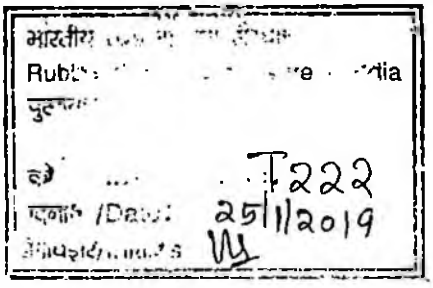
DROUGHT INDUCED LOW MOLECULAR WEIGHT PROTEIN AS A MARKER FOR SCREENING STRESS TOLERANCE IN HEVEA BRASILIENSIS

Thesis Submitted to
The University of Kerala
For the award of the degree of
DOCTOR OF PHILOSOPHY
In
BIOTECHNOLOGY

By
S. PRAMOD

RUBBER RESEARCH INSTITUTE OF INDIA
RUBBER BOARD
(Ministry of Commerce & Industry, Govt. of India)
KOTTAYAM-686 009, KERALA, INDIA

November 2017


Rubb
T222
25/11/2019

DECLARATION

I hereby declare that the Ph.D thesis entitled **"DROUGHT INDUCED LOW MOLECULAR WEIGHT PROTEIN AS A MARKER FOR SCREENING STRESS TOLERANCE IN HEVEA BRASILIENSIS"** is an independent work carried out by me under the supervision of Dr. A. Thulaseedharan, Joint Director (Biotechnology) (Retd.), at the Rubber Research Institute of India, Kottayam and it has not been submitted anywhere else for any other degree or diploma elsewhere.

S. Pramod

Kottayam
27th November, 2017

Dr. A. Thulaseedharan
[Joint Director (Biotechnology)(Retd.)
Rubber Research Institute of India]

CRA- A11, TC 11/1658, Charachira Road,
Kowdiar (PO),Thiruvananthapuram-695003,
Kerala Mob: 91-9447040851
Email: thulaseedharanat@gmail.com,
atncuk@gmail.com

27th November, 2017

CERTIFICATE

This is to certify that the work embodied in the thesis entitled, **"DROUGHT INDUCED LOW MOLECULAR WEIGHT PROTEIN AS A MARKER FOR SCREENING STRESS TOLERANCE IN HEVEA BRASILIENSIS"** has been carried out by **Mr. S. Pramod** under my supervision and guidance at Rubber Research Institute of India, Kottyam-9, for the award of the degree of **Doctor of Philosophy in Biotechnology** under the Faculty of Applied Sciences, University of Kerala, Thiruvananthapuram, Kerala. It is also certified that the work presented in this thesis has not been submitted earlier for any other degree or diploma elsewhere.

Dr. A. Thulaseedharan

Former:
- Principal Scientist and Joint Director (Biotechnology),
  Rubber Research Institute of India, Kottayam-686009, Kerala, INDIA
- Dean, School of Biological Sciences, Central University of Kerala, Kasaragod, India

ACKNOWLEDGEMENT

By the grace of God, on successful completion of thesis work, I take it as a privilege to express my deep sense of gratitude and veneration to all the people who helped me during the course of my research work.

This research work would not have seen the light of the day without the consistent support and encouragement of my respected supervisor's ***Dr.A.Thulaseedharan,*** *Former Joint Director, Biotechnology Division and* ***Dr. K. Annamalainathan,*** *Joint Director, Crop Physiology Division of Rubber Research Institute of India, Kottayam, Kerala. Both of them have always been a constant source of inspiration and working under their supervision, creative suggestions, constant encouragements and support has been an enriching experience for me.*

I also express my deep sense of gratitude to ***Dr. James Jacob,*** *Director of Research for providing me an opportunity to work in a place like Rubber Research Institute of India, and for his encouragement and support throughout the work. His advice and insight have been of great value to me throughout my studies.*

I am extremely grateful to ***Dr.R.Krishnakumar,*** *Former Joint Director, Climate Change and Ecosystem Studies, and to* ***Dr. Molly Thomas,*** *Principal Scientist, Crop Physiology Division, RRII. Their encouragement and support at various stages in these past years is duly acknowledged.*

I am extremely thankful to ***Dr. Jayanta Sarkar*** *and* ***Dr. K.V.Sumesh*** *who were most supportive and helpful at each and every stage of my studies towards the Ph.D. program.*

I am grateful to ***Dr. Shaji Philip, Dr. M.B. Mohamed Sathik, Mr. R. Anantharamanan*** *and* ***Dr. Pramod Sivan,*** *for their timely help and the technical support they had rendered in various aspects of this work.*

I would like to express my gratitude to ***Dr. Jayashree Gopalakrishnan, Dr. Sreelatha. S, Mr. Pradeep Kumar, Dr. D. Bhuvanendran Nair, Dr. Sheela .P. Simon*** *and* ***Mr. Harikumar,*** *scientists and all other staff members of Crop Physiology Division, and also to all other scientists of Biotechnology Division of Rubber Research Institute of India, Kottayam.*

I am grateful to my dear friend ***Dr. Saifudeen Ismail,*** *Post Doctoral Fellow, University of Tennessee, Health Science Center, Memphis, USA, for giving me constant inspiration, guidance and support throughout the work.*

I am delighted to express my sincere affection towards my colleagues at RRII for their support, encouragement and constant companionship of ***Dr. Amith Abraham, Dr. Satheesh P.R., Dr. Mrudula .P. Musthapha, Dr. Ambily P.K., Dr. Linu Kuruvilla, Dr. Lisha .P. Luke , Mrs. Smitha.. M. Xavier, Dr.R. Supriya, Mrs.Vineetha Mohan*** *and* ***Dr. Suni Anie Mathew*** *.*

I am thankful to ***Mr. Aneesh,*** *Assistant Statistician, Rubber Research Institute of India, for the help in statistical data analysis. I thank all administrative members of RRII for their invaluable help and for providing various facilities. I also convey my thanks to staff members of library, computer section and instrumentation section of RRII, Kottayam.*

Words fail to express my affection to my dear friend ***Unni*** *for the immense help and encouragement that he rendered during the course of my work.*

I sincerely acknowledge the support extended by all my colleagues of Food Corporation of India, Kollam, for the successful completion of my doctoral thesis.

I want to express my heartiest gratitude and acknowledgement to my beloved family. They have always taken all the pains to provide me with the best throughout my life. I bow down before them for their moral support, immense patience and loving care that made me more confident.

There are numerous friends and relatives whose name I might have missed; also have provided a patient ear, advice and encouragement during my Ph.D thesis.

The chain of my gratitude would be definitely incomplete if I would forget to thank the first cause of this chain, ***The Almighty, The Prime Mover,*** *whom I have great faith in and by whose blessings I have been able to reach this milestone. I pray to Him to bless me always and enlighten me towards the path of a meaningful and successful life, and also to be a good human being.*

Pramod

Contents

Chapter IV
Identification and Purification of a Low Molecular Weight (LMW) Chloroplast Stress Protein from Young Rubber Plants

Chapter V

Developing a chloroplast LMW protein marker for screening drought tolerance in young *Hevea* plants

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ANOVA	:	Analysis of variance
ATP	:	Adenosine triphosphate
bp	:	base pair
BSA	:	Bovine Serum Albumin
cDNA	:	Complementary DNA
Ct	:	Threshold cycle
2-DE	:	Two dimensional gel-electrophoresis
DEPC	:	Diethyl pyro-carbonate
dist. H_2O	:	Distilled water
dNTPs	:	deoxyribonucleoside triphosphates
ExPASy	:	Expert Protein Analysis System
GAPDH	:	Glyceraldehyde 3 phosphate dehydrogenase
GRAVY	:	Grand average hydropathy
H. brasiliensis	:	*Hevea brasiliensis*
IEF	:	Iso electric focussing
IPG strip	:	Immombilized pH gradient strip
LC-MS	:	Liquid Chromatography-Mass Spectrometry
mRNA	:	messenger RNA
NaCl	:	Sodium Chloride
NCBI	:	National Center for Biotechnology Information
NC membrane	:	Nitrocellulose membrane
NR	:	Natural rubber
nt	:	nucleotide
ORF	:	Open Reading Frame
PAGE	:	Polyacrylamide gel electrophoresis
PB	:	Prang Besar, Malaysia
pI	:	Isoelectric Point
PVP	:	Poly vinyl pyrrolidone
PS II	:	Photosystem II

qPCR	:	quantitative PCR
RH	:	relative humidity
RNA	:	ribonucleic acid
ROS	:	Reactive oxygen species
RRII	:	Rubber Research Institute of India
RRIM	:	Rubber Research Institute of Malaysia
RT-PCR	:	Reverse transcription PCR
SDS	:	Sodium dodecyl sulphate
sHSP	:	Small heat shock protein
TEMED	:	Tetramethylethylenediamine
Tjir	:	Tjirandji, Indonesia
Tris	:	Tris (hydroxymethyl) aminoethane

Units:

%	:	percentage
°C	:	degree Celsius
g	:	gram(s)
hr	:	hour(s)
kDa	:	Kilo dalton
l	:	litre(s)
M	:	molar
min	:	minutes
mol	:	mole(s)
rpm	:	revolutions per minute
v/v	:	volume per volume
w/v	:	weight per volume

Prefixes:

K	:	kilo
M	:	milli
μ	:	micro

Chapter I

General Introduction

Natural rubber is a unique biopolymer derived from latex, a milky colloid produced by some plants. Although nearly 2000 plant species confined to 300 genera of seven families viz., Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae synthesise natural rubber (Heywood, 1978; Backhaus, 1985; Lewinsohn, 1991; Cornish *et. al.,* 1993), around 99% of the world's commercial natural rubber is made from the latex that comes from a tree species called *Hevea brasiliensis* Muell. Arg., widely known as the Para rubber tree. By making a controlled incision in the bark of the tree termed 'rubber tapping' the colloidal milk-coloured latex which is the cell-sap of the tree is harvested and then refined into usable rubber. Particles of rubber hydrocarbon and non rubber substances dispersed in an aqueous serum phase together constitute the natural rubber latex. Non rubber constituents, amounting to more than 5%, include proteins, fatty acids, resins and inorganic materials (Subramaniam, 1995). The purified form of natural rubber is *cis*-1,4-polyisoprene with a formula of $(C_5H_8)_n$.

The history of rubber is much fascinating. Columbus in 15th century had given the first description of rubber and de la Condamine, an astronomer was the first to send samples of the elastic substance "caoutchouc" from Peru to France in 1736 with all details regarding the habit and habitat of the trees and procedures for processing (Dijkman, 1951). The first scientific paper on rubber was presented in Académie in 1751 (published in 1755) by François Fresneau making the first systematic observations on rubber. Except English the name for 'rubber' in most Indo-European languages is derived from the Amerindian name for rubber trees: cachuchu: 'weeping wood'. The English name is attributed to Joseph Priestley, the British Scientist in 1770 who observed that a piece of the material was extremely good to rub out pencil marks on a paper and hence the name 'rubber' (Jones and Allen, 1992). Natural rubber is used extensively in large number of applications and products owing to the superior properties attained as a result of special molecular structure and high molecular weight such as resilience, elasticity, abrasion resistance, efficient heat dispersion and impact resistance than artificially produced polymers. To make it a versatile material raw rubber needs to be processed. Raw rubber is first made soft through the process of mastication where they are pressed between mechanical rollers. Pliable, water proof, mouldable rubber was then made possible following the

discovery of vulcanization by Charles Goodyear in 1839 by heating the raw product with sulphur making it less sticky with superior mechanical properties. A vast array of materials is made with vulcanized rubber including heavy-duty tyres for trucks, busses and airplanes and many latex products for the medical profession and hence natural rubber cannot be replaced by synthetic materials in many of its applications. Rubber tree has attracted attention as a substitute for the tropical rain forest as a resource of wood which is a good source of timber for furniture industry and other applications. Rubber wood is 'environmentally friendly'; since it makes use of plantation trees that have already served a useful economic function. Natural rubber also contributes to the global environment preservation due to its role as an efficient carbon sequesters and mitigating emissions at some extent (Rahman and Shivakumaran, 1998; Jacob, 2005; Annamalainathan *et. al.,* 2015).

Hevea brasiliensis, the rubber tree belonging to the family Euphorbiaceae and the most economically important member of the genus *Hevea* is indigenous to South America and remained as the main source of the limited amount of latex rubber used during much of 19th century. In 1876, Sir Henry Wickham collected 70,000 Para rubber tree seeds from Rio Tapajo'z region (Amazone, Brazil) and delivered to the Kew Gardens, England (Dijkman, 1951). Seedlings were then sent to Ceylon, Indonesia, Singapore and many south East Asian countries. The first introduction of rubber to India was from Ceylon (Priyadarsan, 2011). Currently Thailand is the highest producer of natural rubber and India is one among the top ten rubber producing countries. In India, Kerala is the leading rubber plantation state where the first commercial plantation was started by European planters who formed the "Periyar Syndicate" in 1902 at Thattekadu (George *et. al.,* 1988).

Ten inter-crossable species exist in the genus of *Hevea* but only three species within the genus yield usable rubber, *H. brasiliensis* (Fig 1.1), *H. benthamiana* and *H. guianensis.* Among the three species *H. brasiliensis* is the only species planted commercially and yielded the major source of natural rubber since latex in other species has a high ratio of resin to rubber (Wycherley, 1992). In addition to the Para rubber tree, two species belonging to the family Asteraceae are also known to produce large amounts of rubber with high

molecular weight: Russian dandelion (*Taraxacum koksaghyz*) and a shrub named guayule (*Parthenium argentatum* Gray) (Mooibroek and Cornish, 2000).

Fig 1.1. Plantation of *Hevea brasiliensis* (Para rubber tree)

Para rubber trees being naturally adapted to the Amazonian tropical climate they can perform to its best in climate closely resembling that in its centre of origin. Hence, wet equatorial type of climate is ideal for rubber cultivation characterised by warm temperature, ample rainfall and mild breezes round the year (Bradshaw, 1977). In India traditional rubber growing region falls in the hinterlands of South West coast comprising the Kerala state and adjoining Kanyakumari district of Tamil Nadu. The attributes ideal for rubber cultivation include a maximum temperature around 28 ± 2 °C, high atmospheric humidity, 2000-4000 mm rainfall and bright sunshine amounting to about 2000 hours per annum (Rao and Vijayakumar, 1992). As the global demand for natural rubber

increased, plantations have been extended to suboptimal regions beyond the traditional latitudes, in India, China, Burma and Brazil (Dijkman, 1951; de Barros *et. al.*, 1983; Pushparajah, 1983; Sethuraj, 1985; Sethuraj *et. al.*, 1989). Various agro-climatic studies conducted have been useful to find out areas suitable for rubber cultivation in different parts of the country so as to accelerate the production to meet the increasing demand of rubber. The exclusive objective of rubber breeding is to develop superior clones with improved rubber yield as well as wood. Other desirable secondary characteristics include high initial vigour, smooth and thick bark with good latex vessel system, good bark renewal, high growth rate after initiation of latex harvest, tolerance to major disease and wind (Annamma *et. al.*, 1990; Varghese *et. al.*, 1993). Developing climate resilient clones with tolerance to abiotic stresses such as drought, high temperature, cold, etc. has also been given importance recently (Thulaseedharan *et. al.*, 2000).

