STUDIES ON COLD TOLERANCE OF HEVEA BRASILIENSIS (MULL.ARG) UNDER CONTROLLED CONDITION

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Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled, "**Studies on cold tolerance of *Hevea brasiliensis(mull.arg)* under controlled condition**" is an authentic record of the project work done by MS.Remya.B at Rubber Research Institute of India, Kottayam, under the guidance of Dr.Jayanta Sarkar, Scientit B, Crop Physiology Division, Rubber Research Institute of India, Kottayam, in partial fulfillment of the requirement for the award of the Degree 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.

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This is to certify that the project work entitled "**Studies on cold tolerance of *Hevea brasiliensis* (Muell. Arg.) clones under controlled conditions**" submitted to Mahatma Gandhi University, Kottayam by **Ms. Remya B** in partial fulfillment for the award of the Degree of **Master of Science in Biochemistry** was carried out under my supervision and guidance in the Crop Physiology Division, Rubber Research Institute of India, Kottayam, Kerala. It is also certified that this work has not been presented for any other degree or diploma elsewhere.

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DECLARATION

I do hereby declare that the dissertation entitled **"Studies on cold tolerance on *Hevea brasilliensis (Mull.Arg.)*clones under controlled condition"** 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. Jayanta Sarkar, Scientist B at Crop Physiology Division, 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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CONTENTS

ABSTRACT

Shortage of available land ensuing from competition with other crops led rubber plantation to be extended to marginal and subtropical environments that are prominently located in northeast India. Low temperature strongly affects the development and latex production of rubber trees in addition to low growth. In the present study four *Hevea brasiliensis* clones viz., RRII 105, RRIC 100, SCATC 88/13 and Haiken 1 were exposed to cold stress under controlled growth chamber conditions. Morphological symptoms were notice very prominently like yellowing and drying of leaves in RRIC100 followed by RRII 105 and less prominent on SCATC 88/13 and Haiken 1. Clone Haiken 1 followed by SCATC 88/13 protected the photosynthetic pigments from bleaching and less destruction of carotenoids noticed in Haiken 1. Maximum photochemical efficiency of PSII (Fv/Fm) was recorded in SCATC 88/13 followed by Haiken 1. Maximum photosystem II activity (ΦPSII) was noticed in SCATC 88/13 followed by Haiken 1 after cold exposure for 72 hours. Maximum lipid peroxidation as a result of ROS activity was found in RRII 105 and RRIC 100, which indicates that these two clones are susceptible to cold stress. Two stress proteins were found in the chloroplast protein profile of Haiken 1 (control and cold exposed) and SCATC 88/13 (control) that may be playing any role in conferring tolerance to these two clones.

INTRODUCTION

INTRODUCTION

Hevea brasiliensis, the Pará rubber tree, often simply called rubber tree, is a brevi-deciduous tree belonging to the family Euphorbiaceae and is the most economically important member of the genus *Hevea*. It is the most important commercial source of natural rubber – a product of vital importance recovered from its sap-like extract, latex. Natural rubber however, has been found in the latex of over 2000 species of plants belonging to 311 genera of 79 families. It is a native of the Amazon river basin of South America. It was introduced to tropical Asia in 1876 through Kew Garden in the UK with the seeds brought from Brazil by Sir Henry Wickham. The tree is now grown in the tropical regions of Asia, Africa and America

The rubber tree is sturdy, quick growing and tall. It grows on many types of soils, provided they are deep and well drained. A warm humid climate (21–35°C) and a fairly distributed annual rainfall of not less than 200 cm are necessary for the optimum growth. It however grows successfully under slightly varying conditions also. Rubber trees have a well developed tap root and laterals. The bark on tapping yields latex occurs in latex vessels in the bark, mostly outside the phloem. These vessels spiral up the tree in a right-handed helix which forms an angle of about 30 degrees with the horizontal, and can grow as high as 45 ft. The cambium, in between the wood and the bark, is responsible for the increase in girth of tree including bark renewal

The young plants show characteristic growth pattern of alternating period of rapid elongation and consolidated development. The leaves are trifoliate with long stalks. Normal annual leaf fall of mature tress known as wintering occurs during the period of December to February in South India. Refoliation and flowering follow wintering. Pollination is by insects. The fruits mature in about five months after pollination. They are three seeded, each weighing 4 to 6 g. The seeds belonging to a clone have characteristic size, shape and seed coat mottling which are helpful in their identification (Rubber Grower's Companion, 2011). The economic life period of rubber trees in plantations is around 32 years – up to 7 years of immature phase and about 25 years of productive phase. Rubber has various uses which include eraser, shoes, tires and surgical instruments, mechanical and engineering goods etc. Thailand is the largest land of Rubber plantation in the world. India is one among the top ten rubber producing countries. Kerala state is leading rubber plantation state in India.

Plants experience a variety of environmental stresses during their lifespan. Low temperature is the major factor limiting the productivity and geographical distribution of many species, including important agricultural crops. Shortage of available land ensuing from competition with other crops led rubber plantation to be extended to marginal and subtropical environments that are prominently located in northeast India, highlands and coastal areas of Vietnam, southern China and Southern plateau of Brazil. Low temperature, typhoon and dry periods are frequently encountered in these areas (Priyadarshan and Goncalves, 2003). Among these factors, low temperatures (between 0 to 10 °C) strongly affect the development and latex production of rubber trees in south central China and northeastern states of India (Priyadarshan et al., 2005). In addition to damage to low growth, the lowest temperatures are responsible for the stopping of latex production for 1-3 months per year (Rao et al., 1998; Jacob et al., 1999). Therefore, high attention should be paid to the selection of plants with enhanced tolerance to low temperature. However, the lack of methods for early evaluation of the cold tolerance and the time-cost of breeding programmes of tree such as rubber are challenging.

Hence, keeping these points in view, the present study was undertaken to find out the basis of cold tolerance of four *Hevea* clones under controlled environmental conditions with the following objectives.

1. To study the level of cold tolerance of *Hevea* clones under controlled conditions mimicking the cold prone non-traditional regions of rubber cultivation.

2. To find out the basis of cold tolerance based on physiological and biochemical parameters.

3. To study the level of expression of chloroplast stress protein and its role in cold tolerance of young plants of *Hevea*.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Rubber tree is grown for the production of natural latex. Commercial plantations displayed over several continents of the world: Asia, Africa and South America, but the main region is growing South-East Asia. Expansion of rubber growing in the traditional areas, the country has been compelled to move into non-traditional, low and mid country intermediate zones, where effects of moisture constraints, high and low temperatures can be still more serious to rubber productivity.

Abiotic stress

Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment which is produced by natural factors such as extreme temperatures, wind, drought, and salinity with little control over abiotic stresses. It is very important for us to understand how stress factors affect plants and other living things so that we can take some preventative measures.

Abiotic stress is essentially unavoidable, which affects animals, but plants are especially dependent on environmental factors, so it is particularly constraining and is the most harmful factor concerning the growth and productivity of crops worldwide. Research has also shown that abiotic stressors are at their most harmful when they occur together, in combinations of abiotic stress factors.

Abiotic stress comes in many forms. The most common of the stressors are the easiest for people to identify, but there are many other, less recognizable abiotic stress factors which affect environments constantly. The most basic stressors include: high winds, extreme temperatures, drought, flood, and other natural disasters, such as tornados and wildfires. The lesser-known stressors generally occur on a smaller scale and so are less noticeable, but they include rock content and pH, high radiation, compaction, contamination, and other, highly specific conditions like rapid rehydration during seed germination.

