007).

The thermal effects of photosynthesis and respiration are related to membrane function and membrane integrity. In general, heat stress influences membrane fluidity, induces membrane leakiness, and influences the integrity of protein and membranes. Thylakoid membranes are especially sensitive to drought and heat stress; hence, disturbances in photosynthesis are among the first indicators of drought and heat stress. Under drought stress, photosynthesis decreases before the decrease of respiration, resulting in decrease in the ratio of photosynthesis and respiration and also increase in photorespiration. This often suggests that drought can cause starvation and lead to plant death. However, plants are more likely to suffer greater damage to shoots from the metabolic effects of drought rather than from lack of carbohydrates.

The amount of chlorophyll has been slightly affected by water stress. The reduction in photosynthetic pigments might be due to the changes in the thylakoid organization as well as the biosynthesis of chlorophyll in control and water stressed plants. It has been previously reported that the chloroplast integrity has been damaged under stressful conditions (Kaiser *et al.,* 1981). The surface area of the chloroplast inner-membrane shrinks as the volume of the

chloroplast decreases under water stress. It was presumed that structural rearrangements in the chloroplasts might become necessary under water stress conditions. Isolated intact chloroplasts have also shown a similar trend in the pigment content, when subjected to water stress.

Heat stress also inhibits synthesis and promotes degradation of cytokinins, important hormones for regulation of growth and development processes, such as cell division, leaf senescence, and root growth (Xu *et al.*, 2010). Shashidhar *et al.* (1996) have reported that cytokinin delivery decrease drastically in plants with droughted roots. In general cytokinins favour stomatal opening, this also might account for a decrease in the rate of photosynthesis, due to partial stomatal closure. Xu et al. used transgenic *Agrostis stolonifera,* a C3 perennial grass species, to survey protein changes in response to elevated temperatures. CO_2 assimilation by leaves is reduced mainly by stomatal closure, membrane damage and disturbed activity of various enzymes, especially those of CO_2 fixation and adenosinetriphosphate synthesis.

Overall, both drought and heat stress decrease CO_2 uptake either by stomatal regulation (as in case of drought stress) or internal resistance to CO_2 diffusion, both favoring oxygenase activity, leading to increased photorespiration and decreased photosynthesis. Photosynthesis is relatively more tolerant to heat stress compared with drought stress. This differential sensitivity of photosynthesis to drought and heat stress suggests differential interaction effects. The combination of both drought and heat stress may therefore be additive or multiplicative. The limited transpirational cooling under drought stress can exacerbate the effects of already higher air temperatures (Hale and Orcutt, 1987). Some studies suggest that drought stress influences the thermal tolerance of photosynthesis (Havaux, 1992; Lu and Zhang, 1999). In contrast, some studies have reported that drought greatly exacerbates the effects of heat stress on plant growth and photosynthesis (Xu and Zhou, 2006). Critical heat threshold for the reversible and irreversible inactivation of photosynthesis, which correlates with lethal injuries that become apparent later on, can be determined by *in vitro* by chlorophyll fluorimetry.

Photosynthesis in young *Hevea* plants has been reported to be inhibited by drought and high light conditions. The percentage inhibition in photosynthetic O_2 evolution in drought stressed *Hevea* plants as compared to the irrigated controls was as low as 33% in shade (30% light) grown plants and as high as 51% in open light grown plants (Annamalainathan *et al.*, 2006). Total leaf area and leaf number were also decreased in *Hevea* under water stress. (Dey and Vijaya Kumar, 2005). Several studies have shown that various components of photosynthetic metabolism are very sensitive to drought stress in *Hevea* (Jacob *et al.*, 1999, Devakumar *et al.*, 2002).A decrease in the relative water content (RWC) in response to drought stress has been noted in wide variety of plants as reported by Nayyar and Guptha (2006). Photo system II activity and carboxylation process are highly sensitive to water stress in young plants of Hevea. Tezara *et al.*, (1999) have suggested that decreased coupling factor and photophosphorelation are the cause for decreased photosynthesis under water stress. They showed that decrease in net photosynthesis with water deficiency was related to lower Rubisco activity rather than to ATP and RuBP contents (Tezara *et al.*, 2002)

Physiological characterization of plants subjected to a combination of drought and heat stress has several unique aspects such as combining high respiration with low photosynthesis, closed stomata, and high leaf temperatures (Mittler, 2006). Mittler (2006) emphasized the importance of the combination of stresses and indicated that transcript profiling studies of plants subjected to a combination of drought and heat stresses reveal a unique response involving >770 transcripts that are not altered by drought or/and heat stress. Profiling 308 Prasad et al. experiments further illustrate that acclimation responses heat stress are different and that only a small overlap in transcript expression was found between the two responses (Mittler, 2006). Transcript changes in metabolite accumulation were highly specific during combinations of stresses (Rizhsky *et al.*, 2002). These studies were conducted under controlled environmental conditions and in a non-crop species (*Arabidopsis*) under very low light conditions. Therefore, further studies are required to understand the

interactions of drought and heat stress on photosynthesis and respiration for field crops to improve our knowledge and improve crop models.

Drought and temperature stresses

Soil moisture deficit and extreme temperatures are major abiotic stress factors restricting plant growth and productivity in many regions, and they are often occurring simultaneously. Temperature is one of the key environmental factors which influence plant growth. *Hevea bresiliensis,* being a species adapted to moderate temperatures gets affected by extremes in temperature. In south India, temperatures ranges between 23^{0}C to 34^0 C where as in the Konkan region (west coast region) the maximum is 34-40^{0}C during summer. High temperature conditions results in higher rates of evapotranspiration leading to severe soil moisture stress in the absence of rainfall. High temperatures above 37^{0}C coupled with soil moisture stress, result in injury to leaf and drying of leaf margins (Chandrasekhar *et al.*, 1990; Vijayakumar *et al.*, 1994).

Drought has dramatic effect on plant growth and morphogenesis and is likely to be one of the major environmental factors determining plant productivity and species distribution (Woodward, 1987, Wright, 1992). Effect of drought stress in young plants is drastic reduction in photosynthesis followed by reduced growth Drought stress in plans is aggregated by both high solar radiation and increased atmospheric temperature, thereby increasing the magnitude of damage even under a short period of drought.

Natural rubber cultivation in India faces adverse effect of some of these environmental stresses especially in the non- traditional rubber growing areas (Jacob *et al.*, 1999). The severe soil and atmospheric drought combined with high temperature prevailing in the North Konkan and the chilling winter temperature in North Eastern region adversely affect the survival, growth and productivity of rubber plants. In *Hevea* the interactive effects of multiple environmental stresses are more detrimental than individual stresses and high light intensity has been found to aggravate the harmful effects of drought stress (Alam and Jacob, 2002, Annamalainathan *et al.*, 2005). The responses of plants to combination of

stresses are a complex phenomenon and it results in intensification or overlapping of stress effects. Even in traditional areas drought stress is common and this occur concomitant with high temperature and high light during summer (Jessy *et al.*, 2010).

A transient elevation in temperature, 10-15^{0}C above ambient, is typically defined as heat stress (Wahid *et al.*, 2007); however the effects vary with the duration and amount of temperature increase. Plants differ in their abilities to cope with rising temperatures. The onset of heat immediately changes the cellular state, alters membrane fluidity and lipid composition, and initiates the signaling cascades that ultimately lead to transcript accumulation for genes encoding protective and chaperone activities. Loss of activase activity during heat stress is caused by exceptional sensitivity of the protein to thermal denaturation and is responsible in part for deactivation of Rubisco itself.

Scarcity of water is a severe environmental constraint to plant productivity. Drought-induced loss in crop yield probably exceeds losses from all other causes, since both the severity and duration of the stress are critical. Various management strategies have been proposed to cope with drought stress (Farooq *et al.*, 2009). Drought stress reduces leaf size, stems extension and root proliferation, disturbs plant water relations and reduces water-use efficiency. Plants display a variety of physiological and biochemical responses at cellular and whole-organism levels towards prevailing drought stress, thus making it a complex phenomenon. Enhanced metabolite flux through the photorespiratory pathway increases the oxidative load on the tissues as both processes generate reactive oxygen species. Injury caused by reactive oxygen species to biological macromolecules under drought stress is among the major deterrents to growth (Farooq *et al.*, 2009).