Climatologically five main zones are found in India, viz., tropical rain, tropical wet and dry, sub tropical rain, temperate and desert. Among the five zones, first three are found suitable for rubber cultivation. Due to latitude and altitude changes several locations of these zones are counted as non-traditional (Sethuraj *et. al.*, 1989) and these include the north Konkan and north eastern regions of India. Agro-climatic conditions prevailing in marginal areas delineated as non-traditional zones, spread over the states of Maharashtra, Orissa, Tripura, Assam, West Bengal, Meghalaya, and Mizoram. The major environmental constraint which affects the plant in these places includes chilling winter at north eastern region and high temperature and drought at the Konkan region. Various studies have reported that plant development, general performance and latex yield of rubber trees are affected due to drought and cold stresses (Sethuraj *et. al.*, 1984; Jacob *et. al.*, 1999; Sreelatha *et. al.*, 2007; 2011).

Unfavourable environmental conditions that negatively affect plant growth and development is termed as abiotic stress. Climate change has exacerbated the frequency and severity of many abiotic stresses particularly drought and high temperature which remains as the greatest constraint to crop production (Lobell and Field, 2007). Significant reduction in yield is the direct result of abiotic stresses (Acquaah, 2007). To cope with and recover from damaging effects of abiotic stresses, plants have evolved sophisticated and elaborate mechanisms at the physiological, cellular, and molecular levels that

come into play at the onset of stress. Other than soil moisture deficit stress the next key environmental factor that influence plant growth is temperature. Being a species adapted to moderate temperatures, rubber plants get affected by extreme temperatures. Absence of rainfall and increasing temperature alternatively results in higher rates of evapo-transpiration leading to severe soil moisture stress. Chandrashekar *et. al.,* (1990) have reported that temperature beyond 37°C coupled with soil moisture stress results in leaf injury and killing of leaf margins in rubber. Rubber cultivation under sub-optimal environments in nontraditional areas experiences one or more stress situations. Developing new clones with better adaptability to marginal areas can be considered only through long-term perspective. However, evaluation of existing clones in various environments with extensive eco-physiological research will yield quick and fruitful results.

Leaves, the most essential organ for the manufacture of food are the most sensitive part of plant to drought since many physiological processes gets affected with ultimate consequences on the growth and development (Dutta *et. al.,* 2016). One of the main physiological processes that get affected in higher plants is reduction and /or inhibition of photosynthesis (Annamalainathan *et. al.,* 2010; Keyvan, 2010; Bhargava and Sawant, 2013; Nezhadahmadi *et. al.,* 2013). Increased leaf temperature, impaired photosynthetic machinery, premature leaf senescence, decrease in leaf expansion rate and a low leaf surface are some of the reasons for this effect (Farooq *et. al.,* 2009; Zare *et. al.,* 2011). Chloroplast plays a central role in plant stress response and paves light to understand the connection between different stress response and organellar signalling pathways (Kmiecik *et. al.,* 2016; Sun and Guo, 2016).

In response to drought stress, plants synthesize proteins, aminoacids and also accumulate some minerals (Bernacchia and Furini, 2004; Rahdari and Hoseini, 2012). The quantity as well as quality of plant proteins gets affected as a consequence of drought (Chernyad'ev, 2005; Farooq *et. al.,* 2009). Under water deficit conditions the protein content generally decreases due to suppression of their synthesis. Changes in gene expression during drought periods consequently change the synthesis of mRNAs and drought-related proteins (Nezhadahmadi *et. al.,* 2013, Salehi-lisar *et. al.,* 2012). However, enzymes required for the biosynthesis of various osmo-protectants and those involved in the detoxification of ROS, proteases, various proteins such as LEA, protein factors involved in signal

transduction and gene expression increases under drought stress (Farooq *et. al.*, 2009; Xoconostle-Cazares *et. al* ., 2010; Zlatev and Lidon, 2012; Ding *et. al* ., 2013; Labudda and SafiulAzam, 2014).

Regulatory and functional proteins produced as a result of signal cascade helps to re-establish cellular homeostasis, eliminate toxic compounds, and protect and repair damaged structures (Fig 1.2). A series of cell physiological process including the production of metabolites and proteins involved in protective function are involved in adaptation of the plant to abiotic stresses. Hence genes involved in the biosynthesis of these metabolites as well as those coding for proteins involved in protective mechanisms are modulated for obtaining plants via transgenic approach for improving abiotic stress tolerance (Marco *et. al.*, 2015).

Proteomics has proven to be a promising tool to explore biochemical pathways and the complex response mechanism of plants in response to environmental effects (Zhou *et. al.*, 2015). Proteins that get induced in drought are broadly classified into two main groups. The first group (*functional proteins*) probably functions in stress tolerance and includes chaperones (HSPs, LEAs), lipid transfer proteins, proteins involved in repair and protection from damage, defence-related proteins, proteins involved in the synthesis of osmoprotectants, proteins regulated by plant hormones and those involved in its synthesis, reproduction and development-related proteins, respiration and senescence related proteins, etc. The second group (*regulatory proteins*) are involved in regulation of signal transduction and transcription as part of drought response and include transcription factors of multiple gene families, protein kinases and protein phosphatases (Ashoub *et. al.*, 2013; Augustine, 2016).

A group of proteins that are expressed at high levels when exposed to stress are termed as Heat Shock Proteins (HSPs) and these proteins are present in cells under normal environmental conditions also. Since these proteins were first discovered in cells which were exposed to high temperature they were named as heat shock proteins (Augustine, 2016). HSP 100/ClpB, HSP90/HtpG, HSP 70/DnaK, HSP 60/GroEL and sHSP are the five families of HSP that have been described and they are thought to act as molecular chaperones which bind and

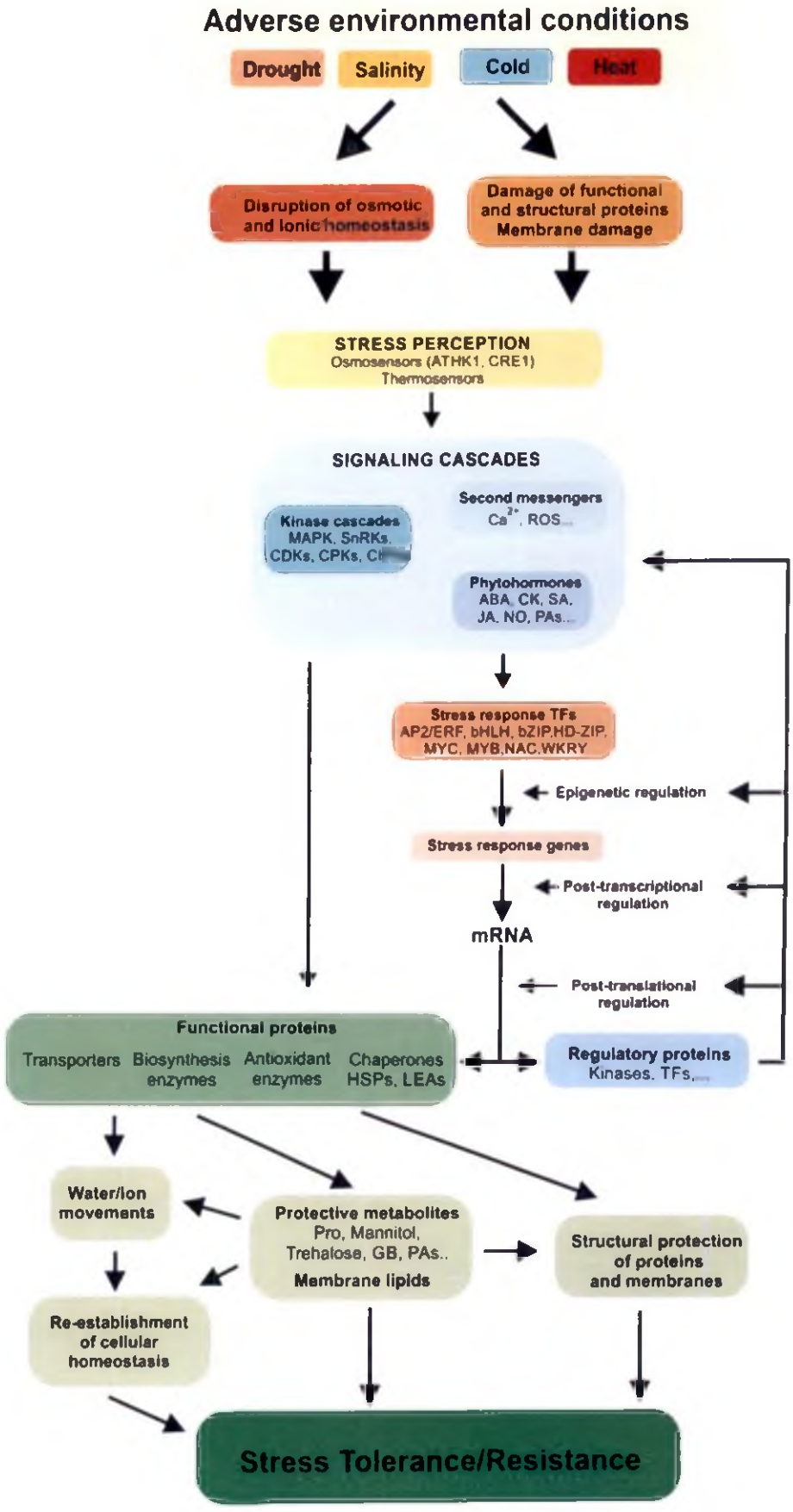


Fig 1.2. Schematic overview of plant molecular response to abiotic stress factors (Marco *et. al.*, 2015)

stabilize proteins at various intermediate stages of its formation and helps in folding, assembly, degradation, and translocation across membranes.

sHSP or LMW HSP class (12-42 kDa) are ubiquitous molecular chaperones which dominate protein synthesis profiles during heat stress (DeRocher *et. al.,* 1991; Hsieh *et. al.,* 1992). A consistently over expressing chloroplast stress protein (~23kDa) was reported and implicated with drought responses in young plants of *Hevea brasiliensis* (Annamalainathan *et. al.,* 2006). However, the pattern of expression of this chloroplast protein was not validated with functional aspects of photosynthetic apparatus and drought tolerance potential of different clones. Breeding and developing new rubber clones with increased tolerance to environmental stress is highly essential, especially in the present scenario of global warming and related climatic changes. In this context, attempts need to be made to identify critical physiological and biochemical traits which characterize the *Hevea* genotypes for drought tolerance. It is felt that establishing specific markers are essential for screening vast number of elite clones, pipeline clones and other ortet selections of *Hevea* for stress tolerance.

Under this scenario, the present study was initiated with an objective to assess the clonal responses of young *Hevea* plants to drought stress and to establish and develop a protein marker for drought tolerance. This protein marker in combination with other crucial physiological parameters can be employed towards screening of new pipelines or germplasm accessions for drought stress tolerance.

Objectives

- To assess clonal responses of young *Hevea* plants to drought stress using specific physiological parameters.
- To identify drought responsive proteins of *Hevea brasiliensis* and validate their functional association with drought stress tolerance.
- To establish and develop the stress protein as a marker for drought tolerance that could be further utilized to select drought tolerant clones of *Hevea brasiliensis.*

Chapter II

Review of Literature

2.1. *Hevea brasiliensis* (Para Rubber Tree)

Hevea brasiliensis (Wild. Ex Adr. De Juss.) Muell. Arg. (Rubber), a member of the family Euphorbiaceae is the primary source of natural rubber, a native to the Amazonian rain forest in Brazil. Due to abundance in high quality latex and convenience of harvesting, *H. brasiliensis* has become the prime source of the modern world's natural rubber (NR) which has descended from seedlings transplanted from Brazil to South and South-east Asia that have undergone several cycles of breeding (Priyadarshan, 2011). The original material referred to as Wickham gene pool form the genetic basis and was introduced by the British in to South-East Asia for commercial cultivation. It was brought under domestication only in 1876 (Wycherley, 1968). Modern clones hence have invariably originated from the very few plants of Wickham's original stock from the banks of the Tapajoz (Imle, 1978). The history of rubber cultivation in India dates back to 1873 when *Hevea* plants were imported from Sri Lanka and were planted in the Nilambur Valley of Kerala state in South India (Haridasan and Nair, 1980). Though, the origin of rubber plant is Amazonian rain forest, the tree grows well in a wide range of agro-climatic conditions. It is a perennial crop mainly cultivated in tropical and some parts of sub-tropical climatic regions. After introduction to tropical Asian and African countries the original wild genotypes subjected to tremendous changes through various breeding programs. Slowly the trees were accustomed to wide range of agro climates and soil conditions.

H. brasiliensis is a fast growing tree with a straight trunk and with an open leafy crown. The bark of the trunk which is usually grey and fairly smooth is the part from where rubber is harvested. Being the tallest species of the genus the trees may grow to over 40 m with a life span of more than 100 years in the wild. Cultivated plants rarely grow beyond 25-30 m in height as a result of latex harvesting by tapping thereby resulting in growth reduction (Webster and Paardekooper, 1989). Rubber trees are deciduous with annual leaf fall. Defoliation-refoliation process during wintering is followed by flowering. Leaves are compound, trifoliate, glabrous and spirally arranged which loops downwards approximately in parallel with petioles which are reddish or bronze in colour and gradually changes to dark green above and light green underneath.

Extra floral nectaries are present in the region of insertion of the leaflets on the long petiole (Premakumari and Saraswathyamma, 2000). The architecture of leaves is varietal characteristics and so clones can be identified by examining the leaves carefully (Mercykutty *et. al.,* 2002). Flowers are monoecious and strongly scented with lateral inflorescence. The mature fruit is a trilocular capsule having a woody endocarp and a thin leathery mesocarp containing three seeds. Seeds are large, ovoid with a hard and shiny seed coat that contains numerous brown or grey-brown mottles or streaks. Dissemination of the species is favoured by a spongy parenchyma tissue inside the seeds which help them to float (Reed, 1976). Root system comprises of a strong taproot and extensive lateral roots together forming about 15% of the total dry weight of a mature tree.

Hevea will perform to its best in a region that resembles the agro-climatic conditions of its native habitat. Ideal agro-climate for rubber cultivation includes well distributed annual rainfall, warm and humid conditions, absence of prolonged drought and plentiful sunshine. To cope up with the increasing global demand of rubber, regions outside the traditional belt have been prompted to focus their attention on the cultivation of rubber (Pushparajah, 1983; 2001). The genetic potential of the planting material, adaptability to the existing environment and the ability to respond to improved agro techniques are the factors responsible for the overall productivity of *Hevea* (Mydin, 2014). Development of improved variety through conventional breeding is slow, time-consuming and labour intensive due to the perennial nature of *Hevea*.

To cope up with the demand for this strategic commodity and its limited scope of expansion in the traditional belts, attempts have been made to elaborate the cultivation to marginally suitable areas like Central and North-East India which are known for its varied climatic constraints. Without compromising on yield and productivity it is essential to identify or develop clones that can withstand such adverse environmental conditions. The major environmental constraints for establishing rubber cultivation in areas such as North Konkan region of India is drought combined with high solar light intensity (Sethuraj *et. al.,* 1989; Devakumar *et. al.,* 1998; Jacob *et. al.,* 1999; Alam *et. al.,* 2005). Hence crop-improvement programmes for the non-traditional belts include screening of clones for various environmental stresses including drought.