Abiotic stress, as a natural part of every ecosystem, will affect organisms in a variety of ways. Although these effects may be either beneficial or detrimental, the location of the area is crucial in determining the extent of the impact that abiotic stress will have. The higher the latitude of the area affected, the greater the impact of abiotic stress will be on that area.

So, a taiga or boreal forest is at the mercy of whatever abiotic stress factors may come along, while tropical zones are much less susceptible to such stressors plant's first line of defense against abiotic stress is in its roots. If the soil holding the plant is healthy and biologically diverse, the plant will have a higher chance of surviving stressful conditions.

Facilitation, or the positive interactions between different species of plants, is an intricate web of association in a natural environment. It is how plants work together. In areas of high stress, the level of facilitation is especially high as well. This could possibly be because the plants need a stronger network to survive in a harsher environment, so their interactions between species, such as cross-pollination or mutualistic, become more common actions to cope with the severity of their habitat. This facilitation will not go so far as to protect an entire species, however. For example, cold weather crops like rye, oats, wheat, and apples are expected to decline by about 15% in the next fifty years and strawberries will drop as much as 32% simply because of projected climate changes of a few degrees. Plants are extremely sensitive to such changes, and do not generally adapt quickly.

Plants also adapt very differently from one another, even from a plant living in the same area. When a group of different plant species was prompted by a variety of different stress signals, such as drought or cold, each plant responded uniquely. Hardly any of the responses were similar, even though the plants had become accustomed to exactly the same home environment. Rice (*Oryza sativa*) is a classic example. Rice is a staple food throughout the world, especially in China and India. Nowadays, due to climate change rice plants experience different types of abiotic stresses, like drought and high salinity. These stress conditions have a negative impact on rice production. Genetic diversity has been studied among several rice varieties with different genotypes using molecular markers.

Cold tolerance depends up on a plant's ability to keep water from leaving its cells and freezing, which severely dehydrate the cells. Adverse environmental conditions such as extreme temperature are detrimental to plant growth and development and thus affect the productivity of various crops around the world.

Many of the important food crops such as corn, sorghum, tomato, soybean and rice, originally of tropical and subtropical origin, are new cultivated in areas were the temperature fall well below optimum required for their normal growth and development. Therefore improvement in vegetative growth and yield performance of any of these crops will require

selection of hybrid that have more efficient growth habit under unfavorable stress conditions (Greaves 1996). However the traditional plant breeding method used to select for cold tolerant varieties are cumbersome and sometimes result in a compromise between selection for efficient growth and high yield.

Any physiological, biochemical, and genetic changes that may be involved in improving cold tolerance in all of these food crops. Increasing evidence suggest that oxidative stress is involved in mediating oxidative stress in mediating chilling injury and tolerant mechanism. Cold or oxidative stress is necessary step towards the eventual goal of achieving cold tolerant plant.

As a consequence of natural selection, plants are natively adapted to the temperature extreme that occur in the environment. Remove them from that environment and they may or may not survive. For example banana kept at low temperature, which suffers an imbalance in their metabolism and kills their cells.

Once the gene of interest are identified and cloned, gene transfer technology can be used to induce foreign gene in to crop plant to improve cold tolerance. To study mechanism of injury or tolerance, most studied have utilized comparison of metabolic differences between chilling sensitive and chilling tolerant varieties as model system (Jhanke, Hull, and Lang,1991;Walker and Mc Kersie 1993, Pinhero et al 1997) The disadvantage with such system is that complexes genetic differences exist between sensitive and (page 61 to be type)

Cold or low temperature stress

Stress in physical term is defined as mechanical force applied to an object. In response to applied stress, an object undergoes change in dimension which is also known as strain. Biological stress is an adverse condition which inhibits normal functioning and well being of a biological system. Cold stress is a serious threat to the sustainability of crop yields. Indeed, cold stress can lead to major crop losses. Various phenotypic symptoms in response to cold stress include poor germination, stunted seedlings, yellowing of leaves (chlorosis), reduced leaf expansion and wilting, and may lead to death of tissue (necrosis). Cold stress also severely hampers the reproductive development of plants.

The major negative effect of cold stress is that it induces severe membrane damage. This damage is largely due to the acute dehydration associated with freezing during cold stress. Cold stress is perceived by the receptor at the cell membrane. Then a signal is transuded to switch on the cold-responsive genes and transcription factors for mediating stress tolerance.

Understanding the mechanism of cold stress tolerance and genes involved in the cold stress signaling network is important for crop improvement. Here, I review cold stress tolerance mechanisms in plants. The major points discussed are the following: (1) physiological effects of cold stress, (2) sensing of cold temperatures and signal transduction, and (3) the role of various cold-responsive genes and transcription factors in the mechanism of cold stress tolerance. Environmental variables, especially those affecting water availability and temperature, are the major determinants of plant growth and development.

Oceans and the low-temperature Polar regions occupy almost 80% of the Earth's surface. Only one-third of the total land area is free of ice, and some42% of this land regularly experiences temperatures below 20°C.In such areas, plants require specialized mechanisms to survive exposure to low temperature. Most temperate plant species have evolved a degree of cold tolerance, the extent of which is typically dependent on a combination of the minimum temperature experienced and the length of exposure to cold stress. Variation in tolerance level can be genetically determined, as well as being affected by plant developmental stage and physiological status at the time of exposure. A number of major temperate crop species are sown in the autumn and winter hardiness – can be broken down into a number of simpler components, one of the most important of which is frost tolerance. Identification of the key genes underlying cold stress has thus become a major priority in the search for improved crop winter hardiness.

A deeper understanding of the regulation of these genes, and of their response to low-temperature stress, would allow clarification of the ways in which plants adjust to the stress. The plant response to low-temperature stress can be divided into three distinct phases. The first is cold acclimation (pre-hardening), which occurs at low, but above zero temperatures. The second stage (hardening), during which the full degree of tolerance is achieved, requires exposure to a period of sub-zero temperatures.

The final phase is plant recovery after winter (Li et al. 2008). Some plants (especially trees) need a combination of short photoperiod and low temperature to fully develop their cold tolerance. In these cases, tolerance can be lost if the temperature is raised above zero and the photoperiod is lengthened. Plant organs differ in their level of tolerance, typically the roots are much more sensitive than the crown. Which is understandable given that the crown is the site of the major meristem responsible for production of new roots and shoots at the end of the cold period.

Cold stress and chlorophyll fluorescence

Chlorophyll fluorescenceis light that has been re-emitted after being absorbed by the Chlorophyll molecules of plant leaves. By measuring the intensity and nature of this fluorescence, plant ecophysiology can be investigated. Light energy that has been absorbed by a leaf will excite electrons in chlorophyll molecules. Energy in photosystem II can be converted to chemical energy to drive photosynthesis (photochemistry). If photochemistry is inefficient, excess energy can damage the leaf. Energy can be emitted (known as energy quenching) in the form of heat (called non-photochemical quenching) or emitted as chlorophyll fluorescence. These three processes are in competition, so fluorescence yield is high when less energy is emitted as heat or used in photochemistry. Therefore, by measuring the amount of chlorophyll fluorescence, the efficiency of photochemistry and non-photochemical quenching can be assessed. The fluorescence emitted from a leaf has a longer wavelength than the light absorbed by the leaf.