The primary targets of thermal damage in plants are the oxygen evolving complex along with the associated cofactors in photosystem II (PSII), carbon fixation by Rubisco and the ATP generating system. Recent investigations on the combined action of moderate light Intensity and heat stress suggest that

moderately high temperatures do not cause serious PSII damage but inhibit the repair of PSII. The latter largely involves *de novo* synthesis of proteins, particularly the D1 protein of the photosynthetic machinery that is damaged due to generation of reactive oxygen species (ROS); resulting in the reduction of carbon fixation and oxygen evolution, as well as disruption of the linear electron flow. The attack of ROS during moderate heat stress principally affects the repair system of PSII, but not directly the PSII reaction center (RC) (Zrobek-Sokolnik, 2012).

Heat stress additionally induces cleavage and aggregation of reaction centre (RC) proteins; the mechanisms of such processes are as yet unclear. On the other hand, membrane linked sensors seem to trigger the accumulation of compatible solutes like glycinebetaine in the neighborhood of PSII membranes. They also induce the expression of stress proteins that alleviate the ROS-mediated inhibition of repair of the stress damaged photosynthetic machinery and are required for the acclimation process (Allakhverdiev *et al.*, 2008).

Cumulative effects of abiotic stresses

The combined physiological and molecular responses of plants for heat and drought stress are quite complex, and it remains extremely difficult to deduce these effects from observing the responses from one stress alone. For example, high leaf temperatures are a result of the combined effect because plants lose the ability for transpirational cooling when water availability is limited. When faced with high temperatures, plants will open their stomata in an effort to cool, however when drought is also introduced plants reduce their stomatal aperture in an effort to reduce water loss, which in turn increases temperatures within the leaf. This increase greatly perturbs cellular homeostasis and the activities of enzymes, membranes, and cellular homeostasis. A recent study in the perennial grass *Leymus chinensis* indicates high temperatures, combined with drought stress, reduces the function of PSII, weakens nitrogen anabolism, increases protein degradation, and provokes the peroxidation of lipids (Xu and Zhou, 2006).

Drought and high light drastically inhibit light reactions and damage the thylakoid membrane proteins in young plants of *Hevea* (Annamalainathan *et al.*, 2006, 2010). It has been reported that a partial shade provides a photo protective role against photo inhibition in young rubber plants during summer (Nair *et al.*, 2002). The percentage inhibition in photosynthetic activity in drought stressed *Hevea* plants as compared to the irrigated controls was as low as around 30% in shade grown plants and as high as 51% in open light grown plants (Annamalainathan *et al.*, 2006). Several studies have shown that various components of photosynthetic metabolism are very sensitive to drought stress in *Hevea* (Jacob *et al.*, 1999, Devakumar *et al.*, 2002).

Photochemical efficiency is commonly affected by stress conditions such as water deficit, low or high temperatures along with high irradiances (Aro *et al.*, 1993, Long *et al.*, 1994). There is more pronounced effect of low temperatures on the photochemical efficiency of plants of tropical origin than those from temperate climate (Allen and Ort, 2001). *Hevea brasiliensis* is a tree species originally belonging to the tropical humid climate and thus being vulnerable to sub-optimal temperatures (Jacob *et al.*, 1999, Alam and Jacob 2002, Devakumar *et al.*, 2002, Ray *et al.*, 2004). Lowering the temperature generally reduces metabolic rates and can therefore limit the sinks for the absorbed excitation energy, particularly CO_2 fixation and other reductive processes including photorespiration (Huner *et al.*, 1998). The highly reduced state of PSII reaction centre can be considerably oxidized if CO_2 assimilation is increased and /or the excess energy is dissipated from the chloroplast as heat (Fryer *et al.*, 1998). Therefore, allocation of photosynthetic electrons to CO_2 assimilation and other reductive processes becomes important under stress.

If the water status of plant is insufficient, the plants experience water deficit, also known as drought or water stress. Water stress also caused due environmental stress like low temperature or salinity stress. All of these different stresses negatively impact on plant productivity. Salinity stress is major factor of abiotic stress which adversely affects crop productivity and quality.

Saline soil is characterized by folic level to chloride and sulfate of sodium (Hirt and Shinozaki, 2004). Salinity stress problem is induced due to irrigation and salt quantity accumulation. It is harmful to plant due to courses nutritional constraints by decreasing uptake phosphorus, potassium, nitrate and calcium, ion cytotoxicity and osmotic stress. Under salinity stress ions like Na^+ and Cl^- interfere with hydration shell of protein (Hirt and Shinozaki, 2004).

Other environmental factors

Heavy metals are defined as metals with a density greater than 5 g cm^{-3}. However, only a limited number of these elements is soluble under physiological conditions and therefore, may become available to living cells. A very small quantity may be used for the metabolism of plants as micronutrients or trace elements (Fe, Mo, Mn, Zn, Ni, Cu, V, Co, W, and Cr) and become toxic when in excess. Some metals in soil such as Hg, As, Ag, Sb, Cd, Pb and U are highly toxic to plants (Hirt and Shinozaki, 2004). There is a growing concern about the increased release of heavy metals in the environment. The sources of heavy metals include traffic, garbage and sewage sludge. The bioavailability of heavy metals in plants is specific and depends on the demand for specific metals as micronutrients and plant capacity to actively regulate the mobilization of metals by exuding organic acids or protons in the rhizosphere. In addition, soil properties influence the chemical mobility of metals, thus regulating its release to the soil solution (Hirt & Shinozaki, 2004).

A common response to exposure to heavy metals is a significant reduction in plant growth (Sanita di Toppi and Gabrielli, 1999). Normal growth is the result of cell division, elongation and differentiation also including programmed cell death in certain tissues such as xylem. The excess of heavy metals affects root functions on various levels and causes the accumulation of abscisic acid (ABA). In the whole plant, roots are the main site access for heavy metals. In general, a large fraction of cadmium (Cd) or copper (Cu) is retained by the roots and only a relatively small amount (about 10%) are transported to the shoot (Liao et al., 2000). The cytokinins act as antagonists to Cd, indicating that the internal hormonal status can critically affect plant tolerance to heavy metals.

Metals like zinc, iron and copper are essential micronutrients required for a wide range of physiological processes in all plant organs for the activities of various metal-dependent enzymes and proteins. However, they can also be toxic at elevated levels. Metals like arsenic, mercury, cadmium and lead are nonessential and potentially highly toxic. Once the cytosolic metal concentration in plant turns out of control, phytotoxicity of heavy metal inhibits transpiration and photosynthesis, disturbs carbohydrate metabolism, and drives the secondary stresses like nutrition stress and oxidative stress, which collectively affect the plant development and growth (Kramer and Clemens, 2005).

Plants have developed a complex network of highly effective homeostatic mechanisms that serve to control the uptake, accumulation, trafficking, and detoxification of metals. Components of this network have been identified continuously, including metal transporters in charge of metal uptake and vacuolar transport; chelators involved in metal detoxification via buffering the cytosolic metal concentrations; and chaperones helping delivery and trafficking of metal ions (Clemens, 2001).

Potassium supplement was found to enhance the tolerance of young plants to transient drought. Plants supplemented with potassium maintained higher leaf water potential and chlorophyll index compared to control when subjected to water stress (Prasannakumari *et al.,* 2010). The response of rubber to K application varied considerably. Earlier studies were conducted in newly cleared forest lands with adequate supply of native K and no response or negative response to K application was observed in these studies. Positive response to K application was recorded at lower levels of K application and under low available K status of the soil, indicating the relatively low K requirement of rubber trees for growth and yield (Jessy, 2011).

Drought tolerance mechanism

Heat shock response is a universal phenomenon characterized by the enhanced expression of a specific evolutionarily conserved genes; highlighted by

accelerated synthesis of defense proteins upon exposure to unfavorable conditions. Plant resistance to drought has been divided into escape, avoidance and tolerance strategies (Turner, 1986). Some major tolerance mechanisms including ion transporters, osmoprotectants, free radical scavengers, late embryogenesis abundant proteins (LEA) and factors involved in signaling cascades and transcriptional control are essentially significant to counteract the stress effects (Wang *et al.,* 2004).

Under drought, the maintenance of leaf turgor may also be achieved by the way of osmotic adjustment in response to the accumulation of proline, sucrose, soluble carbohydrates, glycine betaine, and other solutes in cytoplasm improving water uptake from drying soil, is known as osmotic adjustment. Accumulation of low molecular compounds, such as glycine betaine, sugars, sugar alcohols and proline, is a mechanism aimed at balancing water potential following drought condition (Pilon-Smits *et al.*, 1995).