2.2. Rootstock- scion interaction in *Hevea*

The predominant planting material of rubber is budded stumps raised in polybags or root trainers. A budded plant is genetically heterozygous. Due to the different genetic make-up of the stock and scion, a bud grafted plant is often considered as a multiple genetic system. Although the graft functions as a single metabolic entity, subtle differences may still persist between the rootstock and scion. The physiology of the rootstock can interfere with that of the scion and *vice versa* (Sobhana *et. al.,* 2007). The performance of a scion could be different when grown with its own root system or grafted to a rootstock with another genetic make-up, and the difference depends on the extent of the genetic distance between the stock and scion (Hartman and Kester, 1976; Errea, 1998).

In every plant there exists a dynamic system of metabolic communication between the root and shoot systems. In grafts, this may be more complex given the differences in the genetic make-up between the root stock and the scion. Rootstock-scion communication could result in simple and direct effects of one of the other, or a more complex effect quite different from their individual physiology. Molecular and genetic level effects in scions of bud grafted plants are also reported for several species (Yagishita *et. al.,* 1986, 1990; Degani *et. al.,* 1990). Such effects have also been reported in *Hevea* (Krishnakumar *et. al.,* 1992; Sobhana, 1998). The rootstock can have a positive effect on the scion and *vice versa*. Clonal rootstocks as well as clonal scions are being recommended in some countries (Cardinal *et. al.,* 2007).

Just as the influence of rootstock on growth of scion, scion also exerts influence on the growth of the rootstock in *H. brasiliensis*, but relatively less information is available on this (Sobhana, 1998). The rooting behavior of the rootstocks has obvious effects on the water relations of the scion leaves in *H. brasiliensis*. A scion grafted to monoclonal seedlings of GT 1 and RRIM 623 was better adapted to drought than those grafted to the monoclonal seedlings of RRIM 600 (Bastiah, 1999). This could be due to better rooting of the seedlings of GT1 and RRIM 623 than that of RRIM 600.

Root stocks have been known to influence the cation exchange capacity (CEC) of the roots which influences the mineral uptake. It has been observed

that CEC of lateral roots was significantly influenced by the scion clone in *H. brasiliensis* (Sobhana *et. al.,* 1980). However, the NPK contents in the scion leaf and rootstock had no effect with each other. There has been a positive correlation between magnesium and manganese contents in the rootstock and scion leaves in some clones, but not in others (Sobhana, 1998). No serious efforts have been taken so far towards developing a rootstock for best performance of the scion of *H. brasiliensis*. Always the emphasis has been scion-focused, whether it is high yield or tolerance to environmental stresses. For instance, the drought tolerant plants which have been identified (Sreelatha *et. al.,* 2003) are being multiplied as drought tolerant clones (scions). But from a physiological point of view, it is more likely that these selected clones would perform as better rootstocks than scion vis-à-vis drought tolerance. The lack of successful protocol for large-scale vegetative multiplication of rootstocks prevents such attempts.

2.3. Abiotic stress responses in plants

A variety of environmental factors affect the plant growth and development including availability of water, temperature, light, relative humidity, mineral nutrients, and CO_2 as well as ionizing radiation, pollutants, or wind (Schulze *et. al.,* 2002). Depending upon the quantity or intensity of each abiotic factor the effect varies. A certain quantity of each abiotic environmental factor is required for the optimal growth of plants. Abiotic stress is regarded as any deviation from optimal external conditions whether in excess or deficit in the chemical or physical environment having adverse effect on plant growth and development (Bray *et. al.,* 2000). Abiotic stress conditions include, for example, drought, inadequate mineral nutrients in the soil, excessive soil salinity, extreme temperatures, too high or too low radiation, flooding, metal toxicity, oxidative stress and wind. Of the various environmental stresses as described drought is one of the most important and prevalent stress factors since water accounts for between 80-95% of the fresh biomass of growing plants thereby playing an important role in many aspects of plant growth and development (Salehi-Lisar *et. al.,* 2012). Low rainfall, salinity, high light intensity, extreme temperatures are some of the reasons for a plant to experience water stress. Pseudo-drought or physiological drought is a different condition where enough water is available in

the soil but plants cannot uptake it (Athar and Ashraf, 2009; Salehi-Lisar *et. al.,* 2012; Arbona *et. al.,* 2013). Changes in the physiological, morphological, biochemical, ecological and molecular traits of plants are the immediate outcome of drought and hence it is a multidimensional stress (Shao *et. al.,* 2008, Farooq *et. al.,* 2009, Bhargava and Sawant, 2013). The quantity and quality of plant growth and yield is also negatively affected as a consequence of drought (Jaleel *et. al.,* 2009; Zlatev *et. al.,* 2012; Nezhadahmadi *et. al.,* 2013).

From an agricultural context drought is a term used to define as a period without rainfall (Salehi-Lisar *et. al.,* 2012). The response of plant to water deficit stress depends on plant species, age and developmental stage as well as the length and severity of the water deficiency (Madhava Rao, 2006). In order to tolerate drought stress many plants have developed resistance mechanisms, but these mechanisms are varied and species specific. Plants have several options to tolerate drought stress, including physiological, morphological, developmental, molecular, biochemical and ecological mechanisms. Maintaining cell water homeostasis under drought conditions is the general plan followed by plants in response to drought which is made possible mainly by increasing the water inlet to the cells and prohibiting water loss thereby leading to normal cell functions. Apart from drought tolerance, drought avoidance is another common mechanism to resist drought in annual plants (Madhava Rao, 2006; Athar and Ashraf, 2009; Salehi-Lisar *et. al.,* 2012). Improving plants capacity for drought tolerance is a tedious process as each method employed for improving plant's drought tolerance capacity has certain limitations and problems due to the complexity of drought effects on plants and the response of plants towards drought.

In order to overcome the deleterious effect of drought stress the strategies followed by plants are broadly classified into drought escape, avoidance and/or tolerance (Fig. 2.1). Drought escape is made possible by a shortened life cycle which helps plants to reproduce before the onset of drought (Bray, 2007; Farooq *et. al.,* 2009; Akhtar and Nazir, 2013). Drought avoidance mechanism results from an overall morphological change in plants for the preservation of a high water potential. This is mainly achieved by reducing water loss by low stomatal conductance, an extensive and prolific root system to increase water uptake from soil, cuticle and hairy leaves etc. (Bray, 2007; Farooq *et. al.,* 2009). Tolerance

mechanism includes physiological and molecular mechanisms such as accumulation of compatible solute and osmotic adaptation, alteration in metabolic pathways and induction of antioxidant system (Salehi-Lisar, and Bakhshayeshan-Agdam, 2016). At the molecular level, response towards abiotic stress, such as drought, involves many stress regulatory networks, comprising the participation of signaling molecules, transcription factors, stress-responsive genes coding for proteins with protective roles against stress, including peroxidases, aquaporins, chaperones, LEA proteins and various hormones (Golldack, 2014). Further for the development of transgenic plants tolerant to drought, candidate genes were selected among those involved in the stress regulatory networks (Todaka *et. al.,* 2015).

2.3.1. Drought stress responses of rubber plants

In a perennial crop such as *H. brasiliensis* yield is retrieved throughout the year and the factors governing yield are intricate due to intrinsic attribute of latex production, as latex is the end product of several biochemical steps. So cultivation of rubber in non-marginal areas for yield and secondary attributes hence become more challenging. The response to prolonged drought stress in plants leads to severe problems including decreased water flux, closure of stomata and lesser rate in carbon dioxide fixation. Hydraulic failure and carbon starvation can result in the death of plants (Zeppel *et. al.,* 2013). Common features under drought stress in many plant species is the inhibition of photosynthesis and energy dissipation reflecting the thermostability of PSII and changes in electron transport (Zhou *et. al.,* 2007; Brestic *et. al.,* 2012; Yan *et. al.,* 2013; Zivcak *et. al.,* 2014). Osmotic adjustment is another mechanism associated with plant anti-drought character which involves the accumulation of compatible solutes (low-molecular-weight organic osmolytes), such as proline, fructans, sorbitol, mannitol, glycine betaine, oligosaccharides and sucrose (Rhodes and Hanson, 1993). Maintenance of osmotic equilibrium and protection of membranes as well as macromolecules is in turn attained due to the accumulation of these large compounds (Hoekstra *et. al.,* 2001; Couee *et. al.,* 2006). Reactive oxygen species get generated doubtlessly as a consequence of drought stress in mitochondria and chloroplasts (Apel and Hirt, 2004; Asada, 2006), so enzymes involved in ROS scavenging play important roles in drought tolerance responses.

Superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), catalase (CAT), monodehydroascorbate reductase (NADH), etc are some of the ROS scavenging enzymes.

Growth retardation is the immediate result of drought stress in rubber tree seedlings and mature tapping trees. Shortening of tapping period, blocking latex flow for low water supply, decrease in dry latex contents, increase in occurrence of TPD (tapping panel dryness), and under severe drought conditions even death may occur in mature tapping trees (Huang and Pan, 1992). In order to select and breed for drought-tolerant rubber tree clones many strategies and indices were used, such as leaves with more epicuticular wax (Gururaja Rao *et. al.,* 1988), drought tolerant rootstock (Ahamad, 1999) etc. For explaining drought tolerance mechanism in rubber tree, hydraulic mechanisms were also used (Ayutthaya *et. al.,* 2011). The research on drought tolerance mechanism research on rubber tree focussed mainly in the area of physiological response and anatomy (Nair *et. al.,* 1996). During the growth and developmental stages of rubber tree the effect of drought varied on different physiological metabolism (Devakumar *et. al.,* 1988). Osmoregulation, laticifer turgor pressure (Ranasinghe and Milburn, 1995), transpiration coefficient (Nair *et. al.,* 1996), membrane integrity, low solute potential (Ayutthaya *et. al.,* 2011) were related to the drought tolerance potential in rubber trees. In plant seedlings drought significantly reduced the relative growth rate and RWC, and inhibited photosynthesis (Li *et. al.,* 2011). Drought stress adaptation was reported to be a complex process involved in antioxidative enzymes, energy biosynthesis and osmoregulation related genes in chloroplasts and mitochondria in rubber tree seedling (Li-feng Wang, 2014).

The impact of uncertain weather pattern will be more pronounced during the early establishment and growth of young rubber plants. Monsoon season is the ideal planting season in the traditional rubber growing region in South India. In recent years uncertainty in rainfall and high temperature are making difficult for scheduling various farm operations like planting. Occurrence of unexpected dry spells and bright sunny days with high temperature during monsoon season increased casualty in young plantations. Increasing temperature and soil moisture deficit during summer in traditional rubber growing areas are major constraints for survival of young plants. In a recent survey conducted in central part of

Kerala it was observed that in addition to the recommended management practices like mulching and shading, life saving irrigation was being increasingly practiced. During summer (2010 March/April) almost 18% holdings in which planting were taken up in 2009 were irrigated (Jessy *et. al.,* 2010). Life saving irrigation was not a usual practice in the traditional rubber growing regions until very recently. However, in recent times farmers started practicing life saving irrigation to save their young plants from adverse drought conditions. Chlorophyll bleaching and leaf scorching were observed in unirrigated plants whereas growth was much better in irrigated plants.

Various physiological, biochemical and molecular studies were conducted in different clones of rubber to identify the stress tolerant clones and also to identify the factors contributing to drought tolerance. Most of the studies on mechanism of drought tolerance in rubber plants were carried out in young plant. The stress tolerance traits in one-two year's old plants were analyzed by various photosynthetic parameters. There was a significant reduction in photosynthetic oxygen evolution rate, maximum potential quantum yield of photosystem II (PS II), effective quantum yield of PS II (ΦPS II) and photosynthetic electron transport rate in the leaves of drought imposed plants (Annamalainathan *et. al.,* 2006). However, the clones, such as RRII 430 and RRIM 600 showed relatively small inhibitions in ΦPSII and photosynthetic rate as compared to other clones (Annamalainathan *et. al.,* 2010). This is attributed to their inherent tolerant characters. Gas exchange and fluorescence studies also revealed that the clone RRII 430 is more likely to endure drought stress than the other RRII 400 series (Sumesh *et. al.,* 2011). The optimum temperature for photosynthesis in *Hevea* ranged from 27 to 33°C and there was very severe inhibition in photosynthesis at temperatures below 10°C and above 40°C (Zongdao and Xuequin, 1983). Water use efficiency is another parameter studied in many clones. RRIM 600 and RRII 430 are physiologically better adapted and can withstand water stress for a relatively longer period of time.

In grown up trees the drought induced yield reduction was more pronounced in non-traditional areas than traditional rubber growing regions in India. The summer yield depression in traditional areas was reported to be 30-50% of annual yield in the clone RRII 105. In Konkan region, a very drought

prone area in India where rubber is cultivated, rubber yield during summer months was only 10% of the total yield obtained for the whole year (Chandrashekar *et. al.,* 1990).

Severe inhibition of photosynthesis, transpiration and increased stomatal resistance were observed due to soil moisture deficit in sub-humid rubber growing regions (Mohankrishna *et. al.,* 1991). High light intensity inhibited photosynthesis in the drought stressed *Hevea* leaves. Leaf photosynthesis rates were higher when measured at sub-saturating than at saturating light intensities in the cold stressed and drought stressed *Hevea* leaves (Devakumar *et. al.,* 1999). Therefore, it appears that the shaded leaves in the canopy contribute to the total carbon balance of the plantation more than the exposed leaves during stressful environmental conditions (Devakumar *et. al.,* 1999). Shading young *Hevea* plants exposed to severe environmental stresses is therefore highly advisable.

Severe inhibition in metabolic activity of clone RRII 105 during drought stress was reported on biochemical investigations by Sreelatha *et. al.,* (2007). Sumesh *et. al.,* (2011), by measuring gas exchange parameters under drought stress reported RRIM 600 to be less inhibited and RRII 414 to be severely affected. Gene expression studies of few drought responsive transcripts in young *Hevea* clones identified MAPK to exhibit a strong association with drought tolerance (Luke *et. al.,* 2015). Recent expression studies on MicroRNAs (miRNA) of *H. brasiliensis* under drought stress identified a novel miRNA, HbmiRn_42 that got upregulated in tolerant clones indicating the strong association of it in drought stress tolerance (Kuruvilla *et. al.,* 2016).