Low temperature is one of the most important factors that may limit photosynthetic activity. This is evident in high altitudes and latitudes, where winter conditions impose severe restrictions to plant function and growth (Schaberg *et al.*, 1995; Hansen *et al.*, 1996).

Cold stress and photosynthesis

Photosynthesis is the corner stone of physiological process and the basis of dry production in plants. Photosynthetic rate is an important parameter characterizing the plant because it is a determinant of crop yield and light use efficiency. Photosynthesis is regulated through the control of leaf area and leaf senescence and daily duration and extent of stomatal opening. These provide a crop with substantial flexibility. Plants have a spare capacity for

photosynthesis as protection against multiple environmental stresses. Among internal factors, leaf photosynthetic rate is the basis but not the sole factor. Leaf area is an important factor determining crop yield. a close association with leaf area and yield is observed, when planting density is not high. Many species have a significant negative correlation between leaf size and photosynthetic rate per unit leaf area. Leaf functional duration is also important factor affecting yield.

When the photosynthesis yield relation is analyzed, the partitioning of the photosynthesis to different organs, expressed as partitioning coefficient or harvest index which is defined as the ratio of yield biomass to the total cumulative biomass at harvest must be considered. The size of respiration loss is closely related to crop yield selection for low leaf respiration has led to yield increases in various crop (Barber 1998) for most crops more than half of the economic yield is derived from photosynthesis after flowering.

Therefore, photosynthesis at the reproductive stage is more directly related to yield size. The positive correlation between photosynthesis and yield is observed mostly at the stage. Example of photosynthetic processes affected by low temperatures include enzymatic step of the electron transport chain in chloroplast thylakoids (Bruggemann and Dauborn 1993), coupling to photo phosphorulation, enzymes in the carbon reduction cycle and transport mechanisms of the photosynthetic products from the chloroplasts (Long, 1983; Oquist, 1983). It has been demonstrated that chilling of sensitive plants in light is much more damaging to the photosynthetic apparatus than chilling in darkness(Wise and Nylor , 1987 ;Krause, 1988; Mishra, and Singhal ,1993).Chilling under even moderate light intensities is more damaging to plants than chilling in darkness is primarily attributed to the process of chilling induced photoinhibition. Under non-chilling condition, photoinhibition occur with over reduction of the primary electron acceptor of photosystem II by strong illumination.

Lipid peroxidation

Lipid peroxidation is widely used stress indicator of plant membrane. Malondidaldehyde (MDA) concentration is a widely used method to analyse lipid peroxidation in biological materials. In plant tissue, however, certain compounds (anthocyanin, carbohydrate) may interfere with measurements which may leads to an over estimation of the MDA level. The major lipid class of plant tissue is sterols, steryglucosides,

glucocerebrocides, and phospholipids, and the proportions of free sterols and phospholipids increase during cold acclimation .Qualitative changes in the levels of fatty acids also occur with cold acclimation, leading to unsaturation of the fatty acids and the subsequent protection. The method of plant membrane against freezing stress. It is widely used as a stress indicator of plants. A widely used stress described by Health and Packer is the basic protocol used or adapted in numerous studies dealing with lipid peroxidation. This method is very convenient assay which is both quick and easy to perform. It is based on Malondialdehyde (MDA) production during the oxidation of poly unsaturated fatty acids. The reaction between MDA and thiobarbituric acid (TBA) yields a reddish colour, which peaks at 532 nm.

It is also known that ROS trigger lipid peroxidation in cellular membranes (Fridovich, 1978; Halliwell and Gutteridge, 1986). Unsaturated fatty acids are easily peroxidized by hydroxyl radical that are converted from super oxide (Fridovich,1978; Halliwell and Gutteridge, 1986 ; Cadens,1989). Reduced cellular and membrane damage (lipid peroxidation)in plants has been linked to increased antioxidant defense system activity against ROS (Dhindsa and Mattowe, 1981; Senartna, McKersie and Borochov, 1987; Leprince et al., 1990).

Correlative evidence from different studies also suggests that the chilling sensitivity of plants increase with increased lipid peroxidation (Shewelft and Erickson,1991; Prasad,1996). In maize seedling, lipid peroxidation was increased by about two fold in non acclimated seedling, compared to the control or acclimated seedling, during 4°C stress and recovery (Prasad, 1996). Since seedling pretreated with Hydrogen peroxide and menadione, a superoxide generating compound, also increased lipid peroxidation, it was suggested that the induced lipid peroxidation was due to the accumulated ROS in non-acclimated seedling during chilling stress (Prasad, 1996).

On the other hand, reduced lipid peroxidation in acclimated seedling was due to an increased antioxidant defense system that scavenged ROS during acclimation followed by chilling stress (Prasad, 1996). All of these studies suggest that ROS – induced lipid peroxidation was at least in parts, responsible for increased chilling sensitive in maize seedling and, perhaps, all of the chilling sensitive crop plants. If the stress were too strong, the defense system of plants could not remove the more production of ROS effectively and result in severely damage to plants or even death.

Both biotic and abiotic stresses are known to induce oxidative stress, which increases lipid peroxidation in plants (Kondo, Miyazawa, and Miitzutani, 1992; Prasad, 1996). Methyl jasmonate and other derivatives of lipids peroxidases are known to act as intracellular signals that induce plant defense mechanisms (Doke et al., 1994; Dorases et al., 1995; Rickauer et al., 1997). Methyl jasmonate was shown to induce chilling tolerance in rice seedling (Lee et al., 1996). Treatment of tobacco suspension solutions with high doses of SA resulted in rapid lipid peroxidation that in turn, was responsible for PR – 1 gene expression (Conrath, Silva, and Klessing, 1997). These studies suggest a potential role for lipid peroxidase in mediating gene expression in relevance to plant defense mechanisms.

Heat shock proteins related to low temperature stress

Heat shock proteins (HSP) are a class of functionally related protein involved in the folding and unfolding of other proteins. Their expression is increased when cells are exposed to elevated temperatures or other stress. This increase in expression is transcriptionally regulated. The dramatic up-regulation of the heat shock proteins is a key part of the heat shock response and is induced primarily heat shock factor (HSF).

HSPs are found in virtually all living organisms, from bacteria to human. Heat- shock proteins are named according to their molecular weight. For example Hsp60, Hsp70 andHsp90 (the most widely-studied HSPs) refer to families of heat shock proteins on the order of 60, 70, and 90 kilodaltons in size, respectively. Small 8-kilodalton protein ubiquitin which marks proteins for degradation also has features of a heat shock proteins.

Maintaining proteins in their functional conformations and preventing the aggregation of non-native proteins are particularly important for cell survival under stress- Heat-shock proteins (Hsps) chaperones are responsible for protein folding, assembly, translocation and degradation in many normal cellular processes, stabilize proteins and membrane, and can assist in protein refolding under stress conditions by producing heat shock proteins (HSPs), conferring thermo tolerance, playing the role of molecular formations re establishing normal protein conformation and thus cellular homeostasis.