Among solutes, proline is the most widely studied because of its considerable importance in the stress tolerance, can act as a signaling molecule to modulate organelle functions, influence cell proliferation or cell death and trigger specific gene expression, which can be essential for plant recovery from stress (Szabados and Savoure, 2009). It influences protein solvation and preserves the quarternary structure of complex proteins, maintains membrane integrity under dehydration stress and reduces oxidation of lipid membranes or photo inhibition (Demiral and Turkan, 2004). Furthermore, it also contributes to stabilizing sub-cellular structures, scavenging free radicals, and buffering cellular redox potential under stress conditions (Ashraf and Foolad, 2007). Dehydration avoidance is considered to be an adaptive strategy whereby plants decrease transpiration and modulate water extraction in order to retain water in the tissues and in the soil (Blum, 2009). These processes are mainly co-ordinated by non-hydraulic signals, such as abscisic acid (Parent *et al.*, 2009; Tardieu *et al.*, 2010). ABA stimulates osmotic adjustment (Ober and Sharp, 1994), induces the synthesis of protective proteins (*LEA* and related proteins) (Bray, 1993) and it

has also been shown to induce the expression of various water stress-induced genes.

Stress proteins

Generally the induced proteins called stress proteins, ranging in size approximately from 15-110 KDa in molecular weight. Some of these proteins are constitutive, while others have been found to be expressed under a variety of cellular stresses including heavy metals, high temperature, drought and light mediated oxidative stress. The paramount functions and consequences of the production of stress proteins comprise repair of the induced damage and protection of the host organism from stress. These defensive proteins are known as the heat shock proteins (Hsps) due to their initial discovery in cells exposed to hyperthermia, represents a group of specific proteins which are synthesized primarily in response to heat shock in almost all the biological systems. HSPs are generally divided into two classes: high-molecular-mass HSPs (60–110 kDa) and low-molecular-mass HSPs (15-30 kDa) (Vierling, 1991; O'Connell, 1994). The low-molecular-mass HSPs, which are encoded by a large gene family, are the most abundant HSP class found in plants (Vierling, 1991; Waters *et al.,* 1996).

The vast majority of stress proteins are molecular chaperons that maintain the structural integrity of proteins and enzymes by directing proper folding of nascent and partially unfolded protein or timely degradation of grossly damaged protein. According to Boudet *et al.,* (2006) dehydrins, a group of heat-stable plant proteins produced during late embryogenesis and believed to play a protective role during cellular dehydration (Campbell and Close, 1997), stabilize membranes (Suprunova *et al.,* 2004) act as chaperons or by other means buffer the altered solvent properties inside water stressed cells.

When a cell is under stress the amount of molecular chaperons increased and they bind the exposed surface and protect damaged protein from aggregation and loss. Rokka *et al.,* (2001) have suggested that Rubisco activase protein protects chloroplast protein synthesis from drought stress as a molecular chaperone. In accordance with Medrano *et al.,* (1997) the amount of

Rubisco protein is slightly affected by moderate and even prolonged severe drought. Heat shock is characterized by responses that are mediated at the level of transcription (Sun *et al.*, 2002) leads to the synthesis of HSPs, while synthesis of most other normal proteins is suspended.

The optimal condition for HSP induction in higher plants is a drastic temperature shift to 37-40^{0}C. However, HSP also can be induced if there is a gradual temperature increases. HSP synthesis can be detected within 20 minutes of heat shock and the increase in transcript level of some HSP genes within 3 to 5 minutes (Key *et al.*, 1985). Heat shock transcription factor (HSF) activation that facilitates transient production of HSPs is well-characterized process in acquired thermo tolerance (Larkindale *et al.*, 2005). In plants high temperature induces SUMO 1/2 conjugation to peptide inferring that sumoylation may be a response to high temperature (Miura *et al.*, 2005).

The small heat shock proteins (sHSP) are low molecular mass HSPs (12-40 KDa). In plants sHSPs form a more diverse family than other HSPs/chaperons with respect to sequence similarity, cellular locations and functions. Small HSPs are synthesized ubiquitously in prokaryotic and eukaryotic cells in response to heat and other stresses, whereas some sHSPs are expressed during certain developmental stages. Small HSPs share a conserved 90-aminoacid C-terminal domain called the α-crystalline domain (ACD) related to a domain from the vertebrate α-crystalline proteins of the eye lenses. They have the high capacity to bind non-native proteins, probably through hydrophobic interaction, stabilize and prevent non-native aggregation there by facilitating their subsequent refolding by ATP- depending chaperons such as the Dna K system. Recent studies indicate that sHSPs play an important role in membrane quality control and thereby potentially contribute the maintenance of membrane integrity especially under stress conditions.

A consistently over expressing chloroplast sHSP has been reported in Hevea (Annamalainathan *et al.*, 2006). The amino acid sequence revealed that *Hevea* sHSP (23 KDa) is a novel protein and does not reported in other species as the sequencing is only partial with other reported sHSPs (Annamalainathan *et al.*,

2006). In chloroplast, the sHSP have been implicated in protecting chloroplast from photo inhibitory and photo oxidative stress by preventing aggregation and stabilizing thylakoid membrane (Torok *et al.*, 2001). It has been demonstrated that the chloroplast sHSPs play a direct role in stabilizing PS II oxygen evolution complex protein during heat stress and there by promote the maintenance of PS II electron transport.

Early reports of heat shock proteins in higher plants demonstrated HSP in tobacco and soybean cells grown in solution culture (Barnett *et al.*, 1980) to late the incorporation of radioactive precursors into plants. HSP has been identified in barley (Belangert *et al.*, 1986), peas (Mansfield and Key, 1987), tomato (Nover *et al.,* 1983), carrot (Pitto *et al.,* 1983), mung bean (Chen, Kamisaka and Masuda, 1986), barley (Belatiger, Brodl and Ho, 1986; Mansfield and Key, 1987), *Tradescantia* (Xiao and Mascarenhas, 1985), *Gladiolus* cormels (Ginzburg and Salomon, 1986), *Lilium longiflorum* (Hong-Qi, Croes and Linskens, 1984), corn (Cooper and Ho, 1984; Mansfield and Key, 1987),cotton (Burke *et al.,* 1985), wheat (Key *et al.,* 1983; Mansfield and Key, 1987), millet (Key *et al,* 1983; Mansfield and Key, 1987), sunflowers (Schoffl and Baumann, 1985), sorghum (Ougham and Stoddart, 1986), rice and *Panicum miliaeeutn* (Mansfield and Key, 1987).

The optimal induction temperature for the heat shock response varies between species, but generally occurs from 10^0C to 15^0C above the temperature required for optimal plant growth. The primary structure of HSP is highly conserved during evolution, providing the basis for their important function in normal cells physiology as well as in stress condition. Some HSPs are known as the molecular chaperons that reduce protein denaturation, target denatured protein for proteasome degradation, facilitate protein folding necessary for proper maturation or renaturation, and regulate activity of HSPs to control HSP gene expression during thermo tolerance acquisition (Lee and Vierling, 2000; Kim *et al* ., 2002).

Thermal adaptation responses include membrane compositional changes necessary for maintenance of functional integrity and production of heat shock proteins (HSPs) necessary for cellular protection (Larkindale *et al.*, 2005). It is

natural and widely held assumption that the purpose of the heat shock response is to protect the organism from damaging effect of heat and other forms of stresses. There is a great deal of evidences to support this notion. Several appropriate role of heat shock proteins have been suggested (Neha–Sahir *et al.*, 2005). The alteration of membrane functions at elevated temperature has been suggested (Cooper *et al.*, 1984).

The involvement of HSP (Ubiquitin) in proteolysis is one of best known function of HSPs. The role of HSP in induction of cross-tolerance in plants has been studied. Moderate heat shock has been found to protect against many other kinds of stress in a number of species (Kuznestov *et al.*, 1999). Chloroplastic sHSP, HSP21 protects photo system II under oxidative stress conditions but it also involved in plastid development (Nata-Sahir *et al.*, 2005). Recent study has reported that chloroplast sHSPs also protect photosynthetic electron transport from inhibitory effects of heavy metals (Heckathorn et *al.*, 2006).

The magnitude of the expression level of a sHSP (23 KDa) was relatively higher in drought tolerant *Hevea* clones that indicate probable role of this protein in abiotic stress tolerance (Annamalainathan *et al.*, 2010). The stress tolerance traits in these rubber clones have been analyzed by various photosynthetic parameters. There was a significant reduction in photosynthetic oxygen evolution rate in the leaves of drought imposed plants. On the contrary dark respiration of leaf was increased during early drought period. Further the maximum potential (PSII) and effective quantum yield of PSII (ΦPSII) and electron transport rate were drastically inhibited in drought imposed plants. However, the clones RRII 430 and RRIM 600 had recorded relatively a small inhibition in ΦPSII and photosynthetic rate as compared to other clones and the finding has been attributed to their inherent drought tolerant characters. The clones RRII 105 and PB 260 were shown to be drought susceptible as determined from their photosynthetic parameters and expression level of sHSP (Annamalainathan *et al.*, 2010).