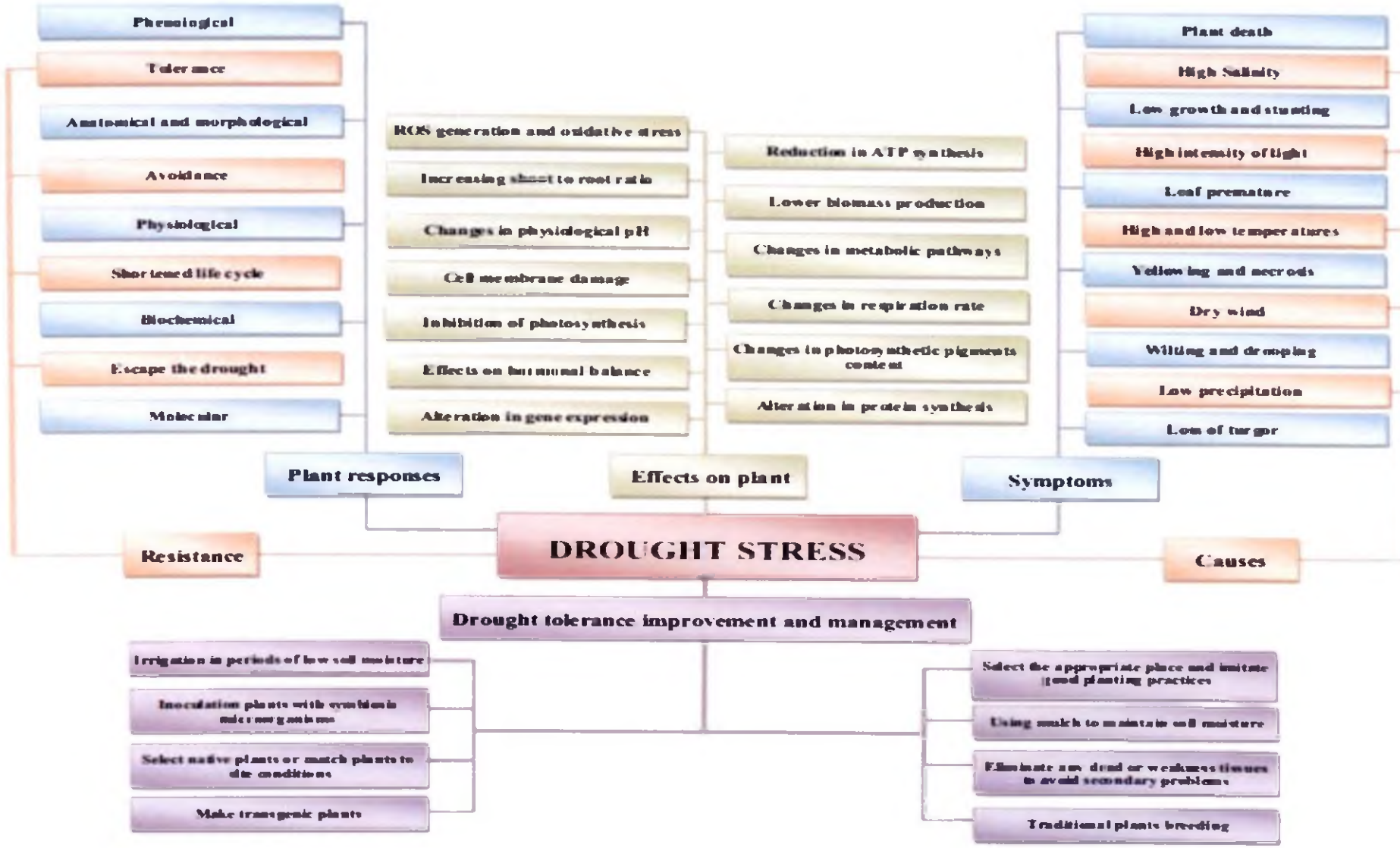


Fig 2.1. Causes of drought and its effects on plants, symptoms of water stress, plant responses to drought and mechanisms involved in resistance, and some useful strategies for drought management (Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

2.4. Proteomic acclimation to drought stress in plants

Profound changes in proteome composition occur while plants acclimatize to environmental stress. Being directly involved in plant cell structure and metabolism proteins represents a crucial component of plant stress response. Proteins are products of genes and they are much closer to the resulting phenotype as they act as direct effectors of phenotype. The changes that occur in the cells of plants that are subjected to stress ultimately depend on the synthesis, modifications and interaction of proteins participating in various metabolic, biosynthetic, degradation and signaling pathways (Hakeem *et. al.,* 2012; Han *et. al.,* 2014; Hu *et. al.,* 2015). Crops may modulate the abundance of candidate proteins, either by synthesizing novel proteins or by increasing their expression primarily related to plant defense system in response to a stress. To unravel the possible relationships between protein abundance and plant stress acclimation, functional analysis of translated regions of the genome is necessary and one of the best options to make it possible is through proteomics techniques. Studies on the gene expression pattern, proteomics and transcriptomics have identified the regulation and activation of several drought stress-related transcripts and proteins, which are broadly classified into two groups *viz.* functional proteins and regulatory proteins. Proteins that probably function in protecting cells from dehydration are included in the group of functional proteins while those involved in regulation of signal transduction and transcription as part of drought response are included in regulatory proteins (Shinozaki *et. al.,* 2003). From a molecular point of view drought tolerance mechanism involves major regulatory and functional proteins. Functional proteins involve protective proteins such as LEA and heat-shock proteins and enzymes associated with the synthesis of osmotically-active compounds, ROS (reactive oxygen species) scavengers, osmolytes like fructan, proline, trehalose, glycine betaine, polyamines and mannitol (Wani *et. al.,* 2016). Differential expression of proteins occurs in different plant organs under drought which show distinct responses (Hao *et. al.,* 2015). Further studies to elucidate molecular regulatory mechanism of drought tolerance are also made possible through proteomics.

2.4.1. Protective Proteins: LEAs, HSPs and other Chaperones:

LEA proteins constitute a diverse group of families of hydrophilic protective proteins whose expression was first described during seed maturation (Galau *et. al.,* 1986). Their intracellular accumulation occurs in response to abiotic stress condition such as drought and cold (Battaglia *et. al.,* 2008). LEA-type proteins are encoded by ERD (early responsive to dehydration), RD (responsive to dehydration), COR (cold regulated), KIN (cold inducible) and RAB (responsive to ABA) genes in different plant species (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). LEA is assumed to perform their protective role due to their hydrophilic nature which give these proteins with the capacity of sequestering ions that are concentrated during cellular dehydration and also to perform chaperone-like function by retaining water molecules which helps in preventing protein aggregation and protecting cellular components from denaturation and inactivation during water deficit situation (Bartels and Sunkars, 2005; Vinocur and Altman, 2005; Goyal *et. al.,* 2005).

Heat shock proteins were first discovered by the Italian Scientist R. Ritossa on gene expression of the puffing in the chromosomes of *Drosophila melanogaster* after exposure to heat (Al-whaibi, 2011). On the basis of their approximate molecular weight heat shock proteins in plants have been classified into (i) Heat shock proteins 100 kDA i.e., HSP 100, (ii) HSP 90, (iii) HSP 70, (iv) HSP 60 and (v) small heat shock proteins (sHSPs) where the molecular weight ranges from 15 to 42 kDa (Schlesinger, 1990; Schoffl *et. al.,* 1998; Kotak *et. al.,* 2007). The HSP family in plants is larger in comparison to all other kingdoms probably due to the reason that plants have better adapted to a wide range of stresses. Heat shock proteins production is one of the prominent responses of virtually all cells under high temperature conditions. These proteins were first discovered in cells that were exposed to high temperature and hence were named as heat shock proteins. HSPs expression is transcriptionally regulated mostly by Heat Shock Factors (HSF) (Augustine, 2016; Marco *et. al.,* 2015).

Table 2.1. Five major classes of plant HSPs/molecular chaperones and their subfamilies (Wang *et. al.*, 2004)

Classes	Representative members	Intracellular Localization	Major functions	Reference
HSP70Subfamily:			Preventing aggregation, assisting refolding, protein import and translocation, signal transduction, and transcriptional activation	Boston *et. al.*, 1996 ; Vieriing 1991 ; Morimoto 1998.
Dnak	Hsp/Hsc70	Cytosol		
	Hsp70	Chloroplast, Mitochondria		
	Bip	Endoplasmic Reticulum		
HSP110/SSE	Hsp91	Cytosol		
Chaperonin/ HSP60 Subfamily:			Folding and assisting refolding	Boston *et. al.*, 1996 ; Hartl 1996 ; Morimoto 1998 .
Group I	Cpn60	Chloroplast, Mitochondria		
Group II	CCt	Cytosol		
HSP90	Hsp90		Facilitating maturation of signaling molecules, genetic buffering	Boston *et. al.*, 1996 ; Young *et. al.*,2001; Krishna and Gloor 2001 .
	AtHsp90-1	Cytosol		
	AtHsp90-5	Chloroplast		
	AtHsp90-6	Mitochondria		
	AtHsp90-7	Endoplasmic Reticulum		
HSP100/Clp Subfamily:	Hsp100		Disaggregation, unfolding	Schirmer *et. al.*, 1996 ; Goloubinoff *et. al.*, 1999
Class I:	ClpB, ClpA/C			
	ClpD	Cytosol, Mitochondria		
Class II:	ClpM, ClpN	Chloroplast		
	ClpX, ClpY	Chloroplast		
sHSP Subfamily:			Preventing aggregation, stabilizing non-native proteins	Waters *et. al.*, 1996 ; Boston *et. al.*, 1996 ; Vierling 1991
I	Hsp17.6	Cytosol		
II	Hsp17.9	Cytosol		
III	Hsp21	Chloroplast		
	Hsp26.2			
IV	Hsp22	Endoplasmic Reticulum		
V	Hsp23	Mitochondria		
VI	Hsp22.3	Membrane		

Molecular chaperones is another name given to heat shock proteins, which bind and stabilize proteins at various intermediate stages of its formation and also helps in assembly, folding, degradation and translocation across membranes. The role of HSPs in heat tolerance also been shown in mutant or transgenic plants (Kotak *et. al.,* 2007). Induction of HSPs by water stress in several plants has also been reported (Bartles and Sunkar, 2005) and it has also been reported that plants vary greatly in the type of HSPs as well as in the amount of expression (Hamilton *et. al.,* 1996). Table 2.1 summarizes the five major classes of HSPs and their subfamilies in respect to plant tolerance to various stresses.

In addition to the major families certain other proteins are also reported with chaperone functions such as protein disulfide isomerase and calnexin/calreticulin assisting in protein folding in the endoplasmic reticulum (ER). Molecular chaperones/HSPs are located in both cytoplasm and organelles, such as mitochondria, chloroplast, nucleus and ER (Augustine, 2016).

2.4.2. Role of heat-shock proteins

The function of any protein is determined by virtue of its formation and folding into 3D structures (Levitt *et. al.,* 1997). For the formation of 3D structure 50% of principle amino acid sequence is required (Dobson *et. al.,* 1998). This shows the importance of the role of HSPs in folding of other proteins. HSPs are induced by heat or any other stresses at any stage of plant growth and are usually cytosolic proteins which play a major function in various intracellular processes. The key steps for the survival of a cell under stress rely on maintaining the proper conformation of the protein and also by preventing the aggregation of non-native proteins. HSPs in normal cellular processes are responsible for protein folding, degradation, translocation and assembly, stabilizing proteins and membranes, and during stress condition, they assist in protein refolding. Stresses including high temperature make it more difficult for proteins to form proper tertiary structures and it may also result in unfolding of some already structured proteins. When left uncorrected it may form aggregates resulting in the death of the cell. To deal with these HSPs gets induced rapidly at high levels (Wang *et. al.* 2004). Re-establishment of protein conformation and thereby cellular

homeostasis is achieved by HSPs and thus playing a crucial role in protecting the plants against various stresses. Based on earlier reports the role of HSPs can be categorized into three: (i) refolding of denatured proteins; (ii) participation in the finalization of newly synthesised proteins; (iii) removal of aggregated protein (Trent, 1996). Moderate to low level expression of HSPs under normal condition is also observed because of their essential roles they play in protein maintenance, such as proper folding of newly synthesised protein. Studies have also proved that HSPs are involved in maintenance of membrane integrity during stress (Tsvetkova *et. al.*, 2002). Altering the biochemical process is the way by which HSPs gets involved in drought adaptation (Iba, 2002). An illustration of part of chaperone machine that operate in the cytosol is depicted in (Fig 2.2).

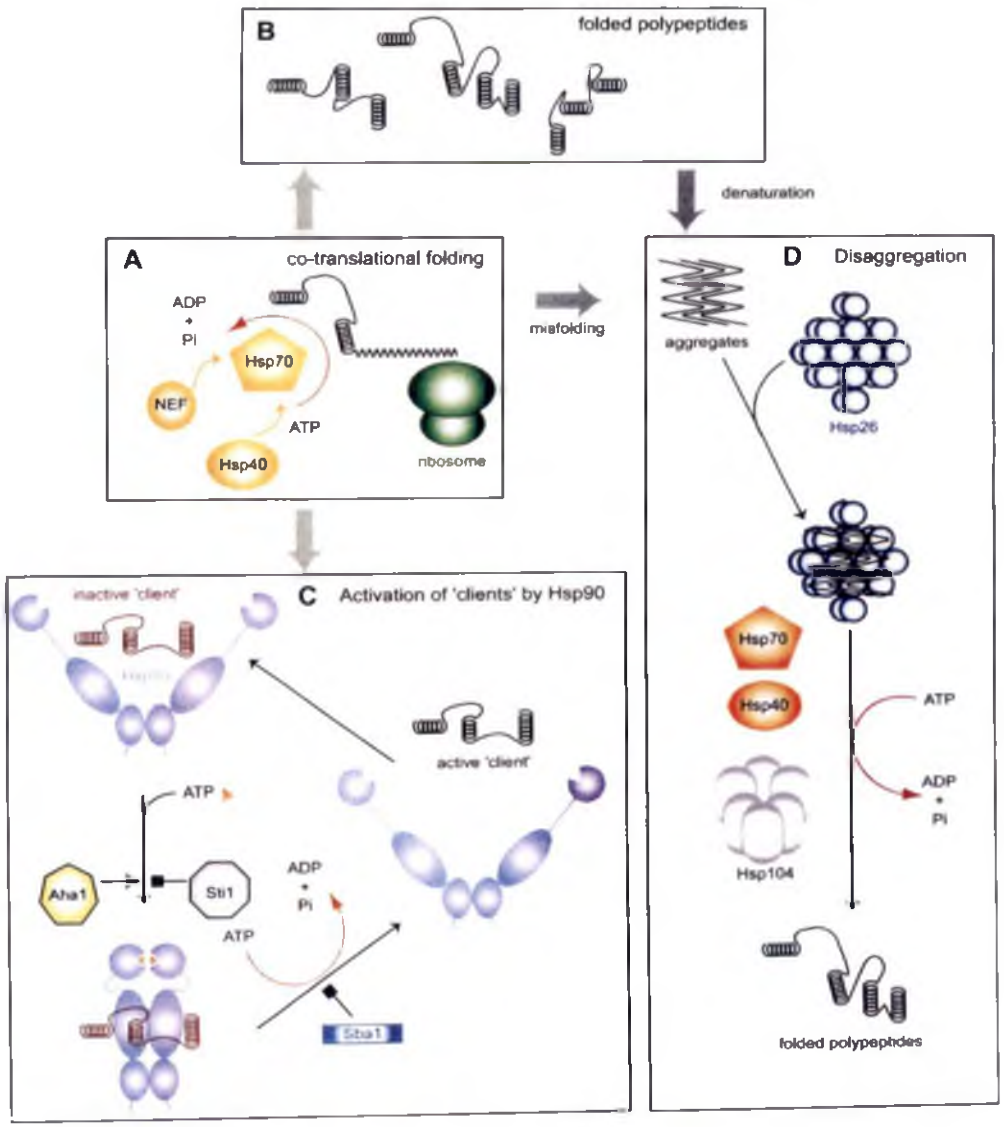


Fig 2.2. Illustration of part of the chaperone machines that operate in the cytosol (Panaretou and Zhai, 2008).

2.4.3. Discoveryof sHSPs:

Small heat shock proteins (sHSPs) were identified initially as ones having a relatively small subunit sizes in *Drosophila melanogaster,* in comparison with those 'larger' ones such as HSP60, HSP70, HSP90 or HSP100 family members. sHSPs were actually also detected during initial attempts to identify newly synthesised proteins from the tissues or individuals of the fruit fly *Drosophila* upon heat shock treatment (Tissieres *et. al.,* 1974; Lewis *et. al.,* 1975; Koninkx, 1976). By fingerprinting analysis of trypsin-cleaved peptides four different forms of small heat shock proteins of 27, 26, 23 and 22 kDa were then clearly distinguished from *Drosophila* tissues (Mirault *et. al.,* 1978). The DNA fragment encoding these four HSP genes located at chromosomal subdivision 67B were isolated and sequenced and it was revealed that their predicted amino acid sequences were homologous to each other and also to that of the mammalian α-crystallin proteins known to be richly present in the eye lens and to form large oligomers (Ingolia and Craig, 1982).