Plants vary greatly in the amount of expressed Hsps as well as their type (Hamilton *et al.*, 1999). The most studied species of plants is *Arabidopsis thaliana* where the response to heat shock treatment occurs through the participation of a number of different Hsps. Five major families of Hsps/chaperones are conservatively recognized the Hsp70 (DnaK) family; the chaperonins (GroEL and Hsp60); the Hsp90 family; the Hsp100 (Clp) family; and the small Hsp (sHsp) family. Higher plants have at least 20 sHsps and there might be 40 kinds of these sHsps in one plant species. It is believed that this diversification of these proteins reflects an adaptation to tolerate the heat stress. Transcription of heat-shock protein genes is controlled by regulatory proteins called heat stress transcription factors (Hsfs). Plants show at least 21Hsfs with each one having its role in regulation, but they also cooperate in all phases of periodical heat stress responses (triggering, maintenance and recovery). There are more than 52 plant species (including crop ones) that have been genetically engineered for different traits such as yield, herbicide and insecticide resistance and some metabolic changes. Major heat-shock proteins have some kind of related roles in solving the problem.

Presence of Hsps in higher plants was discovered in tobacco and soybean using cell culture technique (Barnett et al., 1980). When soybean was subjected to 40 °C for four hours, ten new proteins were found, but disappeared after 3 h treatment 28 °C (Key *et al.*, 1981). Studying the gene expression of Hsp90 in rice plant (*Oryza sativa*) indicated that the heat shock protein Hsp87 was present after 2 h of heat shock (from 28 to 45 °C), and its quantity was high and stable even after long heat stress (4 h) and the return to normal conditions (no stress). It was found, also, that Hsp90 (Hsp85 and Hsp87) could be induced by other kind of stress such as salinity, drought, and cold. This study reported the accumulation of different levels of these proteins in fifteen wild species of rice (Pareek et al.;1998)

Mechanism of cold tolerance

Species adapted by natural selection to cold environments have evolved a number of physiological and morphological means to improve survival in the face of extended cold periods (Guy 1999). Typically, these species – herbs, grasses and ground shrubs – are of short stature, have a low leaf surface area and a high root/shoot ratio. Their growth habit takes full advantage of any heat emitted from the ground during the day and minimizes night chilling, since air temperature is maintained most effectively near the soil surface . Cold-adapted plants tend to be slow growing, have the C3 mode of photosynthesis and store sugars in

underground tissues. Plants well adapted to cool environments have evolved an efficient respiration system, which allows them to rapidly mobilize stored reserves during the short growing season. The timing of developmental and physiological responses to environmental stress is under strict genetic control (Guy 1999). The two distinct strategies taken by plants to combat low temperature stress are avoidance and tolerance.

Overwintering temperate plant species acclimatize during autumn, during which their metabolism is redirected towards synthesis of cryoprotectant molecules such as soluble sugars, sugar alcohols (sorbitol, ribitol, inositol) and low-molecular weight nitrogenous compounds (proline, glycine betaine). These, in conjunction with dehydrin proteins (DHNs), cold-regulated proteins (CORs) and heat-shock proteins (HSPs), act to stabilise both membrane phospholipids and proteins, and cytoplasmic proteins, maintain hydrophobic interactions and ion homeostasis, and scavenge reactive oxygen species (ROS); other solutes released from the symplast serve to protect the plasma membrane from ice adhesion and subsequent cell disruption (Hare et al. 1998; Iba 2002; Wang et al. 2003; Gusta et al. 2004; Chen & Murata 2008). The process of solute release, especially of vacuolar fructans, to the extracellular space is a vesicle-mediated, tonoplast-derived exocytosis (Valluru & Van den Ende 2008).

MATERIALS AND METHODS

MATERIALS AND METHODS

Plant materials

Budded stumps of four clones of *Hevea* viz., RRII 105, RRIC 100, SCATC 88/13 and Haiken 1 were planted in large size (35 cm x 65 cm) polythene bags and raised at the Rubber Research Institute of India, Kottayam. The plants were grown under normal nursery conditions in open sunlight. One set of plants of each clone was maintained at a maximum temperature of 28°C during day time with a light intensity of 600μmol.m^{-2}.sec^{-1} and minimum night temperature of 20°C as control in growth chamber (CONVIRON make) and the relative humidity was maintained at 80% for 72 hours and after chlorophyll fluorescence measurements, leaf samples were collected. Another set was of each clone was exposed to cold stress. The cold exposure was given to the plant gradually over a period of five days and brought a low temperature of 20°C (max.) at day time and 8°C (min.) at night time with a RH of 50% for four days (Table 1). Chlorophyll fluorescence was measured and leaf samples were collected after 72 hours on fourth day of exposure. The leaves of these plants were kept for biochemical and chloroplast protein analysis.

Table 1: Growth chamber conditions: maximum and minimum temperatures and relative humidity of the treatments

Events	Maximum Temperature (°C)	Minimum Temperature (°C)	Relative Humidity (%)
Control	28	20	80
Gradual decrease	27	18	75
	26	16	70
	25	14	65
	24	12	60
	22	10	55
Cold exposure (Day 1)	20	8	50
Cold exposure (Day 2)	20	8	50
Cold exposure (Day 3)	20	8	50
Cold exposure (Day 4)	20	8	50

Measurement of chlorophyll fluorescence and PSII activity

The chlorophyll measurements were made following stand and technique as proposed by Schreiber et al. (1998). Chlorophyll florouscence parameters such as maximum fluroscence under light exposure (Fm), steady state flourescence at any given time (ΦPSII) and minimal flourescence immediately after light exposure (Fo). Effective PSII quantum yield (F PsII) efficiency of excitation energy capture by open PSII reaction were measured by using PAM 2100 (Walz, Germany).

Estimation of chlorophyll

Total chlorophyll contents were estimated by the method of Arnon (1949). The chlorophylls were extracted in acetone : dimethyl sulphoxide (1:1v/v) solution. Leaf discs of 100 mg were weighed and put into acetone : dimethyl sulphoxide (DMSO). It was allowed to stand overnight with frequent shaking. Filtered supernatant was read at 645 and 663nm.

Calculations:

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

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

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

Estimation of carotenoids

The carotenoid contents were estimated by the method of Lichenthaler (1987). The Total carotenoids were extracted in acetone : dimethyl sulphoxide (1:1) solution. It was allowed to stand overnight with frequent shaking. Filtered supernatant was read at 470nm.

Calculations:

Carotenoids: $((1000A_{470})-(1.82Ca)-(85.02Cb))/198$

Estimation of lipid peroxidation

Lipid peroxidation of each sample was estimated by malondialdehyde method.

Reagents:

50Mm K_2HPO4 and 50Mm KH_2PO4; PH adjusted to 7.0

8.1% SDS (Sodium dodecyl sulfate)

20% CH_3COOH (Acetic acid); PH adjusted to 3.5 with NaOH

0.8% TBA (ThioBarbituric Acid)

Extraction

0.3g leaf sample was extracted with 2.5ml potassium phosphate buffer and added little Buttylated hydroxyl toluene, centrifuged at 8000rpm for 20 minutes and the supernatant was taken for the test.

Procedure

Taken 0.7ml of extract, in to it add 0.2ml of 8.1 SDS, 1.5ml of 20% acetic acid and 1.5ml of 0.8%TBA. Heated at 95 for 60 minutes and taken the OD at 532 and 660nm.

Estimation of ascorbic acid

For estimation of ascorbic acid, the following reagents are used

4%Trichloro acetic acid (TCA)

2,4 Dimethyl hydrazine reagent (2%) in 9N H_2SO4

Thiourea (10%)

Sulphuric acid (85%)

Extraction

500mg of leaf sample was crushed with 10ml of 4% TCA and centrifuged at 2000rpm for 10 minutes.