MATERIALS AND METHODS

Plant material and Growth Chamber Condition

The experimental plants were raised in polybag at the Rubber Research Institute of India's (RRII), Kottayam, Kerala. Budded stumps of a popular clone namely, RRII 105 were planted in medium size (25 x 45 cm) polythene bags. The plants were grown under normal field conditions (twenty plants per treatment) in open sunlight for seven months. During eighth month a set of plants was transferred to a plant growth chamber (CONVIRON, Canada) and 50% of plants were imposed with drought stress by withholding irrigation for five days during April-May of the year 2012 and another 50% plants were kept as irrigated controls. Concomitant with drought condition temperature stress was also imposed by keeping different sets of plants under 30, 35 and 40°C for 5 days each. The day time light conditions were 400 μmole m^{-2} s^{-1} for first two hours (6-8 am) in the morning followed by 800 μmole m^{-2} s^{-1} till 2 pm. After noon there was a decline in light intensity to 400 μmole m^{-2} s^{-1} till 6 pm. Night time was maintained without any light inside the growth chamber for 12 hours (6pm-6am). The RH was set at 75% inside the growth chamber throughout the study period. For biochemical and chloroplast protein analyses leaf samples were collected after 5 days of withholding irrigation.

Measurement of Water Potential

The water potential of the leaf was measured before sampling (for pigment analysis and photosynthesis) by using Psypro water potential system-Wescor (435-752-6011). Psychrometer measures the water vapor pressure of a solution or plant sample, on the basis of the principle that evaporation of water from a surface cools the surface. The sample chambers of Wescor system were taken to the growth chamber and the collected leaf discs were immediately transferred to the chambers, transported to lab and then observations were taken.

Estimation of leaf chlorophyll content

Chlorophyll *a, b* and total chlorophyll contents were estimated by the method of Arnon (1949). The chlorophylls were extracted in Acetone: Dimethyl sulphoxide (1:1) solution. Leaf discs of 100mg were weighed and put into 1:1 ratio of Acetone: Dimethyl sulfoxide (DMSO). It was allowed to stand overnight with frequent shaking. Filtered supernatant was read at 645 and 663nm.

Calculations:

Chlorophyll *a*: $((12.7 \cdot A_{663})-(2.69 \cdot A_{645}) / 1 \times 1000 \times wt\ (mg)) \times Volume$

Chlorophyll *b*: $((22.9 \cdot A_{645})-(4.68 \times A_{663}) / 1 \times 1000 \times wt\ (mg)) \times Volume$

Total Chlorophyll: $((20.2 \times_{645} +8.02 \times_{663}) / 1 \times 1000 \times wt\ (mg)) \times Volume$

Estimation of carotenoids

The carotenoids contents were estimated by the method of Lichenthaler (1987). The total carotenoids were extracted in Acetone: Dimethyl sulphoxide (1:1) solution. The following calculations were done using the formula:

$((1000 \times A_{470})-(1.82 \times Ca)-(85.02 \times Cb))/198$

Statistical analysis

The values between irrigated control and drought imposed samples were tested for significance by Student's test'.

Estimation of proteins

The protein content was estimated by the method of Lowry *et al* (1951). The following reagents were used:

Solution A: 2% Na_2CO_3 in 0.1 N NaOH.

Solution B: 0.5% $CuSO_4$ solution in 1% NaK (Sodium Potassium Tartarate.)

Solution C: 50 ml of Sol. A + 1ml of Sol. B.

Solution D: Foiln: phenol reagent (1:1).

Procedure:

Alkaline copper reagent (Solution C) was taken in a test tube and added a known aliquot of protein in buffer or SDS. Then 0.5ml Folin phenol (1:1) reagent was added. Absorbance was read at 660nm. Protein amount was calculated by using BSA as the standard.

Assay of quantum yield of PS II

The chlorophyll fluorescence measurements were made following standard technique as proposed by Schreiber *et al.,* (1998). Chlorophyll fluorescence parameters namely, maximal fluorescence under light exposure (Fm), steady state fluorescence at any given time (Fs) and minimal fluorescence immediately after light exposure (Fo), effective PSII quantum yield(ΦPS II) efficiency of excitation energy capture by open PS II reaction centre were measured by using PAM 2000 (Walz Germany), (Schreiber *et al.,*1998).

Isolation of Chloroplasts

Type II broken chloroplast were isolated by the method of Reeves and Hall (1973). Fresh leaf sample was ground with liquid nitrogen in a mortar and pestle. The powdered leaf sample was added with 5 ml of grinding buffer and transferred to a centrifuge tube. The homogenate was centrifuged at 500g for 2 minutes. The pellet represented unbroken cells and tissue was removed and the supernatant was spun at 3500g for 5 minutes and the resulting pellet was suspended in 1 ml of Tris buffer as chloroplast suspension.

Protein preparation for SDS-PAGE:

Chloroplasts were precipitated with 10% TCA and left on ice for 30 min before centrifugation to collect the pellet. A trace of TCA left behind in the pellet was removed by three washing in ice cold acetone. The final pellet was air dried and solublized in a small amount of 10% of SDS to which equal volume of sample buffer was added. The samples were boiled for 2 min and centrifuged at 3000 x g for 5min to remove unsolublized materials. Chloroplast proteins were dissolved in 10% SDS and quantified by the method of Lowry *et al.,* (1951).

SDS-PAGE analysis of proteins

Analysis of chloroplast protein was carried out by SDS-PAGE according to the method of Laemmle (1970) using a 10% linear gel. The composition of the various solutions is as follows.

a. Sample buffer (for 10ml)

0.5 M Tris-Hcl, pH 6.8	2.5 ml
Beta-mercaptoethanol	2.5 ml
Glycerol	2.5 ml
1% bromophenol blue	1.25 ml
Distilled water	1.25 ml

b. Separation gel buffer (for 30ml)

Acrylamide (30%)	12 ml
0.5 M Tris-HCl, pH 8.8	7.2 ml
Distilled water	10 ml
10% SDS	0.3 ml
10% APS	0.15 ml
TEMED	10 µl

c. Stacking gel buffer (for 10ml):

Acrylamide (30%)	1.35 ml
0.5 M Tris-HCl, pH 6.8	3.0 ml
Distilled water	5.5 ml
10% SDS	0.1 ml
10% APS	0.05 ml
TEMED	5 µl

d. Acrylamide stock (30%):

Acrylamide	30.0 g

N, N-methylene bisacrylamide	1.6 g
Distilled water added to make up to 100 ml	

e. Running buffer:

50 mM Tris-Hcl, pH 8.3	3.0 g
Glycine	14.3 g
SDS	1.0 g

Preparation of separating gel:

A linear gel of 1.5 mm thickness was prepared by adding 30% of acrylamide solution followed by 0.5 M Tris-HCl, distilled water, 10% SDS, 10% APS and TEMED.

Preparation of stacking gel:

The stacking solution was layered over the separating gel after inserting a comb and was allowed to polymerize. Protein samples were mixed with equal volume of sample buffer and heated to 100 ^{0} C for 3 min. After cooling to room temperature the samples were centrifuged at 10, 000 g for 2 min. The supernatant was loaded on the gel and was run at 50 V till the samples cross the stacking layer. Then the voltage was increased to 120 V. Electrophoresis was carried out at 20 ^{0} C.

Staining and Destaining:

The gel after electrophoresis was immersed in staining solution. The stain was prepared by dissolving 500 mg of coomassie brilliant blue (sigma) in 80 ml of methanol, 100 ml of distilled water and 20 ml of glacial acetic acid. The gel was stained for 6 h and destained with 40% methanol and 10% acetic acid mixture for 12 h. The destained gel was preserved in 7% acetic acid solution.

The destained gel was documented with the help of Bio Imaging system. The relative intensity of the stress protein bands in the drought samples were compared with respective control chloroplast samples.

RESULTS AND DISCUSSION

Young plant establishment in marginally suitable non-traditional rubber growing areas like central India and Konkan region is a major constraint. In the present scenario of changing climate young rubber plants are more likely to be subjected to abiotic stresses like soil moisture deficit, high temperature and high solar light conditions due to failure or delay of monsoon even in tradition rubber belts. Therefore, a study was taken up to understand the interactive effects of drought and high temperature stresses in young rubber plants. The present study was conducted during summer season of 2012 at RRII, Kottayam. Young plants of a popular high yielding rubber clone namely, RRII 105 were planted in medium size poly bags in the field. During the study period plants were transported to a growth chamber (CONVIRON, Canada) and kept at different temperature regimes; 30, 35 and 40^{0}C. Drought was imposed in one set of plants by withholding irrigation for 5 days at each temperature regimes while another set was irrigated at saturated level.