Later proteins homologous to the fruit fly sHSPs and mammalian α-crystallins were subsequently identified from such species as bacteria (Booth *et. al.,* 1988; Nerland *et. al.,* 1988; Verbon *et. al.,* 1992; Lee *et. al.,* 1992; Allen *et. al.,* 1992), higher plants (Key *et. al.,* 1981; Nagao *et. al.,* 1985), and animal (Russnak *et. al.,* 1983). Though amino acid sequences among HSP70s and HSP60s showed high level of conservation that for sHSPs was found to be far less pronounced (de Jong *et. al.,* 1993; Sauer and Durre 1993). sHPS from different sources have been commonly found to be present as large insoluble heat shock granules which are resistant to detergent extraction in the nucleus under heat shock conditions, they were found to be soluble and perinuclear-located before or after the heat shock treatment (Collier *et. al.,* 1988; Arrigo *et. al.,* 1988). These proteins were found to form large dynamic oligomers (Arrigo and Welch, 1987; Behlke *et. al.,* 1991) and to suppress the aggregation of unfolded client proteins they exhibit chaperone-like activities under *in vitro* conditions (Horwitz 1992; Jakob *et. al.,* 1993; Chang *et. al.,* 1996). Nevertheless, as for many proteins in living organisms, the actual function of this family of proteins, has been elusive.

2.4.4. sHSPs from plants:

All organisms respond to heat stress by elevating the transcription and translation of genes coding for 'heat shock proteins' (HSPs). In higher plants the first observations of HSP synthesis were reported for tobacco and soybean (Barnett *et. al.,* 1980; Key *et. al.,* 1981). From the initial studies on HSPs in plants it was known that plants synthesise a very large number of HSPs with molecular weights ranging between 15 and 25 kDa (sHSPs) when compared to *Drosophila*, *Saccharomyces cerevisiae*, bacteria and humans. In plants, the mRNAs that code for sHSPs were produced at much higher levels during heat stress, that cDNAs encoding these sHSPs were among the first gene sequence cloned from plants (Schoffl and Key, 1982). Along with the numerous sHSP mRNAs the accumulation of mRNAs encoding members of the HSP100/ClpB, HSP90 and HSP70 families is apparent. This abundance and diversity of small heat shock proteins in plants has made their function and evolution of considerable interest. In plants the first sequences of sHSPs were obtained in 1985, and the data revealed that the proteins were homologous to sHSPs that had already been characterized from *Drosophila, Xenopus* and *C. elegans* (Nagao *et. al.,* 1985). The common feature of all sHSPs is a 90-100 amino acid conserved C- terminal domain called the α-crystallin domain (ACD), related to a domain from the vertebrate α-crystallin proteins of the eye lens (Ingolia and Craig, 1982). Now sHSPs are known to be present in all domains of life. Knowledge on the origins and diversity of the sHSPs in plants has been gained in subsequent decades of research. sHSPs in metazoans are found primarily in nucleus and cytosol while plant sHSPs are also present in these compartments, and in addition, nuclear-encoded plant sHSPS have been characterised that are targeted to chloroplasts (Vierling *et. al.,* 1988; Waters and Vierling, 1999a; Van Aken *et. al.,* 2009), mitochondria (Lenne *et. al.,* 1995; Van Aken *et. al.,* 2009), endoplasmic reticulum (Helm *et. al.,* 1995), the nucleus, and peroxisomes (Scharf *et. al.,* 2001; Ma *et. al.,* 2006). Drosophila is the only organism other than plants known to have organelle specific sHSP which is reported to localize to mitochondria (Wadhwa *et. al.,* 2010). Cytosolic sHSPs of angiosperms are further grouped into five or more families that originated hundreds of millions years ago and show evidence of continued diversification (Waters and Vierling,

1999b; Waters *et. al.,* 2008; Siddique *et. al.,* 2008; Lopes-Caiter *et. al.,* 2013). The evolution of the diverse land plant sHSPs has been driven by the sessile nature of plants preventing them from escaping unfavourable climatic constraints potentially counteracted by sHSP function (Santhanagopalan *et. al.,* 2015). All sHSPs are grouped together as ATP-independent molecular chaperones which are proposed to prevent irreversible aggregation of stress-sensitive proteins is exactly how they function and what these proteins protect is far from determined (Basha *et. al.,* 2012).

The sHSPs are not capable to refold non-native proteins (Ehrnsperger *et. al.,* 1997; Lee *et. al.,* 1997; Veinger *et. al.,* 1998; Lee and Vierling, 2000) but have a high capacity to bind non-native proteins, probably through hydrophobic interactions (Reddy *et. al.,* 2000), and thereby to stabilize and prevent non-native aggregation, facilitating their subsequent refolding by ATP-dependent chaperones such as DnaK system or ClpB/DnaK (Mogk *et. al.,* 2003). sHSPs are the most prevalent in plants among the five conserved families of HSPs (Vierling, 1991). There are six recognised sub families of sHSPs in plants which are highly expressed (Waters *et. al.,* 1996). Multiple sHSPs are synthesised by plants encoded by these six nuclear multigene families; and each gene family encodes proteins found in a distinct cellular compartment such as cytosol, mitochondria, chloroplast and endoplasmic reticulum (Waters *et. al.,* 1996). 13 different sHSPs are grouped into six classes in *Arabidopsis* based on their intracellular localization and sequence relatedness. Multidomain proteins that contain one or more regions with homology to the ACD containing proteins were also identified and these were coded by six open reading frames (Scharf *et. al.,* 2001).

sHSPs in plants respond to a wide range of abiotic stresses including heat, cold, salinity, drought and oxidative stress. Hence it is suggested that there exists a strong correlation between sHSP accumulation and tolerance of plants to stress. *Arabidopsis thaliana* heat shock protein 25.3 (At25.3), was the first organelle-targeted sHSP recognized (Vierling *et. al.,* 1986; 1988), and was considered as the only chloroplast targeted protein. However, recent studies report that At23.5 and At23.6, which were originally defined as mitochondrial proteins is to be targeted to chloroplasts as well (Van Aken *et. al.,* 2009).

Mitochondrial sHSP in maize (msHSP) were shown to be involved in the protection of NADH: ubiquinone oxidoreductase acivity (Complex I) during salinity stress with improved mitochondrial electron transport, though they failed to protect enzymes associated with Complex II (Hamilton and Heckathorn, 2001). Chloroplast sHSPs importance was reported in *Agorstis stolonifera* grass, from which sHSP26.2 was isolated from a heat-tolerant variant, an identical sHSP, sHSP26.2m, with a point mutation that generated a premature stop codon was isolated from the heat sensitive variant that failed to accumulate upon heat stress (Wang and Luthe, 2003). sHSPs which were discovered to be induced by heat stress, the patterns of expression of sHSP are much complex and vary between organisms and developmental stages. Though majority of sHSP genes in *Arabidopsis* show dramatic increases in transcription at elevated temperatures, At14.7, At15.4 and At21.7 show no response at all to heat (Siddique *et. al.*, 2008; Waters *et. al.*, 2008). Data on gene expression studies increases in specific sHSP transcripts under other abiotic stress conditions, in response to biotic stress, in organ specific patterns and in normal development (Siddique *et. al.*, 2008; Waters *et. al.*, 2008; Giorno *et. al.*, 2010; Lopes-Caiter *et. al.*, 2013). sHSP expression in the absence of stress during seed development where transcription and accumulation of class I proteins has also been observed (Almoguera and Jordano, 1992; Coca *et. al.*, 1994; Wehmeyer *et. al.*, 1996; Wehmeyer and Vierling, 2000; Reddy *et. al.*, 2014). But till date it has been difficult to link sHSPs directly to any form of stress tolerance in seeds (Tejedor-Cano *et. al.*, 2010; Personat *et. al.*, 2014). SP family of proteins (Wang *et. al.*, 2002), another class of stress-associated proteins was also discovered which shares some of the features of sHSPs, yet have additional characteristics and functions. sHSPs being abundant in plants which functions in binding and stabilizing denatured proteins suggest that these sHSPs play an important role in plant-acquired stress tolerance (Waters *et. al.*, 1996; Sun *et. al.*, 2002; Wang *et. al.*, 2003).

2.4.5. Functions of plant sHSPs:

Various studies have been conducted to test if plant sHSP expression can provide stress tolerance *in vivo*, both in *E.coli* and plants. From a variety of species the cytosolic class I proteins are reported to enhance survival of *E.coli* cells treated at 50°C for varying periods of time (Table 2.2). Further studies have extended these observations to include some degree of tolerance to cold, osmotic and salinity stress. A recent study has also reported that the expression of a mitochondrial sHSP in *E.coli* imparts with tolerance to salt and arsenic (Table 2.2).

Table 2.2. Phenotypes of *Escherichia coli* cells expressing plant sHSPs (Santhanagopalan *et. al.*, 2015)

Plant species	sHSP	Class	Phenotype	Reference
Oryza sativa (rice)	Os16.9	I	Heat tolerance	Yeh *et. al.*, (1997)
Castanea sativa (chestnut)	Cs17.5	I	Tolerance at 50 °C and 4 °C	Soto *et. al.*, (1999)
Nicotiana tabacum (tobacco)	Nt18.3	I	Tolerance to 50 °C	Joe *et. al.*,(2000)
Nicotiana tabacum (tobacco)	Nt18.0	I	Tolerance to 50 °C	Smykal *et. al.*,(2000)
Oryza sativa (rice)	Os16.9	I	Heat tolerance	Yeh *et. al.*, (2002)
Rosa chinensis (rose)	Rc17.8	I	Heat, salt, oxidative stress tolerance	Jiang *et. al.*, (2009)
Daucus carota (carrot)	Dc17.7	I	Enhanced viability at 2 °C	Song and Ahn (2010)
Daucus carota (carrot)	Dc17.7	I	Salt tolerance	Song and Ahn (2011)
Medicago sativa (alfalfa)	Ms23.0	Mito	Salt and arsenic tolerance	Lee *et. al.*, (2012)

Many plant species were transformed to constitutively express specific sHSPs using the 35S promoter to drive expression of the transgene (Table 2.3). Certain reports indicate that the resulting increased level of sHSPs in the absence of stress do not have any major effect on growth of the plants though specific

data are not provided. Many experiments were performed with class I cytosolic proteins, with reports of enhanced tolerance to drought, heat, NaCl, mannitol and H_2O_2. Chloroplast sHSP over-expression has been linked to cold, heat and oxidative stress tolerance, as well as enhanced differentiation of chromoplasts from chloroplasts. Class II cytosolic sHSPs has also been indicated in few studies that contribute to drought, heat, salinity and oxidative stress tolerance (Table 2.3). The tests of stress tolerance in majority of the over-expression studies have been restricted to germination or to young seedlings, and to a very limited range of stress conditions. None of these plants were assessed outside laboratory conditions and to mimic the heat stress that the plants experiences in field or to assess effects on yield were not given full consideration (Santhanagopalan *et. al.*, 2015).

Studies in plants where sHSP expression is inhibited by antisense RNA or studies using sHSP mutants or other methods have been limited. Antisense inhibition of different sHSP expression in different plants such as inhibition of: class I sHSP in carrot cells, chloroplast sHSP in *Arabidopsis*, mitochondrial sHSP in tobacco seedlings was found to have different phenotypic effects. An increase in electrolyte leakage after 50°C was observed in carrot cells (Malik *et. al.*, 1999), decreased viability after 2 h at 39°C in *Arabidopsis* (Chauhan *et. al.*, 2012) although no similar phenotype was reported in the *Arabidopsis* At25.3 mutant (Zhong *et. al.*, 2013) and reduced survival of seedlings after a 2 h stress at 46°C in tobacco (Sanmiya *et. al.*, 2004). All these studies involving inhibition of sHSPs with antisense RNA reports were the results obtained from only a single stress condition. To determine if these sHSPs had overlapping functions in other processes, attempts were also made to generate double mutants but the results were not fruitful. In a nut-shell these *in vivo* studies, along with the diverse patterns of expression of sHSPs and their evolutionary conservation indicate that these proteins have much diverse roles in plants. However, the potential mechanisms that could be responsible for the protective effects observed in plants or cells that over-express sHSPs or the loss-of-function phenotypes in sHSP mutants remains undetermined.

Table 2.3. Studies of sHSP overexpression in transgenic plants (Santhanagopalan *et. al.*, 2015)

Species transformed	sHSP	Class	Phenotypes	Reference
Daucus carota (carrot)	Carrot Dc17.7	I	Reduced electrolyte leakage of leaves incubated at 50 °C (AS[a] – increased electrolyte leakage)	Malik *et. al.*, (1999)
Solanum tuberosum (potato)	Carrot HisDc17.7[b]	I	Reduced electrolyte leakage of leaf pieces at 47 °C, 4 h; more tuber production in vitro at 29 °C	Ahn and Zimmerman (2006)
Oryza sativa (rice)	Rice Os17.7	I	10 day seedlings could re-grow after 6 days no H_2O (soil: −15 MPa), or 3 days in 30 % PEG	Sato and Yokoya (2008)
Arabidopsis thaliana	Rose Rc17.8	I	10 day seedlings, enhanced survival after 45 °C, 2 h; 4 week old seedlings less electrolyte leakage (45 °C,2 h)	Jiang *et. al.*,(2009)
Arabidopsis thaliana	Rose Rc17.8	I	9 day old seedlings had longer roots on mannitol; 4 weeks old plants 15 days no H_2O had more siliques, higher fresh wt on recovery	Jiang *et. al.*,(2009)
Arabidopsis thaliana	*Opuntia streptacantha* Os18.0	I	Enhanced germination rate in NaCl (125–175 mM), glucose or mannitol (6 or 7 %), ABA(3–9 uM); enhanced plant survival of NaCl (150 mM, 14 days), mannitol (5 %, 21 day)	Salas-Munoz *et. al.*,(2012)
Nicotiana tabacum (tobacco)	*Zea mays* Zm16.9	I	Enhanced germination after 40 °C, 10 days or H_2O_2 (5 mM, 20 days); increased root length in 7 day old seedlings 40 °C, 9 h or H_2O_2 ; minor decrease in oxidative stress markers	Sun *et. al.*,(2012)
Oryza sativa (rice)	Rice Os17	I	Enhanced germination, longer roots on NaCl (100–150 mM) or mannitol (50–150 mM); enhanced seedling survival to NaCl (200 mM, 24 h) and air exposure (9.5 h), less oxidative stress on PEG (3 days, 20 %) NaCl (200 mM)	Zou *et. al.*,(2012)
Arabidopsis thaliana	Arabidopsis At17.8-HA[c]	I	Sensitivity of germination and reduced root growth on ABA (3 uM; 12 uM); reduced water loss of detached leaves	Kim *et. al.*,(2013)
Lactuca sativa (lettuce)	Arabidopsis At17.8-HA[c]	I	Sensitivity of germination and reduced root growth on ABA (3 uM, 12 uM) increased dehydration (3 weeks plants, 4 weeks no H_2O) and NaCl tolerance (5 weeks plants, 500 mM NaCl)	Kim *et. al.*,(2013)
Arabidopsis thaliana	Arabidopsis At17.7A	II	Increased plant fresh weight after 17 days w/o H_2O or with NaCl (75 mM)	Sun *et. al.*,(2001)