Procedure

Ascorbic acid standards are taken as 0.2 to 1ml. 1ml 0f supernatant is taken for the test and the volume made up to 2ml with 4%TCA. To this add 0.5ml Dimethyl hydrazine reagent and 2 drop of thiourea. Then it was kept for incubation at 37°C for 3 hours. After incubation ozazone crystals are formed, which are dissolved in 2.5ml 85% H_2SO_4 in cold. These tubes are cooled in ice. Absorbance read at 540nm in spectrophotometer

Isolation of chloroplast protein

Type II broken chloroplast were isolated by the method of Reeves and Hall (1973). Fresh leaf sample (10 g) was crushed with liquid nitrogen in a mortar and pestle. To the powdered leaf sample 15ml chloroplast isolation buffer and a pinch of PVP was added. Then the homogenate was centrifuged at 700-800rpm for two one minutes. The pellet represented unbroken cells and tissue was removed and the supernatant was centrifuged at 3500 rpm for five minutes and the resulting pellet was suspended in 1ml of Tris buffer as chloroplast suspension.

Chloroplast were precipitated with 10% TCA and left on ice for 30 minutes 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 dissolved in small amount of 10% SDS to which equal volume of sample buffer was added. The samples were boiled for 2 minutes and centrifuged at 3000 x g for 5 minutes to remove unsolubulisd materials. Chloroplast proteins were dissolved in 10% SDS and quantified by the method of Lowry et al. (1951).

Estimation of protein

The protein content was estimated by Bradford (1976) method.

Bradford's reagents: 20mg Coomassie brilliant blue G-250 in 10ml of 95% ethanol, to this 20ml of 85% Orthophosphoric acid was added and the volume was made up to 200ml with distilled water and filtered through Whatman no.1 filter paper.

SDS-PAGE analysis of proteins

Analysis of chloroplast protein was carried out by SDS-PAGE according to the method of Laemmli (1970) using 10% linear gel.

The composition of the various solutions is as follows.

a) Sample buffer (10ml)

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

b) Separating gel buffer (30ml)

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

c) Stacking gel buffer (10ml)

Acrylamide(30%)	1.35ml
0.5M Tris-HCl, pH6.8	3.0ml
Distilled water	5.5ml
10%SDS	0.1ml
10% APS	0.05ml
TEMED	5ul

d) Acrylamide stock (30%)

Acrylamide	30g
N,N-methylene bisacrylamide	1.6g
Distilled water	100ml

e) Running buffer

50Mm Tris-HCl, pH8.3	3.0g
Glycine	14.3g
SDS (Sodium dodecyl sulfate)	1.0g

Preparation of separating gel

A linear gel of 1.5mm thickness was prepared by adding 30% of acrylamide solution followed by 0.5M 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 sample were mixed with equal volume of sample buffer and heated to 100°C. for 2 min. After cooling to room temperature the samples were centrifuged at 10,000g for 2 min. The supernatant was loaded on the gel and was run at 50V till the sample cross the stacking layer. Then the voltage was increased to 120v. Electrophoresis was carried out at 20 °C.

Staining and distaining

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

RESULTS AND DISCUSSIONS

In order to elucidate the level and mechanism of cold tolerance of *Hevea brasiliensis* clones based on physiological and biochemical parameters and to find out the level of expression of chloroplast stress proteins and its role in cold tolerance, a study was conducted at Rubber Research Institute of India, Kottayam under controlled conditions mimicking the cold prone non-traditional regions of rubber cultivation. The study was conducted during the month of May 2012 under controlled growth chamber conditions with four clones of *Hevea* namely RRII 105, RRIC 100, SCATC 88/13 and Haiken 1.

Effect of cold on plant morphology

The controlled plants with three replications were maintained at a maximum temperature of 28°C during day time with a light intensity of 600μmol.m^{-2}.sec^{-1} and minimum temperature of 20°C under a CONVIRON growth chamber. The relative humidity was maintained at 80%. The cold exposure was given to the plant gradually over a period of five days and brought a low temperature of 20°C (max.) at day time and 8°C (min.) at night time with a RH of 50% for four days.

Among the clones the visual symptoms of cold exposure started in the clones like RRII 105 and RRIC 100 in the very second day of exposure. The leaves started yellowing and developed brown patches starting from the margins of the leaves and after four days most of the leaves of RRIC 100 completely turned brown (Fig.1). Whereas, the clones like SCATC 88/13 and Haiken 1 still remained green with less visual symptoms of yellowing of leaves. Samples were collected after 72 hours on the fourth day of exposure.

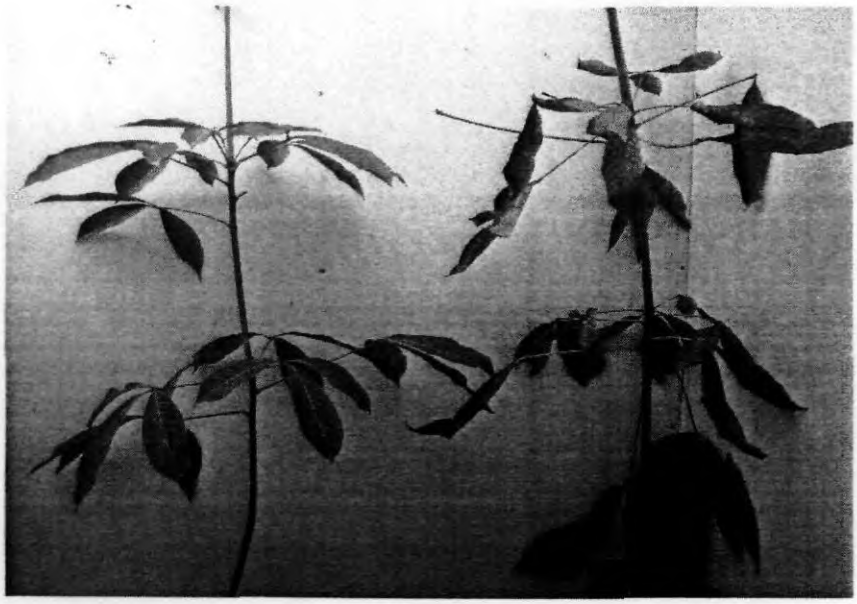

Fig. 1: Controlled and cold exposed (for 72 hours) plants of *Hevea* clone RRIC 100

Photosynthetic pigment contents

The leaf pigments namely chlorophyll a, chlorophyll b, total chlorophyll and carotenoids that are responsible for harvest of light and driving out the photosynthesis process of plants were estimated in control and cold exposed clones of *Hevea*. When compared to controlled plants, the cold exposed plants showed reduction of chlorophyll a in three clones namely RRII 105, RRIC 100 and SCATC 88/13 (Fig. 2). Whereas, Haiken 1 maintained its chlorophyll a content. Similarly, the content of chlorophyll b also found to reduce after cold exposure in all the clones with a maximum reduction in SCATC 88/13 (Fig. 3). The total chlorophyll content was also reduced in three clones namely RRII 105, RRIC 100 and SCATC 88/13. Like chlorophyll a, the total chlorophyll content was better in clone Haiken 1 (Fig. 4).