Effects of drought and high temperature stresses on plant morphology

Different sets of plants were kept at 30, 35 and 40^{0}C with and without irrigation for 5 days. Under 30^{0}C there was no much visible difference in the foliage appearance between irrigated and drought imposed plants except a minor indication of flaccid leaves. Those plants kept at 35^{0}C showed a slight degree of chlorophyll bleaching and drooping of leaves at the end of 5th day of drought. On the contrast plants kept at 40^{0}C showed a drastic bleaching of leaf lamina, drooping and defoliation in the lower whorls (Fig.1). These observations indicated high temperature stress aggravates the drought effects in young rubber plants.

Leaf water potential

Leaf water potential was observed in irrigated and drought imposed plants before photosynthetic measurement and sample collections for biochemical analysis. When compared to the irrigated plants, there was a

decline (more negative) in leaf water potential in drought imposed plants under all temperature conditions (Fig.2). High temperature (35 and 40^{0}C) grown plants recorded a drastic reduction in water potential than ambient temperature (30^{0}C) (Fig.2). This indicates the sensitivity of mesophyll cell's water potential to the increased temperature. Generally high temperature influences the water loss through elevated transpiratory rate, there by more negative tissue water potential. High temperature during day time can have direct damaging effects associated with hot tissue temperatures or indirect effects associated with the plant-water-deficits that can arise due to high evaporative demands. Evaporative demand exhibits near exponential increases with increases in day-time temperatures and can result in high transpiration rates and low plant water potentials (Hall, 2001).

Exposure of plants to drought led to noticeable decreases in leaf water potential and relative water content with a concurrent increase in leaf temperature. The higher leaf water potential and relative water content as well as optimum leaf temperature are associated with a higher photosynthetic rate. Drought stressed plants displayed higher canopy temperature than well-watered plants at both vegetative and anthesis growth stages. Leaf water potential is considered to be a reliable parameter for quantifying plant water stress response. Certain genotypes maintain better leaf water potential under drought periods. Singh *et al.,* (1990) observed significant differences in water potential among wheat genotypes under drought stress.

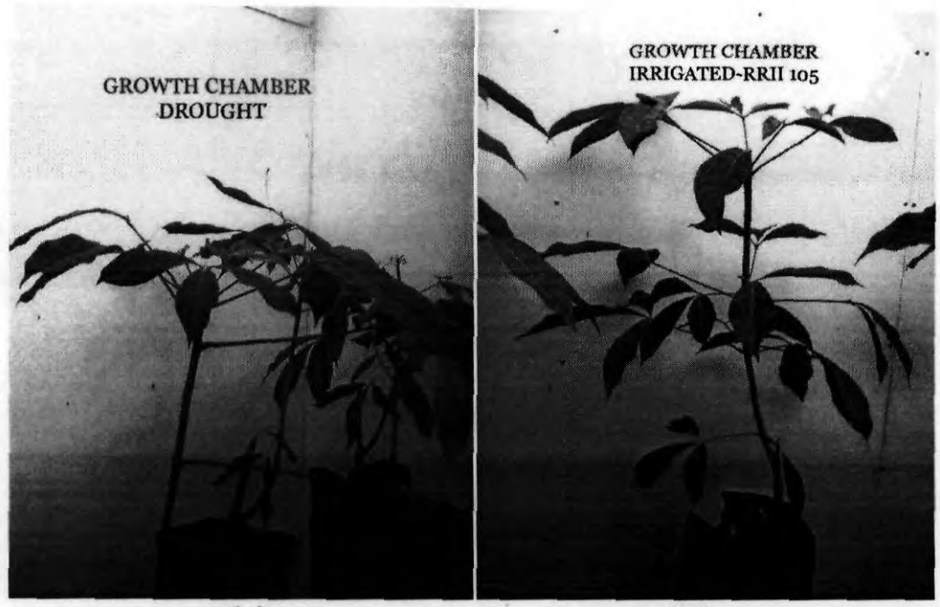

Fig.1

Young plants of *Hevea* (clone RRII 105) grown in poly bags with saturated irrigation (b) and drought imposed (a) at 40°C. Drought was imposed by withholding irrigation for 5 days under growth chamber conditions (Details given in materials and methods).

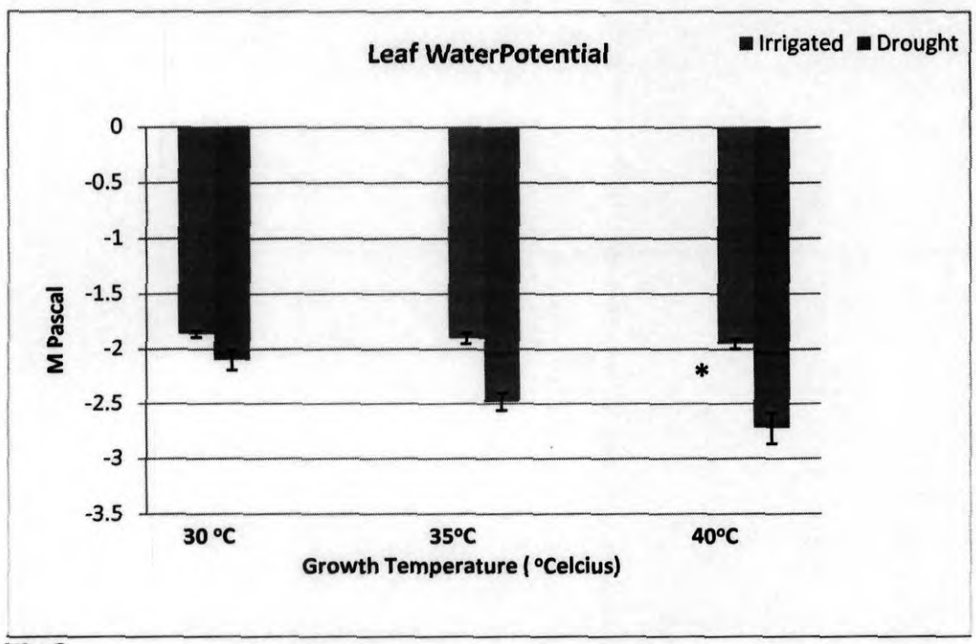

Fig.2

Leaf water potential (M Pa) of irrigated and drought imposed young plants of *Hevea* at different temperature (° C) regimes. Drought was imposed by withholding irrigation for 5 days under growth chamber conditions. * indicates the values are significantly different at 5% level.

Photosynthetic pigment contents

The photosynthetic pigments namely chlorophyll *a*, *b* and total carotenoids were estimated in irrigated and drought imposed plants at 30, 35 and 40^{0}C. Drought imposed plants showed a marginal decline in chlorophyll *a* and *b* content (Fig.3). When the temperature regimes increased from ambient to 35 and 40^{0}C both chlorophyll *a* and *b* contents were drastically reduced in drought imposed plants. The reduction in the level of chlorophyll *a* and *b* was reflected in total chlorophyll content (Fig.4). There was a drastic reduction of chlorophyll pigments content at 40^{0}C than in plants grown at 30 and 35^{0}C. Interestingly there was a marginal reduction of chlorophyll *b* and total chlorophyll contents in plants grown at 40^{0}C even under irrigated conditions. Carotenoids also seem to be more sensitive to drought coupled with high temperature conditions. When the growth temperature increased the magnitude of leaf carotenoid reduction also increased under water deficit condition (Fig.5).

Drought stress coupled with high temperature resulted in photo oxidation of chlorophyll and carotenoids pigment. Drought mediated oxidative stress and production of reactive oxygen species (ROS) and free radicals inflict lipid peroxidation and bleaching of pigments in photosynthetic apparatus (Smirnoff, 1993; Asada, 1999). Carotenoids are the important accessory pigments of photosystems. A vital role of carotenoids on photosynthetic tissues is photoprotection by quenching the triplet state of chlorophyll and scavenging for singlet oxygen. This function is associated with the ability of the carotenoid molecule to participate in photochemical reactions such as singlet-singlet energy, triplet-triplet energy, oxidation, reduction and isomerization (Frank and Cogdell, 1993; Koyama, 1991). A second essential function of carotenoids is that of acting as accessory light-harvesting pigments, as their presence in pigment-protein complexes (PPCs) in the thylakoid membrane (Young, 1993; Frank and Cogdell, 1993).