Table 2.3 Studies of sHSP overexpression in transgenic plants (Continued)

Species transformed	sHSP	Class	Phenotypes	Reference
Arabidopsis thaliana	*Nelumbo nucifere* Nn17.5	II	5 day old seedlings enhanced survival 44 °C, 60–75 min; better germination, growth and higher SOD after accelerated ageing (41 °C, 72 h, 100 % humidity)	Zhou *et. al.*, (2012)
Arabidopsis thaliana	*Lillium davidii* Ld16.45	II	Increased germination after 45 °C (1–2 h) or 1–7 days on NaCl (100–150 mM). Constitutive increase in SOD and CAT in absence of stress. Longer roots after 14 days on H_2O_2 (1–2 mM)	Mu *et. al.*,(2013)
Lycopersicon esculentum (tomato)	Tomato Le21.0	Cplast	Enhanced PSII activity after 4 °C then high light; no PSII protection from heat; enhanced chromoplast differentiation	Neta-Sharir *et. al.*,(2005)
Nicotiana tabacum (tobacco)	*Capsicum annuum* Cp26.0	Cplast	20 % higher O_2 evolution after 42 °C, 2 h or 4 °C, 6 h. ~15 % higher P700 after 4 °C 2 h	Guo *et. al.*,(2007)
Festuca arundinacea (fescue)	Rice Os26	Cplast	6 week old plants, methyl viologen or 42 °C for 12–48 h, 42 °C, reduced electrolyte leakage and lipid oxidation, enhanced PS activity	Kim *et. al.*,(2012)
Arabidopsis thaliana	Wheat Ta26.0	Cplast	35 °C 2 weeks constant, enhanced fresh wt, germination, seed wt, PS. (AS – 2 h 39 °C lethal)	Chauhan *et. al.*,(2012)
Nicotiana tabacum (tobacco)	*Capsicum annuum* Cp26.0	Cplast	4 week old greenhouse plants, 4 °C for 6 h; enhanced PS activities	Li *et. al.*,(2012)
Nicotiana tabacum (tobacco)	Tomato Le25.0	Mito	Increased seedling survival on plates at 48 °C, 2 h. (AS – reduced survival 46 °C, 2 h)	Sanmiya *et. al.*,(2004)
Lycopersicon esculentum (tomato)	Tomato Le21.5	ER	14 day old liquid-grown seedlings recovered from 24 h 10 ug/ml tunicamycin	Zhao *et. al.*,(2007)
Arabidopsis thaliana	*S. cerevisiae* Hsp26	n/a	3 week old seedlings on plates −10 °C, 2.5 h: higher survival, >proline, >soluble sugars, >freezing response gene expression	Xue *et. al.*,(2009)

[a] A S – results from anti-sense inhibition of sHSP expression reported in the same publication
[b] sHSP was introduced with an N-terminal histidine affinity tag
[c] sHSP introduced with a C-terminal HA affinity tag

2.4.6. Mechanism of chaperone function of sHSP:

Based on studies of plant class I sHSPs of wheat (*Triticum aestivum*) Ta16.9 and pea (*Pisum sativum*) homologus protein Ps18.1 the current model for sHSP chaperone activity was defined. Both Ps18.1 and Ta16.9 exist as dodecamers in solution at room temperature like other plant class I sHSPs (Kirschner *et. al.,* 2000; Yoon *et. al.,* 2005; Tiroli and Ramos 2010; Basha *et. al.,* 2010). It is suggested that the sHSP oligomers act as reservoirs of the active dimeric units of the chaperone (van Montfort *et. al.,* 2001; Stengel *et. al.,* 2010). Generally there are four different regulatory stimuli (i) presence of unfolded or partially folded substrates; (ii) changes in the environmental temperature or other stresses; (iii) phosphorylation or more general post- translational modifications and, (iv) formation of hetero-oligomers affecting the association/disassociation equilibrium of sHSPs which lead to their activation (Haslbeck *et. al.,* 2015). Different type of stress can activate the sHSPs by shifting the equilibrium to dimeric form, which can then bind to unfolded or misfolded proteins. The activation mechanism appears to be largely dependent on the ratio of sHSP to substrate and the HSP70/HSP40 system alone is effective in refolding the substrate proteins only if these sHSPs are present at proper or excess concentrations, where soluble and well defined sHSP/substrate complexes form. When substrate proteins are in excess level, sHSPs become incorporated into aggregates of the substrate protein. Later HSP70/HSP40 and members of the HSP100 family becomes necessary for the refolding of the substrates from these aggregate-like sHSP substrate complexes (Lee and Vierling 2000; Mogk *et. al.,* 2003; Haslbeck *et. al.,* 2005; Patel *et. al.,* 2014). This process is passive; indicating that the energy required for performing any conformational changes necessary for their function comes from the ambient environment (McHaourab *et. al.,* 2009). This is different from the usual mechanism of transferring energy used by the majority molecular chaperones, and thereby revealing that sHSPs populate a relatively shallow free-energy surface (Papoian 2008; Zheng *et. al.,* 2012). Fig. 2.3 illustrates how the dimeric units of the sHSP become available to 'stressed' cellular proteins upon activation of the sHSP. A super-transformer model illustrating both the dynamic structures of sHSPs and the variable sHSP-

substrate interactions was proposed recently. According to this model, each sHSP monomer, like a transformer, is able to adapt different conformations, and thereby they can easily assembles into various transformable and dynamic oligomers (super transformer) (Fu, 2015). Hence, sHSPs are an excellent illustration of the idea that protein dynamics are able to regulate and control the protein function and the mechanism seems to be conserved from bacteria to the eukaryotes.

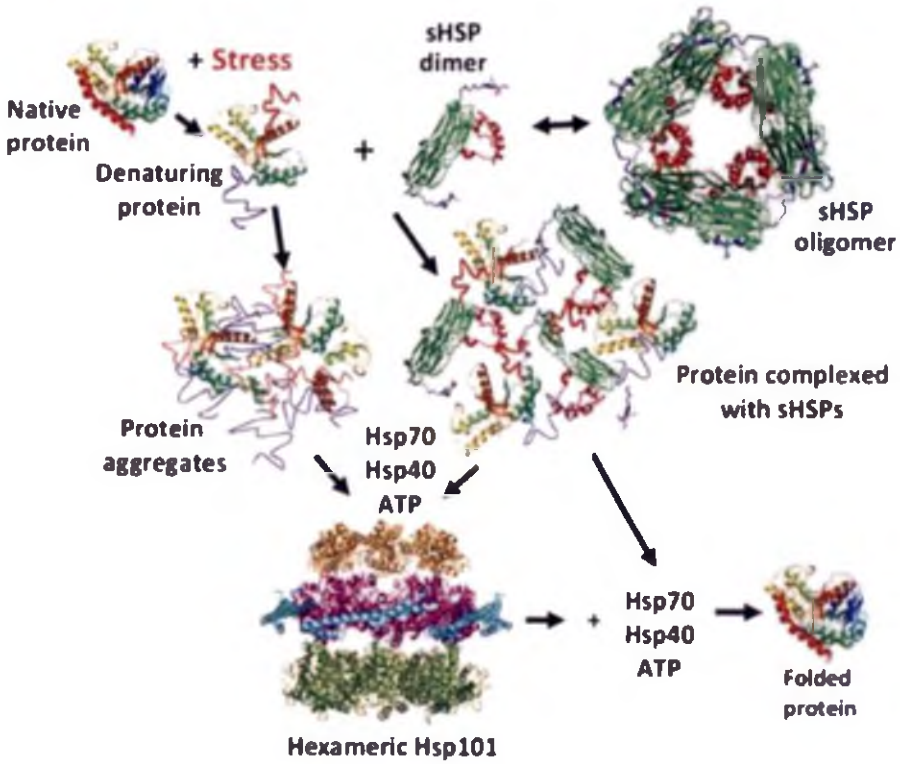


Fig. 2.3. Mechanism of the chaperone function of sHSPs. Under heat stress sHSPs bind non-native/partially unfolded proteins in an energy-independent way and keep them in a folding-competent state in sHSPs/substrate complexes. Subsequently, the substrates are refolded by downstream ATP-dependent chaperone systems, such as HSP70/40, and HSP100

(Santhanagopalan *et. al.*, 2015)

2.4.7. sHSPs based markers

In marker assisted selection, DNA-based molecular markers have been widely explored and implemented in crop improvement programs. Protein-based marker systems also represent molecular markers which were widely used long before DNA markers became popular (Langridge and Chalmers, 2004).

Molecular markers may be coming full circle with protein markers again being proposed as viable genetic markers for marker assisted selection. Reports on application of sHSP as biochemical marker against abiotic stress tolerance is scanty. Sato and Yokoya (2008), over-expressed a rice small heat shock protein gene *sHSP17.7* in the rice cultivar "Hoshinoyume". The expression of the sHSP protein was further analysed by western blotting and reported that the expression of the protein was higher in three transgenic lines which could regrow after rewatering after drought imposition in comparison to wild type. In another study conducted by Chauhan *et. al.*,(2012) wheat chloroplastic sHSP (HSP26) which is highly inducible by heat stress was characterized through transgenic approach using *Arabidopsis*. The heat inducible protein TaHSP26 over-expression was analysed in different transgenic lines by western blot analysis. Through genome-wide expression analysis it was re-confirmed that the tolerance was due to over expression of TaHSP26. Heat shock protein based markers were exploited much in animals, but were less reported in plants.

2.4.8. sHSP in *Hevea*

Annamalinathan *et. al.,* (2006; 2010) studied on the chloroplastic factors responsible for drought tolerance in *Hevea* and reported that sun exposed plants with concomitant drought stress induced a novel 23 kDa chloroplast protein in young plants of rubber. The protein was identified as small heat shock protein by LC/MSMS. The findings indicated that sHSP may play a role in drought tolerance as the more tolerant clones expressed relatively increased amount of the sHSP. Later, Jun *et. al.,* (2015), cloned the sequence of sHSP gene from *Hevea* and the deduced protein showed high identity to chloroplast localized sHSP23.8 and the gene was named as *HbsHSP23.8*. Real Time RT-PCR analysis indicated the expression of this gene was regulated by salinity, drought, low temperature, ethylene and jasmonic acid treatments, indicating the role of *HbsHSP23.8* role in abiotic stress reponses as well as ethylene and jasmonic acid signals in rubber tree. However there are no reports available on the use of sHSP as marker for screening drought tolerance in *H. brasiliensis*.

Chapter III

Studies on Physiological Traits to Identify Stress Tolerance in Hevea Clones

3.1. INTRODUCTION

Natural rubber (*Hevea brasiliensis*) is a tree species native to the Amazon forests. The rubber tree is known as Para rubber plant belonging to the family Euphorbiaceae. It is the most important commercial source of natural rubber accounting around 99% of the world's total natural rubber production. Para rubber plant is a deciduous tree with orthotropic rhythmic growth where the mature tree can attain a height of 25 m and can grow on varying types of soils if deep and well drained. Fairly distributed annual rainfall of not less than 200 cm and a warm equable humid climate where the atmospheric humidity (RH) might be around 70-80% and temperature must be about 20-34° C is necessary for the optimum growth (Jacob *et al.*, 2016).

The traditional Indian rubber plantation industry has been mainly in Kerala and adjoining districts of Tamil Nadu and Karnataka. This region is spread across 80° and 120° N latitude between Arabian sea coast and Western slopes of Western Ghats and their foothills. At present about 25 % of arable land in` Kerala is under this crop and therefore expanding rubber cultivation into newer areas in Kerala is not advisable or feasible. To meet the growing demand for natural rubber it has become necessary to produce more rubber by extending its cultivation to non traditional areas outside the state of Kerala. The Konkan region of the West coast, the Coromandel coast on the East, Northern West Bengal and North Eastern states have been identified as potential areas for rubber cultivation in the country (Hajra and Potty, 1986).

Drought, high solar radiation, low atmospheric humidity, high and low temperatures, high VPD, poor soils etc are the adverse environmental conditions which limit the expansion of rubber cultivation to newer area in many rubber producing countries including India (Sethuraj *et. al.*, 1989; Samarappuli and Yogaratnam., 1998; Jacob *et. al.*, 1999). Stressful environment caused by the above conditions is a limiting factor even in traditional rubber growing belt. The growth and productivity of natural rubber can be influenced by changing climate in the recent past (Satheesh and Jacob, 2011). Plants being sessile organisms often expose to adverse climatic conditions in nature. They must cope with multiple environmental stress factors commonly referred to as "abiotic stress"

which affect every aspects of plant growth; modify plant anatomy, physiology, biochemistry and gene expression. Increasing spell of drought and uncertain weather conditions are reality in this changing climatic regimes. Hence in the present scenario developing smarter and climate resilient clones with increased tolerance to environmental stresses are highly essential.

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) and drought is probably the largest factor which limits the agricultural productivity in general and is the most important factor that prevents the expansion of cultivation of *H. brasiliensis* to new places in habitat available to plants. In India drought and high temperature in the North Konkan and chilling winter in the North East are the two major limiting factors that restrict the growth and productivity of *Hevea* (Jacob *et. al.*, 1999). Summer in the North Konkan can last for more than 6 months from mid-December onwards with practically no rain during this period. Summer in this region is characterized by fast depletion of soil moisture, high temperature and very low relative humidity. 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. Excess light can aggravate the harmful effects of environmental stresses like drought and chilling (Jacob and Nataraja, 1998).

In recent years, it is observed that the stressful environment caused by the above conditions is a limiting factor in the traditional rubber growing areas too. Countries such as India, Thailand and Sri Lanka are experiencing drought stress in the traditional belts and this can be a major constraint in the early stage of establishment of rubber plantations (Samarappuli and Yogaratnam, 1998; Chantuma *et. al.*, 2012; Jessy *et. al.*, 2014). Though, rubber can be grown successfully in non-traditional regions with adequate irrigation during summer period (Vijayakumar *et. al.*, 1998) availability of irrigation water and labor are challenging problems in most of the rubber growing countries.

Manifestations of drought stress include lowering of tissue water content, decline in leaf water potential causing loss of turgidity, 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 (Jaleel *et. al.*, 2009). 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 defence mechanisms. In general most of the damaging effects of irradiation and moisture stress to green leaves occur at the chloroplast membrane and enzyme levels (Oquist *et. al.,* 1995). The PS II and electron transport components of thylakoid membranes are the main targets of photoinhibiton due to the formation of excess active oxygen species during adverse climatic condition (Demmig-Adams and Adams, 1992; Ashraf and Harris, 2013). Drought and high light intensity drastically inhibit light reactions and damage the thylakoid proteins in young plants of *Hevea* (Annamalainathan *et. al.*, 2006). Drought tolerance of crop plants can be considered as the tolerance of moderate level dehydration.