Photosynthetic pigments are more sensitive to cold stress and high light conditions due to photo-oxidation of pigments. Cold 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).

Similar trends were noticed in carotenoids content as that of chlorophyll b (Fig. 5) upon cold exposure. In all the four clones there was a reduction in carotenoids content with a

minimum reduction in clone Haiken 1, which predicts that the clone may be tolerant to cold stress.

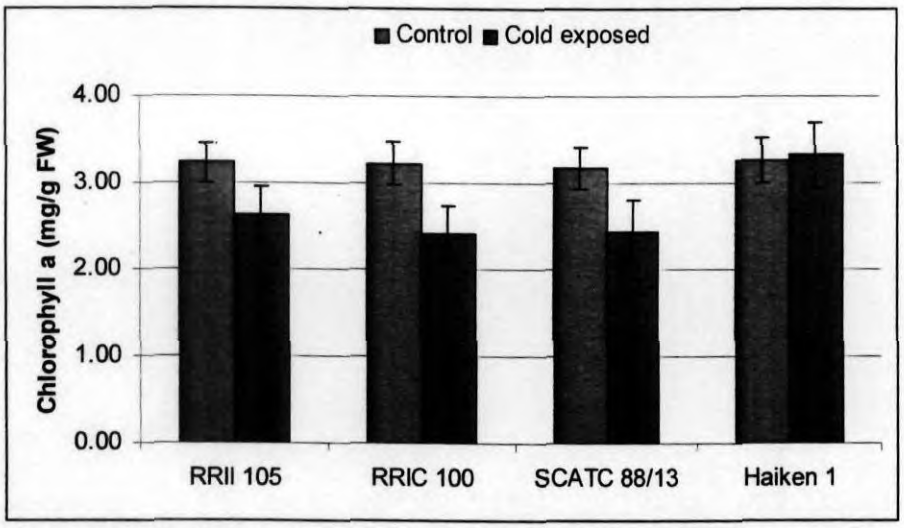

Fig.2: Chlorophyll a content of control and cold exposed clones of *Hevea*

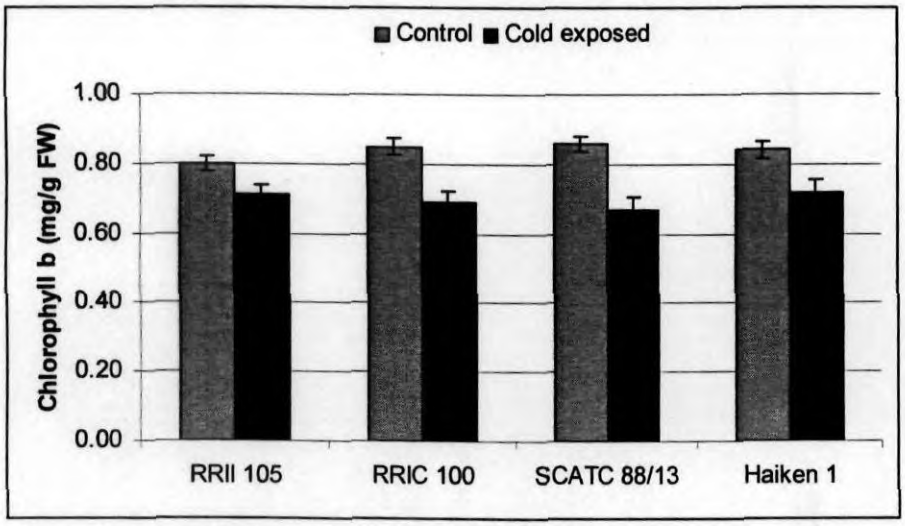

Fig.3: Chlorophyll b content of control and cold exposed clones of *Hevea*

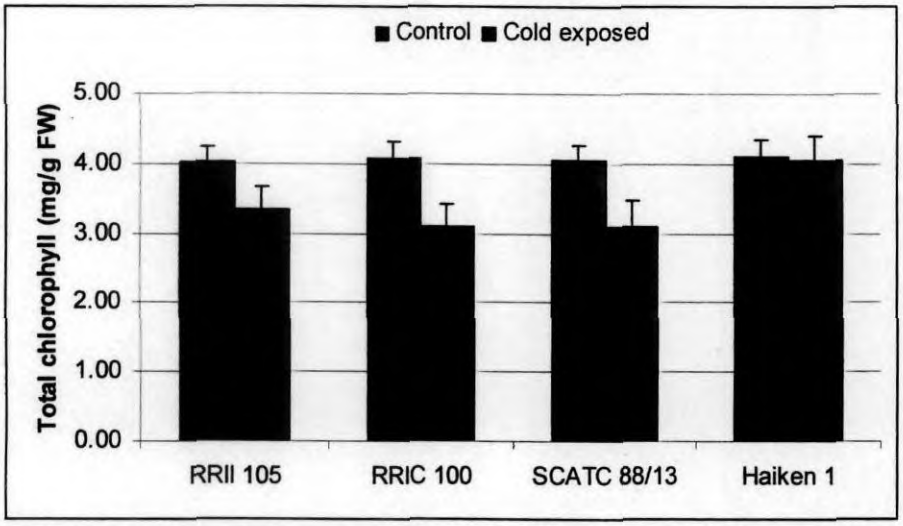

Fig.4: Total chlorophyll content of control and cold exposed clones of *Hevea*

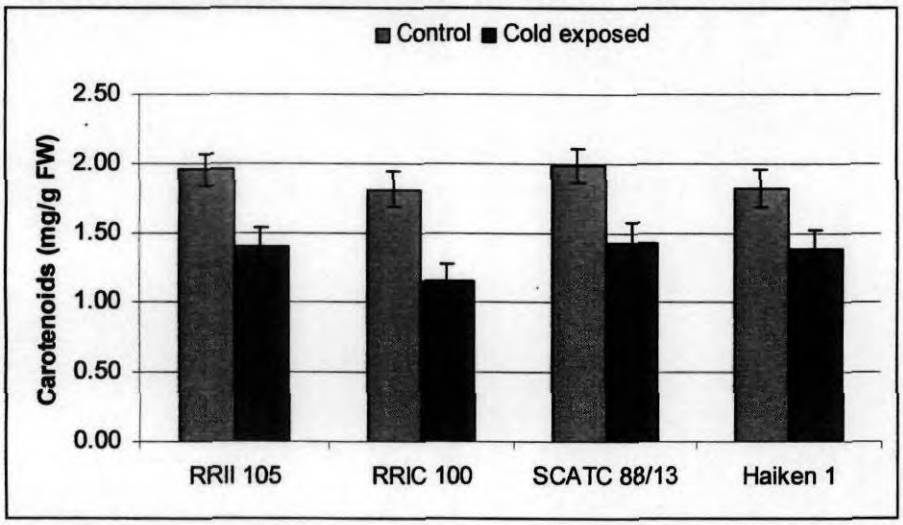

Fig.5: Carotenoids content of control and cold exposed clones of *Hevea*

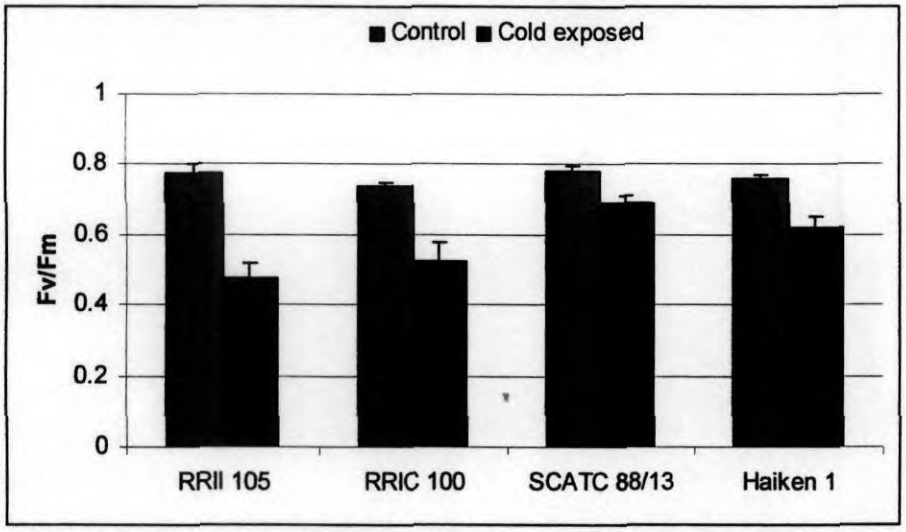

Fig.6: Maximum photochemical efficiency of PSII (Fv/Fm) of control and

cold exposed clones of *Hevea*

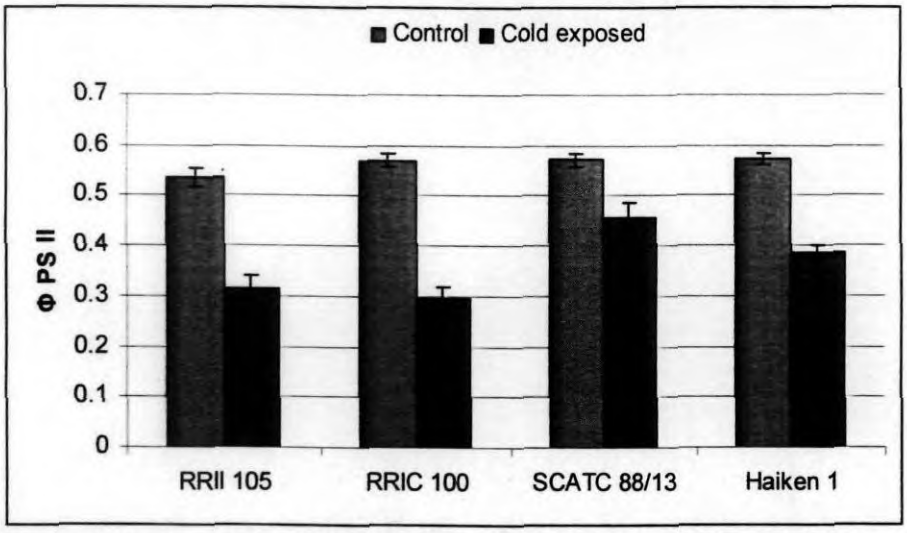

Fig.7: Effective quantum yield of PSII (ΦPSII) of control and cold exposed clones of *Hevea*

Carotenoids are the important accessory pigments pf photosystem involved in light harvesting and photo protection. First, they act as light-harvesting pigments, in coordination with chlorophylls effectively harvesting the solar light and funneling photos to reaction centres of photosynthesis apparatus. Secondly, they perform as essential photo protective role by quenching triplet state chlorophyll molecules and scavenging singlet oxygen nd other toxic oxygen species formed within the chloroplast (Young, 1991). Carotenoids play a major role in dissipation of excess electron 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 centres. Non-photochemical quenching is one of the main ways of protecting against photoinhibition. In higher plants there are three carotenoid pigments that are active in xanthophylls cycles: violaxanthin, antheraxanthin and zeaxanthin. During light stress violaxanthin is converted to zaxanthin 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).

In the present study the degradation of carotenoids were comparatively lesss in SCATC 88/13 and Haiken 1, indicated the relative stability of photosystems in these clones

there by providing better photo protection. Interestingly the reduction in chlorophyll b and carotenoids are very less in Haiken 1. This clone was seemed to be cold tolerant.

Chlorophyll florescence and photosystem II (PSII) activity