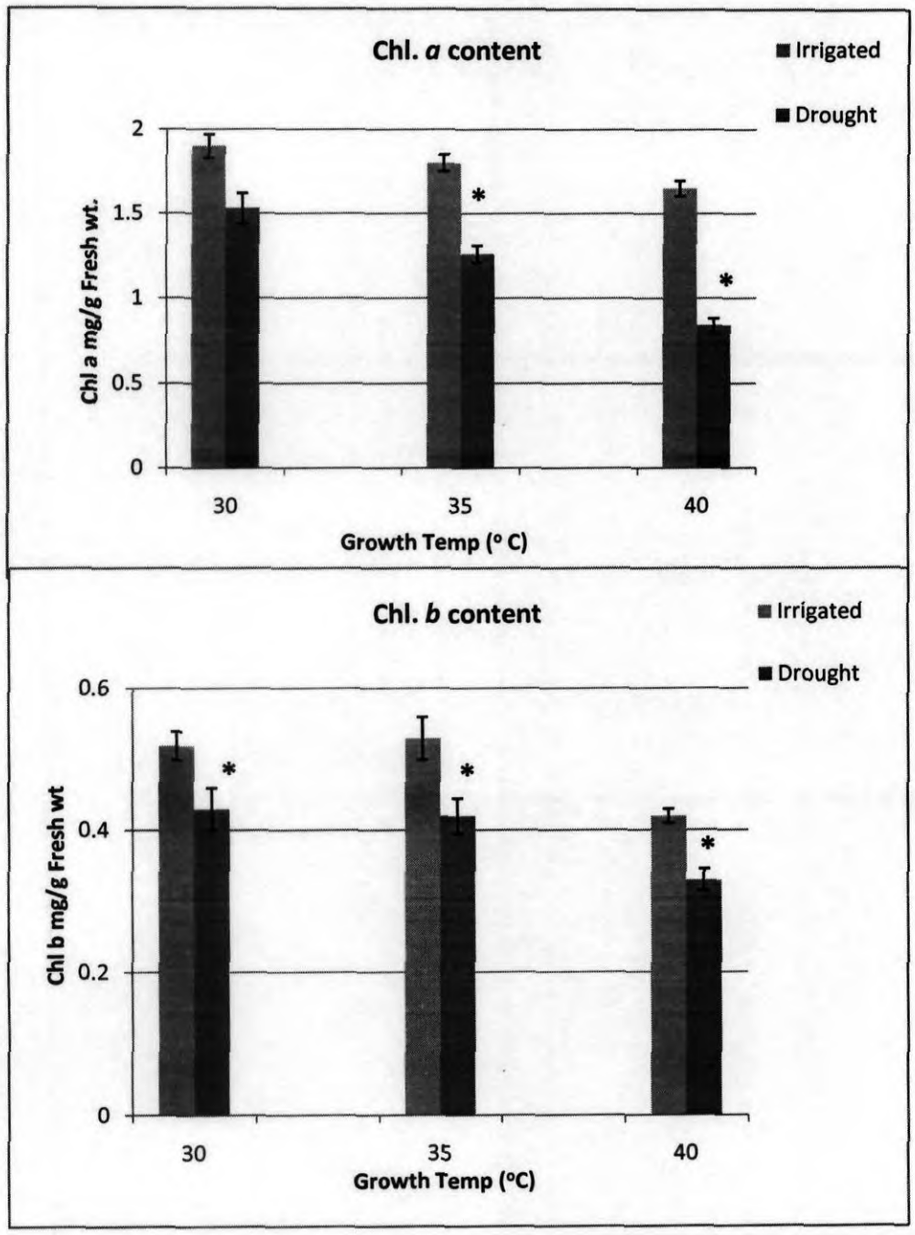

Fig.3

Leaf chlorophyll *a* and chlorophyll *b* contents of irrigated and drought imposed young plants of *Hevea* grown at different temperature (° C) under growth chamber conditions. * indicates the values are significantly different at 5% level.

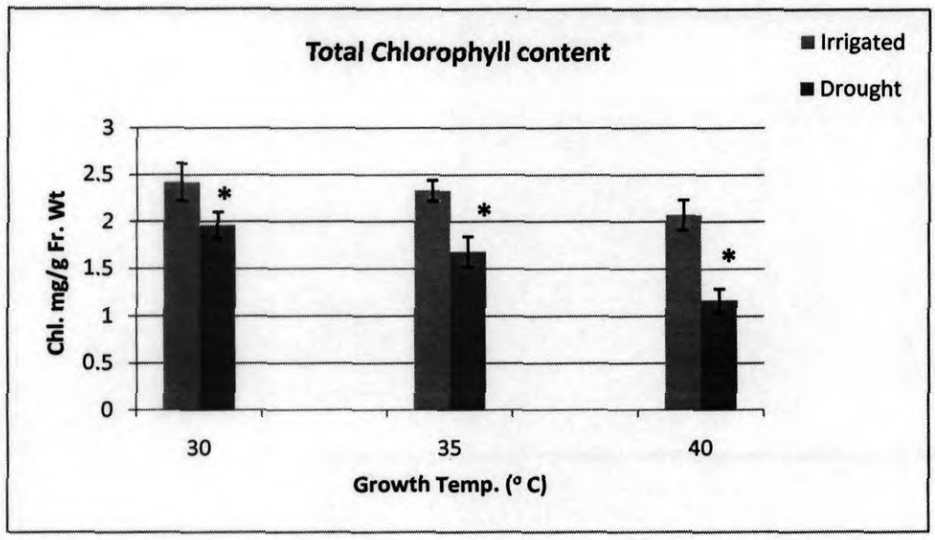

Fig.4

Total chlorophyll contents of irrigated and drought imposed young plants of *Hevea* grown at different temperature (° C) under growth chamber conditions.

* indicates the values are significantly different at 5% level.

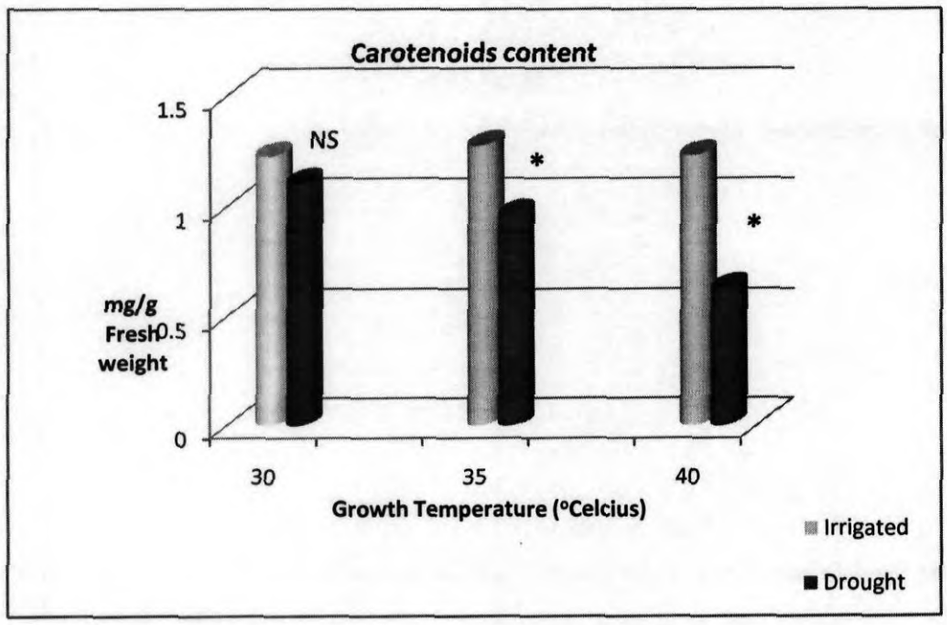

Fig.5

Leaf carotenoids content of irrigated and drought imposed young plants of *Hevea* grown at different temperature (° C) under growth chamber conditions. * indicates the values are significantly different at 5% level.