Estimation of typical physiological parameters helps in identifying clones which can tolerate stress. Chlorophyll content in leaves under stress conditions is a measure of plant adaptability to high light and drought stresses at leaf level. One of the basic machineries of photosynthesis is chlorophyll pigments that exist in all plant tissues (Masinovsky *et. al.,* 1992). Chlorophyll content is a good indicator of plant health as bleaching of chlorophyll is a consequence of drought and accompanied oxidative stress. Degradation of chlorophyll occurs when the absorbed energy is excess than what can be used for carbon reduction (Jacob *et. al.,* 1999). Leaf water potential (Ψ_L) is another important character that indicates plant water relation. Plants maintaining better leaf water potential under stress conditions is a good indicator of stress adaptation. A more negative water potential is indicative of plants undergoing severe water deficit stress. The health of photosynthetic systems within the leaf can be measured through chlorophyll fluorescence by using modulated fluorometers which are designed to measure variable fluorescence of photosystem II. The maximum potential PS II activity (Fv/Fm) tests the photosynthetic efficiency in plants since damage to PSII will

often be the first manifestation of stress in a leaf (Maxwell and Johnson, 2000). The efficiency of PSII photochemistry is also measured by the parameter ϕ PSII (effective quantum yield of PS II) giving the rate of linear electron transport and hence considered as an indication of overall photosynthesis. CO_2 uptake by leaves will be limited in drought-induced stomata closure. In such cases, there is an increased susceptibility to photo-damage due to restricted CO_2 availability (Cornic and Massacci, 1996). Hence, gas exchange measurements along with the technique of chlorophyll fluorescence will help to obtain a full picture of the response of plants towards stress.

Therefore, the objectives of the present study were to assess the clonal response of young *Hevea* plants to drought stress by analysing certain crucial physiological parameters under soil moisture deficit condition like estimation of chlorophyll and carotenoid pigments, leaf water potential (Ψ_L), maximum potential rate of photosystem II (dark Fv/Fm), ϕ PSII activity (effective quantum yield of PS II), photosynthetic oxygen evolution rate of leaf and CO_2 assimilation (P_N) rates.

In order to understand the interactive effects of drought and high temperature stress on photosynthesis, the popular clone RRII 105 was grown under different temperature conditions with and without irrigation inside a plant growth chamber and various physiological parameters was studied.

3.2. MATERIALS AND METHODS

3.2.1. Screening of young rubber clones against drought stress based on physiological responses

3.2.1.1. Plant material and growth condition

The experimental plants were raised in polybags at the nursery of Rubber Research Institute of India (RRII), Kottayam, Kerala. Budded stumps of ten popular *Hevea* clones, namely, RRII 105, RRII 208, RRII 414, RRII 417, RRII 422, RRII 429, RRII 430, RRIM 600, PB 260, and Tjir 1 were grown in polythene bags (35 x 65 cm). The polybags were filled with approximately 30 kg laterite rich garden soil per bag. The plants were grown for one year under normal field conditions (twenty plants per treatment) with open sunlight. One set of plants of each clone were subjected to water deficit stress by withholding

irrigation for 10 days during the rain free summer season for three consecutive years. Mid-day sun light load was around 1800 $\mu mol/m^{-2}/s^{-1}$. A second set of plants kept as controls were irrigated on alternate days up to field capacity. Photosynthetic measurements were made in these one year old plants after 10 days of withholding irrigation.

3.2.1.2. Measurement of leaf Water Potential (Ψ_L)

The mid-day (12:00 noon) water potential of the leaf from sun exposed top whorl of irrigated control and drought imposed plants was measured using PSΨPRO water potential system (Wescor, USA). 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 field and collected leaf discs were immediately transferred to the chambers, transported to lab and then observations were taken.

3.2.1.3. Measurement of photosynthetic oxygen evolution

The rate of photosynthetic oxygen evolution by leaf discs of freshly harvested leaf (with an area of 9.2 cm^2) was measured at 25^0C with a Clark type oxygen electrode (Hansatech LD 2/2, King's Lynn, UK). The measurement light (LED) was achieved using a Hansatech LH 36 light source. To avoid any CO_2 limitation, 2% CO_2 was generated in the closed chamber using 100 mM bicarbonate buffer (pH 9.2). The leaf disc was first acclimatized to complete dark for five minutes to achieve full potential dark respiration. The leaf disc was then exposed to chosen light intensity (500 $\mu mol/m^{-2}/s^{-1}$) using a LED source for 5 minutes and photosynthetic oxygen evolution rate was measured (Walker, 1988).

3.2.1.4. Measurement of photosynthetic CO_2 assimilation

An infra-red gas analyzer (IRGA), Li 6400 (LiCOR, Nebraska, USA) was used to measure the net photosynthetic rate (P_N) of the plants. The required measurement light intensity (500 $\mu mol/m^{-2}/s^{-1}$ red light + 10% blue light), CO_2 concentrations (400ppm) and other environmental parameters were monitored and controlled.

3.2.1.5. Assay of quantum yield of PS II

The chlorophyll fluorescence technique is a simple, non-destructive method widely used to assess the physiological state of photosynthetic apparatus during any environmental stress condition (Krause and Weis, 1991). Chlorophyll fluorescence parameters namely, maximum potential rate of photosystem II (dark Fv/Fm), minimal (Fo') and maximal fluorescence under light exposure (Fm'), effective PSII quantum yield (Φ PS II) efficiency of excitation energy capture by open PS II reaction center after exposure to 316 µmole actinic light and electron transport rate of PS II (ETR) were measured by using PAM 2100 and Dual-PAM 100 (Waltz, Germany), (Schreiber *et. al.*, 1998).

3.2.1.6. Statistical Analysis

Two factor ANOVA was performed to test the difference between clones for various physiological activities. Comparisons were made between clones and drought effect interactions using DMRT (Duncan's multiple range tests).

3.2.2. Studies on interactive effects of drought and high temperature in clone RRII 105 grown under growth chamber conditions.

3.2.2.I. Plant material and growth chamber condition

Budded stumps of a popular clone, 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 one year. The plants were then transferred to a plant growth chamber (M/S 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 in separate experiments. The day time light conditions were 400 $\mu mol/m^{-2}/s^{-1}$ for first two hours (6-8 am) in the morning followed by 800 $\mu mol/m^{-2}/s^{-1}$ till 2 pm. After noon there was a decline in light intensity to 400 $\mu mol/m^{-2}/s^{-1}$ till 6 pm. Night time was maintained without any light inside the growth chamber for 12 hours (6 pm-6 am). The RH was set at 75 % inside the growth chamber throughout the study period.

3.2.2.2. Estimation of chlorophyll

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 100 mg 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*: $((12.7_{A663})-(2.69_{A\,645})$ / 1 x 1000 x wt (mg)) x Volume

Chlorophyll *b*: $((22.9_{A645})-(4.68_{\,A\,663})$/1 x 1000 x wt (mg)) x Volume

Total Chlorophyll: $((20.2_{\,A645}+8.02_{A\,663})$/ 1 x 1000 x wt (mg)) x Volume

3.2.2.3. Estimation of carotenoids

The carotenoids were estimated by the method of Lichtenthaler (1987). The total carotenoids were extracted in acetone: dimethyl sulphoxide (1:1) solution. The following calculations were done using the formula:

Calculation:

$((1000xA_{470})-(1.82xCa)-(85.02xCb))/198$

3.2.2.4. Measurement of Leaf Water Potential (Ψ_L)

The water potential of the leaf of the plants inside growth chamber was measured before sampling by using PSΨPRO water potential system (Wescor, USA). 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.

3.2.2.5. Assay of quantum yield of PS II

The chlorophyll fluorescence measurements were made in plants grown in growth chamber 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 2100 (Walz, Germany).

3.2.2.6. Statistical Analysis

The values between irrigated control and drought imposed samples were tested for significance by student's t-test.

3.3. Results

3.3.1. Plant material

When the budded stumps of different clones were raised in polythene bags under nursery conditions uniform plants were obtained after six months of growth (Fig.3.1).

Fig 3.1 Young bud grafted plants of *Hevea* raised in polybags

3.3.2. Measurement of leaf Water Potential (Ψ_L)

The mid-day Ψ_L among irrigated plants did not show any significant clonal differences. However, Ψ_L showed significant differences between the irrigated and drought exposed plants in all the clones. Drought imposed plants recorded significantly less leaf water potential compared to their irrigated counterparts. There were significant clonal differences existing among the

drought imposed plants (Fig.3.2). Clones such as RRIM 600, RRII 429, RRII 430 and Tjir 1 maintained relatively better Ψ_L after 10 days of withdrawal of irrigation; whereas, clones, RRII 105, PB 260, RRII 414 and RRII 422 showed more negative leaf water potential indicating their fair degree of susceptibility to desiccation stress.

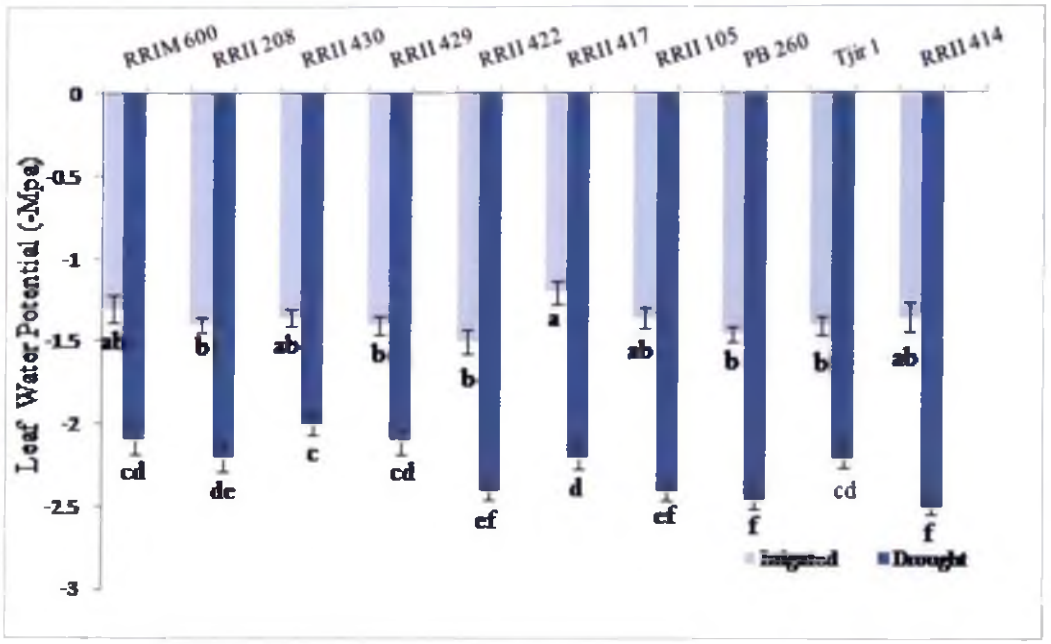


Fig. 3.2. Mid-day leaf water potential of young plants belonging to different clones of *Hevea* grown in polybags. Irrigation water was withheld for 10 days in drought samples. Control plants were irrigated continuously with saturated soil moisture level.

Values are the means ± standard error of ten replicates. Means in a bar graph followed by same superscript letters are not significantly different according to Duncan's multiple range test at P<0.05.

3.3.3. Measurement of photosynthetic oxygen evolution

The photosynthetic oxygen evolution rate was measured in leaves of irrigated and drought imposed plants in the morning hours (9:00 - 10:00 am). The activity was significantly different among the irrigated plants of various clones. The rate of activity was significantly less in PB 260, RRII 430 and Tjir 1 than other clones. Under drought condition, the activity was drastically inhibited compared to their respective irrigated control plants in clones RRII 105, PB 260, RRII 414 and Tjir1. But this was relatively less inhibited in RRIM 600, RRII 430, RRII 208 and RRII 429 (Fig. 3.3). The extent of inhibition was moderate in

clones RRII 417 and RRII 422. Drought exposed plants of RRII 430 recorded comparatively a small reduction from the saturated irrigation level and recorded a stable level of activity indicating its inherent tolerance nature.

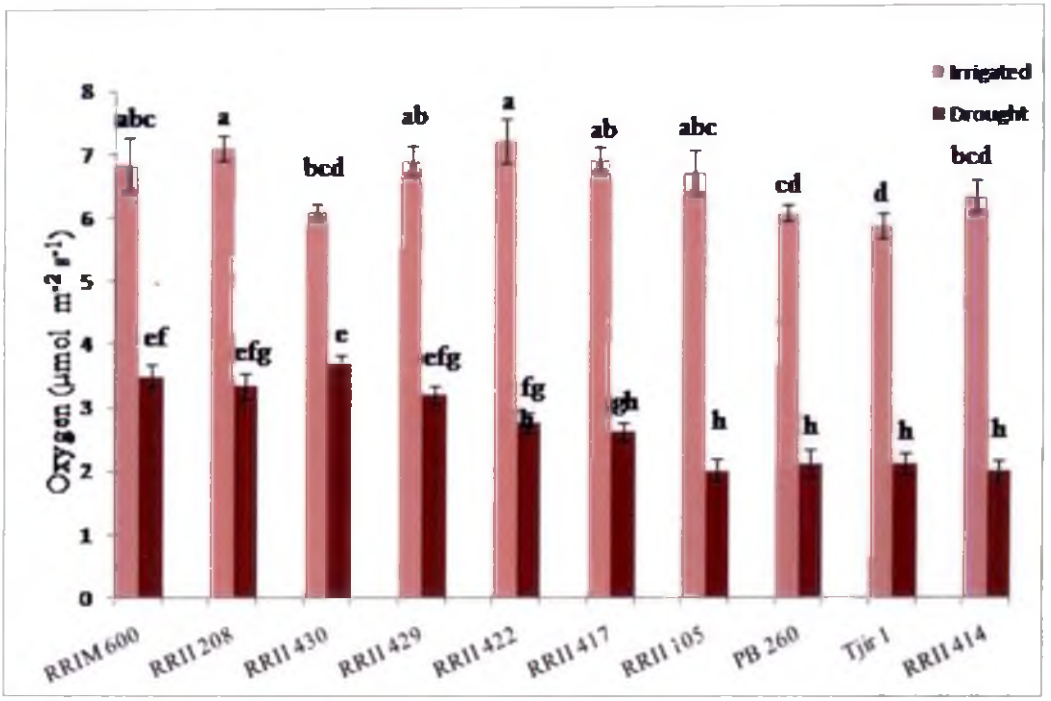


Fig. 3.3. Photosynthetic oxygen evolution rate of leaf discs collected from control (irrigated) and drought imposed (withholding irrigation for 10 days) plants of *Hevea*.

Values are the means ± standard error of ten replicates. Means in a bar graph followed by same superscript letters are not significantly different according to Duncan's multiple range test at P<0.05.

3.3.4. Measurement of photosynthetic CO_2 assimilation

The net photosynthesis rate measured during morning hours (9:00–10:00 am) also showed similar trend as observed for oxygen evolution activity. However, clones such as RRII 417 and Tjir 1 recorded very low P_N after withdrawal of irrigation (Fig 3.4). The drastic reduction in P_N indicated their relative susceptibility to desiccation stress. Although, clones such as RRII 208, RRIM 600 and RRII 430 recorded a significantly lesser rate of P_N than irrigated controls, they maintained comparatively stable level of photosynthesis than other clones even after 10 days of drought in presence of high intensity of solar light.

The drastic inhibition of photosynthetic CO_2 assimilation rate under water deficit condition also substantiated by the results observed on reduced PSII photochemistry in the light-adapted leaves (Fig. 3.4) and such reductions may be a mechanism to down-regulate photosynthetic electron transport to match decreased CO_2 assimilation in water deficit plants.