Chlorophyll fluorescence was measured to find out the maximum photochemical efficiency of PSII (Fv/Fm) and effective quantum yield of PSII (ΦPSII) in controlled and cold exposed plant. In controlled plants, the maximum photochemical efficiency of PSII (Fv/Fm) was highest in SCATC 88/13 followed by RRII 105, Haiken 1 and RRIC 100 (Fig. 6). After cold exposure it was affected in all the clones and maximum effect was noticed in clone RRIC 100 followed by RRII RRII 105 and minimum reduction was noticed in SCATC 88/13 and Haiken 1, which indicated that the latter two clones are most efficient PSII present in their leaves that can operate optimally even under low temperature.

Whereas, the effective quantum yield of PSII (ΦPSII) of control and cold exposed clones were found highest in SCATC 88/13 followed by Haiken 1 (Fig. 7) after cold exposure and lowest in RRIC 100 followed by RRII 105. These indicate that clones like SCATC 88/13 and Haiken 1 are more tolerant than RRIC 100 and RRII 105. PSII activity often declines concomitantly with low temperature stress with high light, suggesting that the activity of the photosynthetic electron chain is finely tuned to that of CO_2 uptake (Genty et al., 1989). The PSII, thylakoid membranes and electron transport components are the main targets of photo-inhibition due to the formation of excess active oxygen species during adverse climatic conditions (Halliwell and Gutteridge, 1999).

So the present study indicated Haiken 1 and SCATC 88/13 were superior to clones like RRII 105 and 100 in terms of cold tolerance.

Aantioxidant and lipid proxidation

The level of ascorbic acid in the leaves is supposed to be acting as antioxidant against the ROS generated during the stress conditions and membrane lipid peroxidation. The content of ascorbic acid was found more in RRIC 100 followed by SCATC 88/13 and Haiken 1 and

minimum in RRII RRII 105 (Fig. 8). After cold exposure, there was a drastic increase in ascorbic cid content in all the three clones mentioned excluding RRII 105.

The content of MDA, one of the most frequently used indicator of lipid peroxidation, was measured in control as well as cold stressed conditions. The MDA production was stimulated during the period of cold treatment (Fig. 9). It became significantly greater than the control plants after cold exposure. The maximum level of MDA was noticed in RRIC 100 followed by RRII 105, SCATC 88/13 and Haiken 1. These indicate that Haiken 1 was most tolerant in respect to lipid peroxidation followed by SCATC 88/13. The clones RRII 105 and RRIC 100 were the most susceptible in respect to lipid peroxidation.

The excess energy which cannot be dissipated by the photochemical or NPQ-procss, would ultimately induce great accumulation of ROS leading to an oxidative stress. MDA production, an indicator of lipid peroxidation, was significantly increased after 72 h, suggesting that an oxidative stress took place in leaf cells. Alam and Jacob (2002) also observed an accumulation of MDA in the leaves of rubber tree cultivated in cool mountains with high irradiance.