Carotenoids also play a major role in dissipation of excess electrons as non photochemical quenching (NPQ) through xanthophyll cycle (Demmig-Adams and Adams, 1992). The mechanism of xanthophyll cycle involves the enzymatic removal of epoxy groups from xanthophylls to create so-called de-epoxidised xanthophylls. This reduces the amount of energy that reaches the photosynthetic reaction centers. Non-photochemical quenching is one of the main ways of protecting against photoinhibition. In higher plants both carotenes and xanthophylls are found in leaves, with the same four major Carotenoids, β carotene, β cryptoxanthin, zeaxanthin, antheraxanthin and lutein 5,6-epoxide. There are only three pigments that are active in the xanthophyll cycle; violaxanthin, antheraxanthin and zeaxanthin. During light stress violaxanthin is converted to zeaxanthin via the intermediate antheraxanthin, which plays a direct photo protective role acting as a lipid-protective antioxidant and by stimulating non-photochemical quenching within light harvesting pigment-proteins. This conversion of violaxanthin to zeaxanthin is done by the enzyme violaxanthin de-epoxidase, while the reverse reaction is performed by zeaxanthin epoxidase (Wright *et al.,* 2011). Carotenoids like β carotene, a key scavenger of reactive oxygen species such as singlet oxygen and so protect thylakoid membrane from oxidative damage (Young, 1991). Environmental factors such as light intensity, including sun/shade adaptation, temperature and photobleaching also have profound effects on carotenoid levels (Young, 1993).

Photosystem II activity

The photosystem II (PSII) activity in chloroplast is known to be a sensitive photochemical reaction influenced by environmental parameters. Therefore, the magnitude of impairment of PSII activity indicated the level of responses of plants to the drought and other abiotic stresses. Among the irrigated plants there was no much reduction in ϕPSII activity when plants were grown under 30 and 35^{0}C (Fig. 6). When the plant growth temperature increased to 40^{0}C there was a significant reduction in ϕPSII even in irrigated plants. After drought imposition the magnitude of reduction of ϕPSII was small at 30^{0}C and very high at 40^{0}C

(Fig.6). This result indicated that PSII activity is more sensitive to water deficit with concomitant occurrence of high temperature. The cumulative effects of these environmental stresses were obviously seen in the present study with young *Hevea* plants.

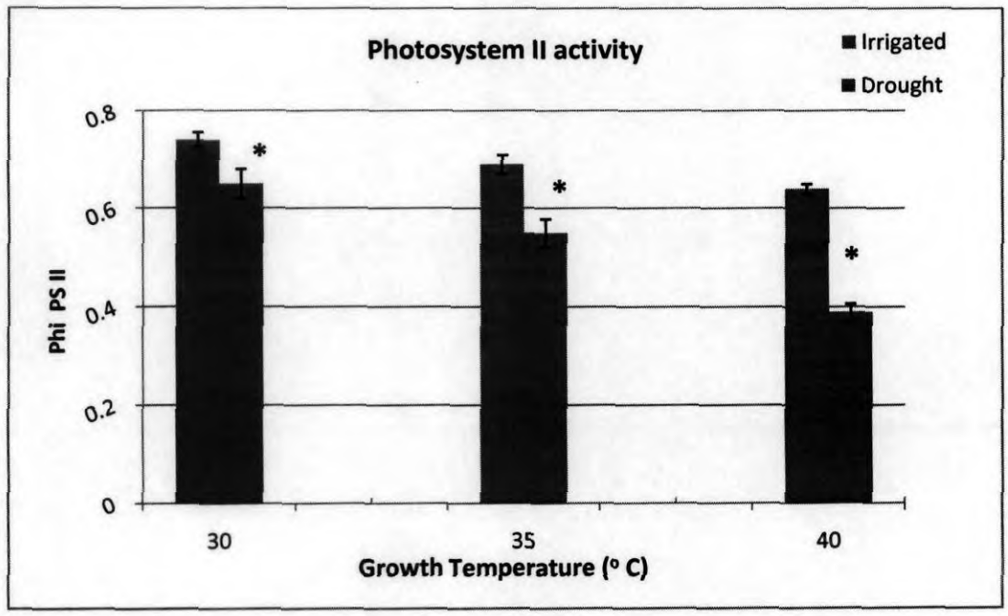

Fig.6

Effective quantum yield of PS II (ΦPS II) in irrigated and drought imposed young plants of *Hevea* (clone RRII 105) grown at different temperature (° C) under growth chamber conditions. * indicates the values are significantly different at 5% level.

Photosynthetic carbon reduction and carbon oxidation cycles are the main electron sink for PSII activity during mild drought (Cornic and Fresneau, 2002). Within PSII the O_2 evolving complex proteins are frequently the most susceptible to heat stress, although both the reaction centre and the light-harvesting complexes can be disrupted by high temperatures as well (Havaux, 1992). Sensitivity of photosynthesis to heat mainly due to damage to components of photosystemII located in the thylakoid membranes of the chloroplast and membrane properties (Al-Khatib and Paulsen 1999). The quantum yield of PSII as related to Calvin cycle metabolism is reduced only under drastic water deficit in some species. Long term drought mediated reduction in water content of

tissue led to considerable depletion of pea PSII core. The decline in PSII efficiency is probably a regulatory mechanism serving a photo protective role. Increased levels of energy dissipation which decrease ΦPSII may help to protect PSII from over excitation and photo damage (Schindler and Lichtenthaler., 1994). It has been reported that there was a significant reduction in photosynthetic oxygen evolution rate in the leaves of drought imposed *Hevea* plants. On the contrary dark respiration of leaf was increased during early drought period. Further the maximum potential (PSII) and effective quantum yield of PSII (ΦPSII) and electron transport rate were drastically inhibited in drought imposed plants. However, certain clones like RRII 430 and RRIM 600 recorded relatively a small inhibition in ΦPSII and photosynthetic rate as compared to other clones and the finding had been attributed to their inherent drought tolerant characters. The clones RRII 105 and PB 260 were shown to be drought susceptible as determined from their photosynthetic parameters and expression level of sHSP (Annamalainathan *et al.*, 2010).

Chloroplast protein profile

SDS PAGE analysis of chloroplast protein from irrigated and drought imposed plants at different temperature was carried out. The protein profile showed induction of thylakoid membrane protein with a molecular mass of 23 KDa under drought condition. The magnitude of induction was lesser at 30^{0}C and it was very prominent at 35^{0}C (Fig.7). The induction of stress protein was seems to be many fold higher at 35^{0}C. In order to compare the response of *Hevea* plants for the stress protein induction chloroplast isolated from plants exposed to different temperature regimes (30, 35 and 40^{0}C) and extracted proteins were resolved in SDS PAGE (Fig.8). In this profile also the drought plants under 35^{0}C showed a greater level of 23 KDa stress protein than at 30 and 40^{0}C. The prominent expression of 23KDa at 35^{0}C indicates the stress protein induction under drought condition was aggravated at high temperature. However, the protein expression was not very clear at 40^{0}C probably due to severe degradation of thylakoidal proteins under very high temperature. The expression of 23 KDa stress protein was reported as chloroplast small heat shock proteins (sHsps) in *Hevea* under drought conditions (Annamalainathan *et al.*, 2006, 2010).

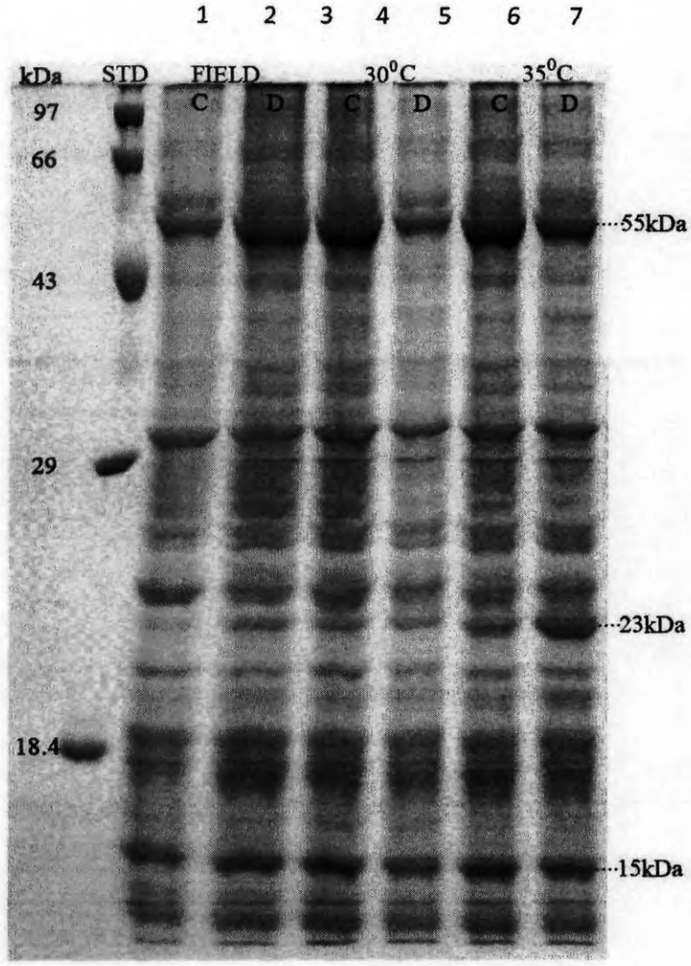

Fig.7

SDS PAGE profile of chloroplast protein from irrigated (C) and drought (D) imposed *Hevea* plants grown under different temperatures, 30 and 35°C inside a growth chamber(Lanes4-7). Drought was imposed by withholding irrigation for 5 days in growth chamber. Lanes 2 and 3 are chloroplast protein profile of polybag plants from an open field with (C) and without irrigation (D). The Rubisco protein (50 and 15 K Da) and 23 K Da chloroplast stress proteins were indicated. The standard molecular weight markers (Lane 1) were noted in the left side.