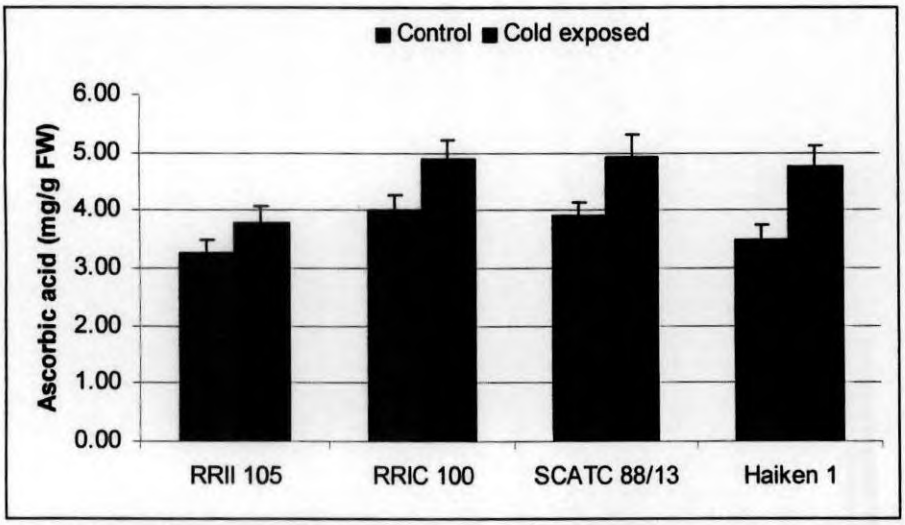

Fig.8: Ascorbic acid content of control and cold exposed clones of *Hevea*

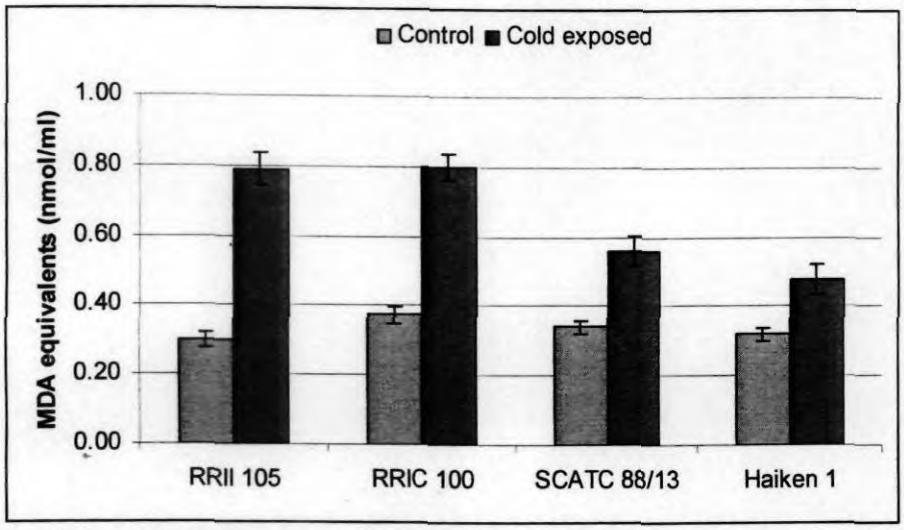

Fig.9: Level of lipid peroxidation expressed as MDA equivalents of control and cold exposed clones of *Hevea*

Chloroplast protein profile

The chloroplast protein profile of four clones of *Hevea* exposed to cold and control conditions were analysed in SDS-PAGE. The profile (Fig. 10) showed presence of one medium and one low molecular weight proteins where as decline in level of certain enzymatic proteins under cold stress conditions. The presence of a 60 kDa and 17 kDa proteins were observed in Haiken 1. The 17 kDa protein was also present in control plants of SCATC 88/13. These two proteins may be heat shock proteins (HSPs). A few enzymatic proteins like subunits of Rubisco and other soluble proteins showed a reduction in their level under cold stress conditions.

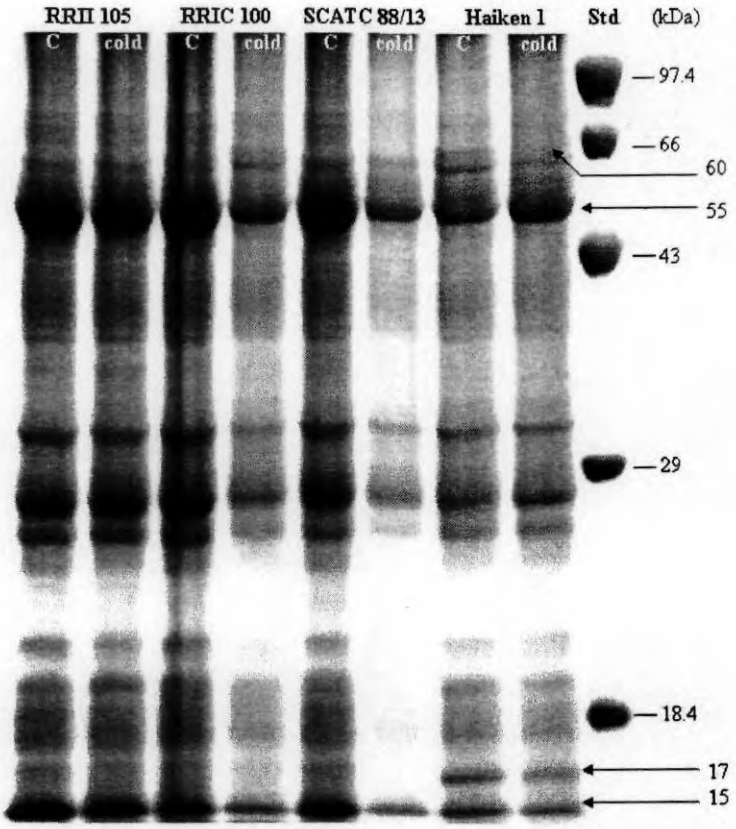

Fig. 10: Presence of a 60 kDa and 17 kDa stress proteins as indicated by the arrow
C indicates control and cold indicated cold exposure for 72 hours

In the present study, the level of expression of HSPs was greater in those clones which show comparatively tolerance to cold stress condition. This indicated that HSPs have a role in stress protection, most probably protection of thylakoid membrane against low temperature induced oxidative stress and membrane damage. Haiken 1 clone with higher level of stress protein also had high level of carotenoids and chlorophyll b content. HSPs and other stress proteins have been known to protect cells against deleterious effects of stress (Feder et al., 1999). HSPs and their cognates are found in very organism at ordinary growth temperature and play an important role in cellular functions related with growth (Lindquist, 1986; 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, 1991). 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 other stresses (Ray, 1999; Iba et al., 2002). In all organisms, the induction of HSPs is remarkably rapid and intense under abiotic stress condition. 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).

The chloroplast HSPs are reported in general in a variety of plant species, including 16 kDa sHSP from tobacco (Lee et al., 1998) and 21 kDa HSP from tomato, Arabidopsis and soyabean (Suzuki et al., 1998). The HSPs are present within chloroplast as large oligomers containing nine more subunits and are actively synthesized during heat stress (Suzuki et al., 1998). In chloroplast the HSPs have been implicated in protecting aggregation and stabilizing the thylakoid memebrane (Torok et al., 2001). It has been demonstrated that the chloroplast HSPs plays a dirct role in stabilizing the photo system II (PS II) oxygen evolving complex (OEC) proteins during stress and they promotes the maintenance of PS II electron transport. Thus HSPs are appear to be general stress proteins in chloroplast that are involved in maintaining function and survival of this organelle during stress of helping in stress recovery.

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