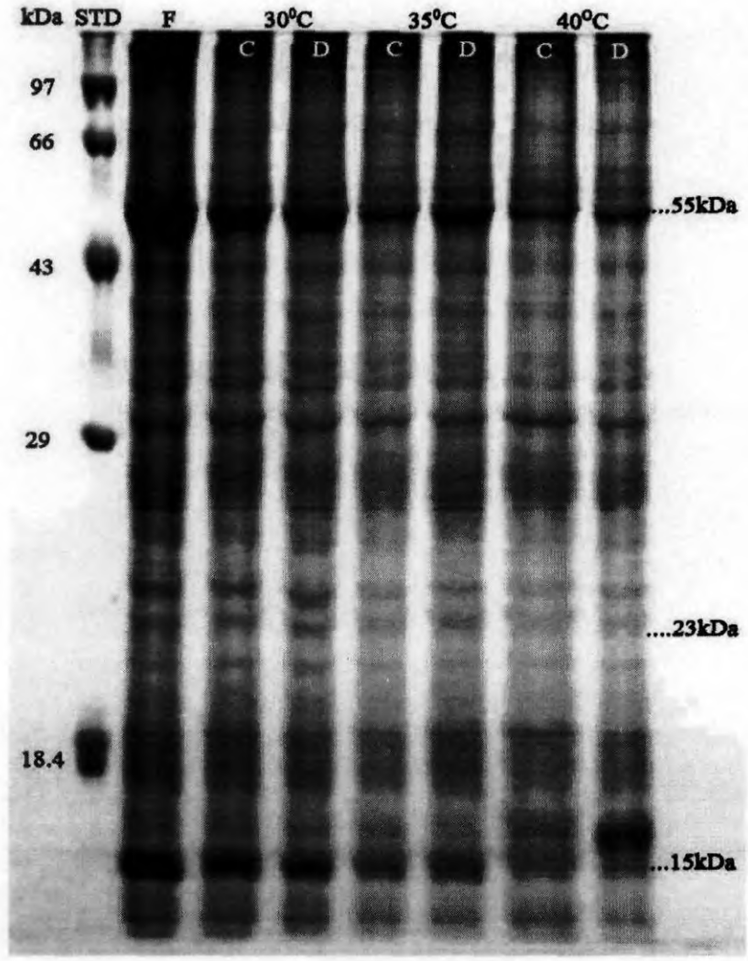

Fig. 8

SDS PAGE profile of chloroplast protein from irrigated (C) and drought (D) imposed young rubber plants grown under different temperatures; 30, 35 and 40°C inside a growth chamber. Drought was imposed by withholding irrigation for 5 days. Lane 2 (F) indicates the chloroplast protein profile of field grown plants (for comparison) during summer. The Rubisco protein (50 and 15 K Da) and 23 K Da chloroplast proteins were indicated. The standard molecular weight markers (Lane 1) were noted in the left side.

When young *Hevea* plants exposed to drought condition an important protein, namely, the large subunit of Rubisco was shown to be degraded even at 30 and 35^{0}C (Fig.7) inside growth chamber. Further high temperature treatment was extended up to 40^{0}C, in that condition both large (LSU) and small sub units (SSU) of Rubisco were shown to be degraded. Even the level of Rubisco enzyme was lesser in irrigated control plants grown at 40^{0}C when compared to ambient 30^{0}C (Fig.8). However, the protein profile showed there was no much reduction in Rubisco content when drought imposed at field condition.

Heat shock proteins (Hsps) and other stress proteins have been known to protect cells against deleterious effects of stress (Feder *et al.*, 1999, Young, *et al.*, 2004). Hsps and their cognates are found in every organism at ordinary growth temperature and play an important role in cellular functions related with growth (Waters *et al.*, 1996). The major stress proteins occur at low to moderate levels in cells that have not been stressed but accumulate to very high levels in stressed cells (Young, *et al.*, 2004). Hsps are characterized as structurally unstable proteins. They serve important physiological functions in plants. These functions of Hsps are closely related to resistance to heat and the other stresses (Ray *et al.*, 1999, Iba *et al.*, 2002). Plants probably synthesize middle level Hsps at mild heat stress conditions at first, but if heat stress continues they synthesize more Hsps (Ahn *et al.*, 2004). A decrease of Hsp expression level after induction is observed with age. The main reason seems to be a lower capacity to up regulate expression at an older age. A chloroplastic 22 kDa Hsp from Chenopodium album, which is localized in thylakoid lumen, interacts specifically with the thermo labile oxygen evolving complex of PSII. Therefore protecting it from heat stress damage but fails to reactivate the heat denatured PSII (Heckathorn *et al.*, 1999, Sun *et al.*, 2002). In experiments with heat stress had been released, the sHsps were shown to be quite stable with half-lives of 30-50 h, further suggesting that sHsps may be important for recovery as well.

Small heat shock proteins are ubiquitous proteins found throughout all plant species. Chloroplast sHsps are a subclass of the sHSP family also present in endoplasmic reticulum, mitochondrion and cytosol. The small heat shock

proteins (sHSP) are low molecular mass HSPs (12-40 KDa). The HSPs are present within chloroplast as large oligomers containing 9 more subunits and are actively synthesized during heat stress (Suzuki *et al.*, 1998). In plants sHSPs form a more diverse family than other HSPs/chaperons with respect to sequence similarity, cellular locations and functions. They are synthesized ubiquitously in prokaryotic and eukaryotic cells in response to heat and other stresses, and some are expressed during certain developmental stages. They have the high capacity to bind non-native proteins, probably through hydrophobic interaction, and to stabilize and prevent non-native aggregation there by facilitating their subsequent refolding by ATP- depending chaperons such as the Dna K system. Recent studies indicate that small heat shock proteins play an important role in membrane quality control and thereby potentially contribute the maintenance of membrane integrity especially under stress conditions.

Heckathorn *et al.*, (2002) have reported that this protein is involved in the protection of PSII when the plants experience abiotic stresses. In chloroplast the sHSPs have been implicated in protecting this organelle from photo inhibitory and oxidative stress by preventing aggregation and stabilizing the thylakoid membrane (Torok *et al.*, 2001). It has been demonstrated that the chloroplast sHSPs plays a direct role in stabilizing the photo systemII (PSII) oxygen evolution complex (OEC) proteins during heat stress and there by promotes the maintenance of PSII electron transport. This protein was also implicated in protective mechanism in plants experiencing oxidative stress by undergoing oxidation dependent conformational changes in the molecular structure. Thus sHSPs are appear to be general stress proteins in chloroplast that are involved in maintaining function and survival of this organelle during stress or facilitating recovery from stress. The amino acid sequence revealed that *Hevea* sHSP (23 KDa) is a novel protein and does not reported in other species as the sequencing is only partial with other reported sHSPs (Annamalainathan *et al.*, 2006).

In the present study the level of expression of 23 KDa proteins was greater in those plants which were subjected to water deficit stress with high temperature. The stress protein was identified as heat shock protein and it was

greatly evident as the magnitude of induction seems to be very high under high temperature. This indicated that sHSP has a role in stress protection, most probably protection of thylakoid membrane against water deficit induced oxidative stress and membrane damage (Heckathorn *et al.,* 1999).

Concluding Remarks

The present study was carried out to understand the cumulative effects of soil moisture deficit and high temperature stresses on the physiology of young plants of natural rubber. The popular *Hevea* clone, RRII 105, when imposed with drought at ambient temperature there was no much reduction in photosynthetic pigments and photosystem II activity. There was no much difference in chloroplast protein profile between control and drought plants also at 30^{0}C. When the growth temperature increased, there was a drastic reduction of chlorophylls, carotenoids and photosystem II activity. A chloroplast stress protein (23 KDa, sHSP) was found over expressed at 35^{0}C under drought condition. The damaging effect of drought on the photosynthetic apparatus was further aggravated by high temperature.

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