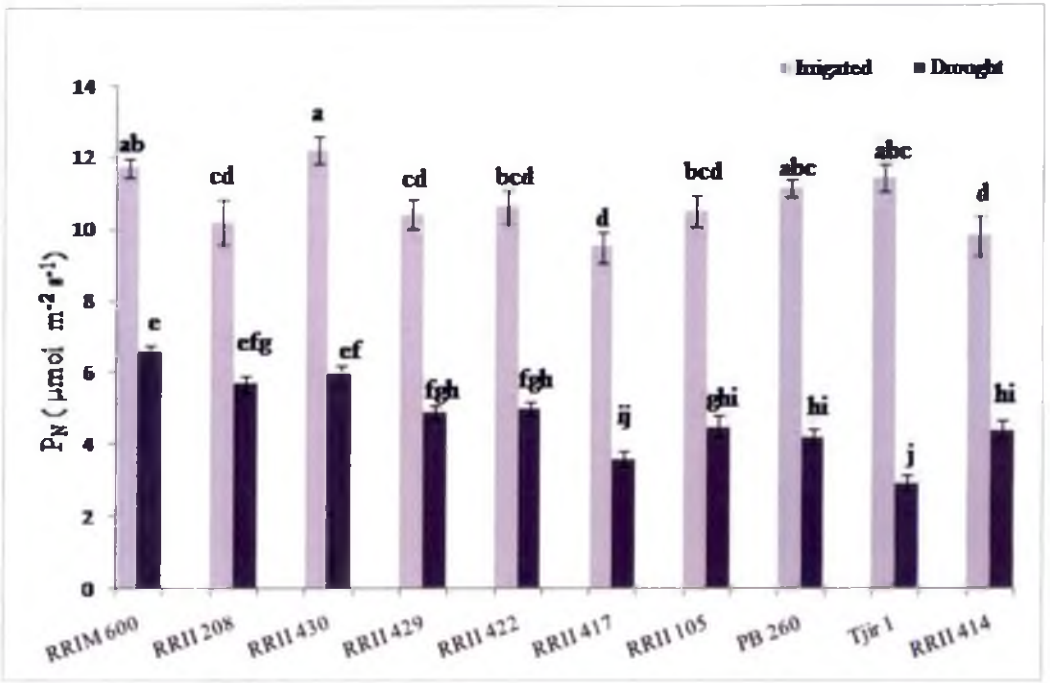


Fig. 3.4. Net photosynthetic rate (P_N) of *Hevea* plants exposed to drought stress for 10 days by withholding irrigation in polybags. The control plants maintained with saturated level of soil moisture condition.

Values are the means ± standard error of ten replicates. Means in a bar graph followed by same superscript letters are not significantly different according to Duncan's multiple range test at P<0.05.

3.3.5 Assay of quantum yield of PS II

In general the effective quantum yield of PS II (ΦPSII) showed a greater decline in all the drought imposed plants when compared to their irrigated counterparts. The magnitude of inhibition was severe in clones PB 260, RRII 105, RRII 414, RRII 417 and RRII 422. On the other hand clones RRIM 600 and RRII 430 showed relatively better stability in PS II activity even after 10 days of drought imposition (Fig.3.5). The extent of inhibition was moderate in RRII 429 and Tjir 1.

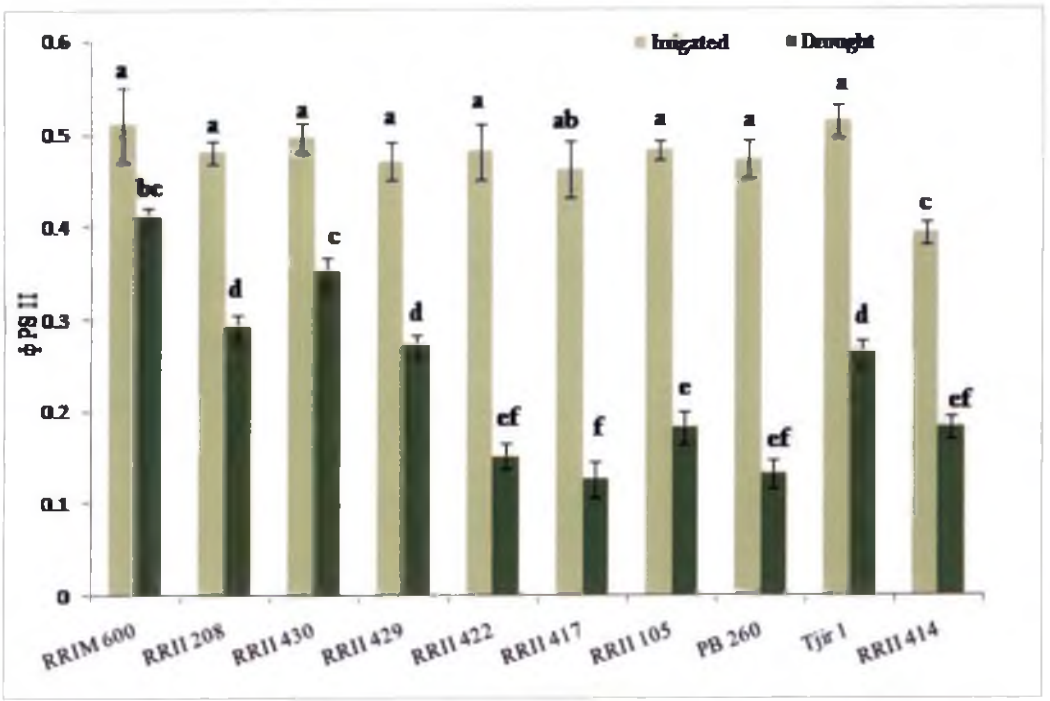


Fig. 3.5. The effective quantum yield of photosystem II (ϕ PS II) in young *Hevea* plants exposed to drought stress for 10 days by withholding irrigation in polybags. The control plants maintained with full saturated level of irrigation.

Values are the means ± standard error of ten replicates. Means in a bar graph followed by same superscript letters are not significantly different according to Duncan's multiple range test at P<0.05.

3.4. Interactive effects of drought and high temperature in clone RRII 105 grown under growth chamber conditions.

3.4.1. 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 contrary plants kept at 40^{0}C showed a drastic bleaching of leaf lamina, drooping and defoliation in the lower whorls (Fig.3.6). These observations indicated high temperature stress aggravates the drought effects in young rubber plants.

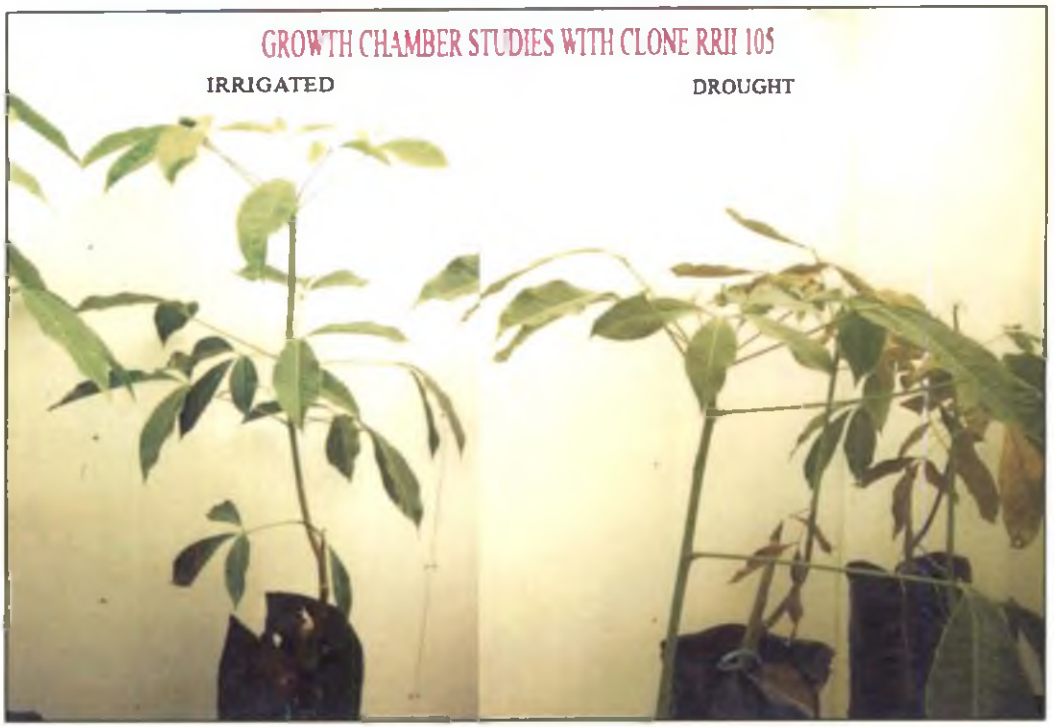


Fig 3.6. Young plants of *Hevea* (clone RRII 105) grown in polybags with saturated irrigation and drought imposed at 40°C. Drought was imposed by withholding irrigation for 5 days inside growth chamber (M/S CONVIRON, Canada)

3.4.2. Photosynthetic pigments content

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. 3.7, Fig 3.8). 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. 3.9). 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. 3.10).

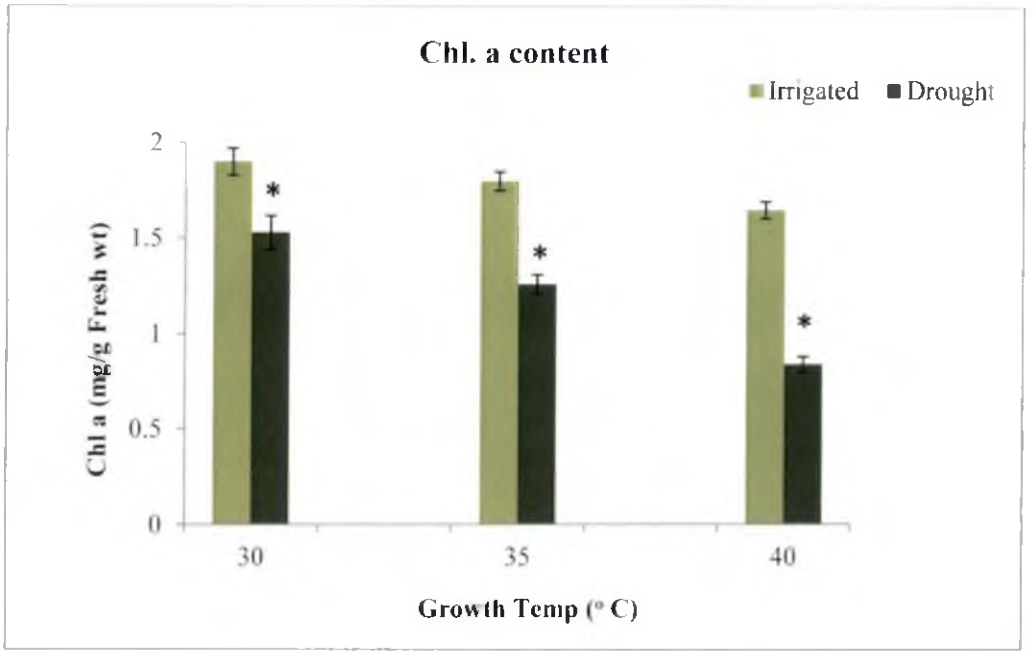


Fig 3.7. Leaf chlorophyll ***a*** content of irrigated and drought imposed young plants of *Hevea* grown at different temperature (° C) under growth chamber conditions.

*Values are the means ± standard error of ten replicates. * indicates the values are significantly different at 5% level.*

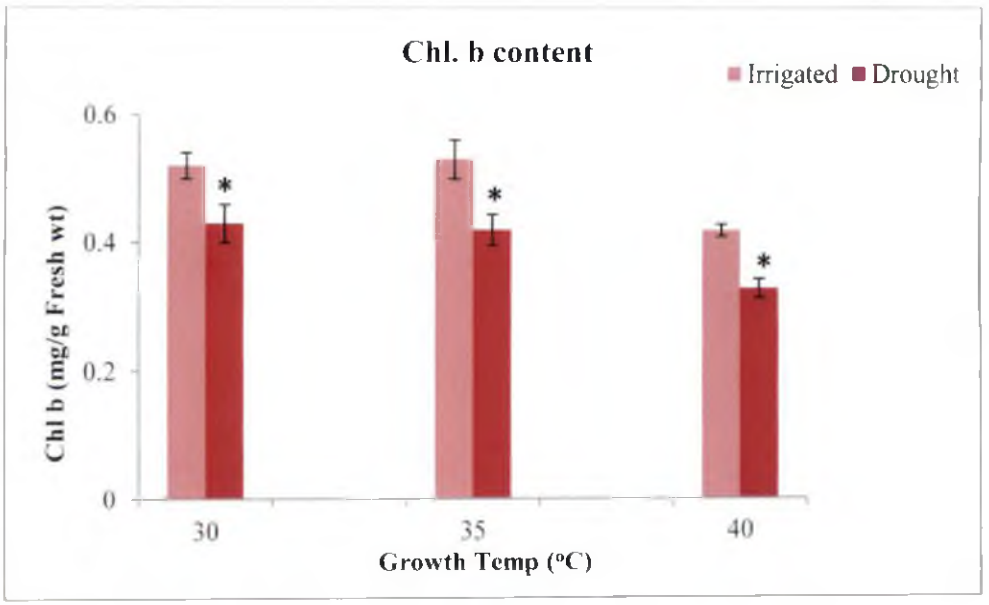


Fig 3.8. Leaf chlorophyll ***b*** content of irrigated and drought imposed young plants of *Hevea* grown at different temperature (° C) under growth chamber conditions.

*Values are the means ± standard error of ten replicates. * indicates the values are significantly different at 5% level.*

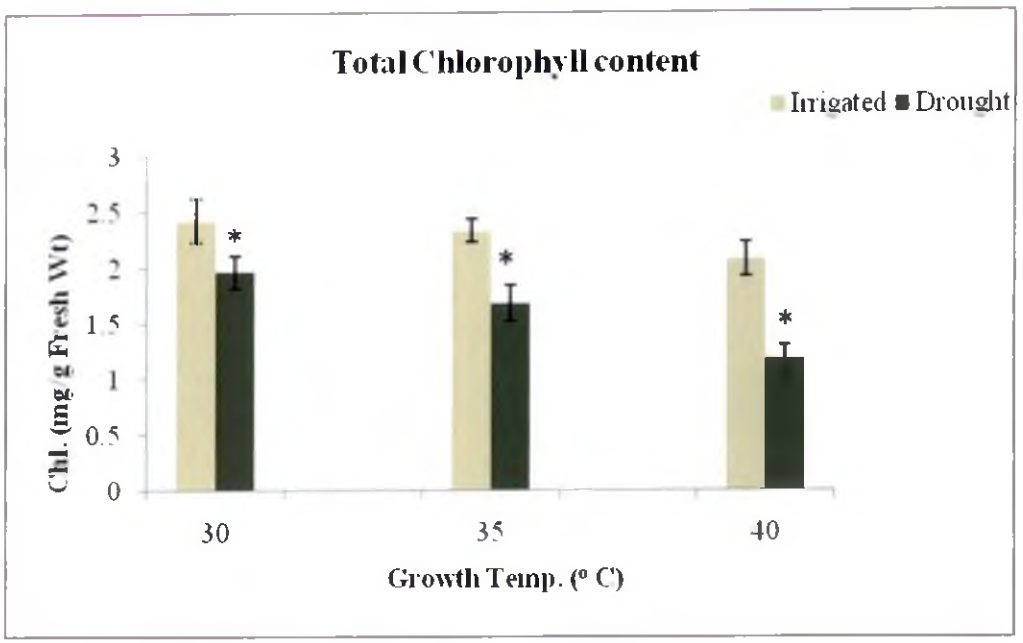


Fig 3.9. Total chlorophyll contents of irrigated and drought imposed young plants of *Hevea* grown at different temperature (° C) under growth chamber conditions.

*Values are the means ± standard error of ten replicates. * indicates the values are significantly different at 5% level.*

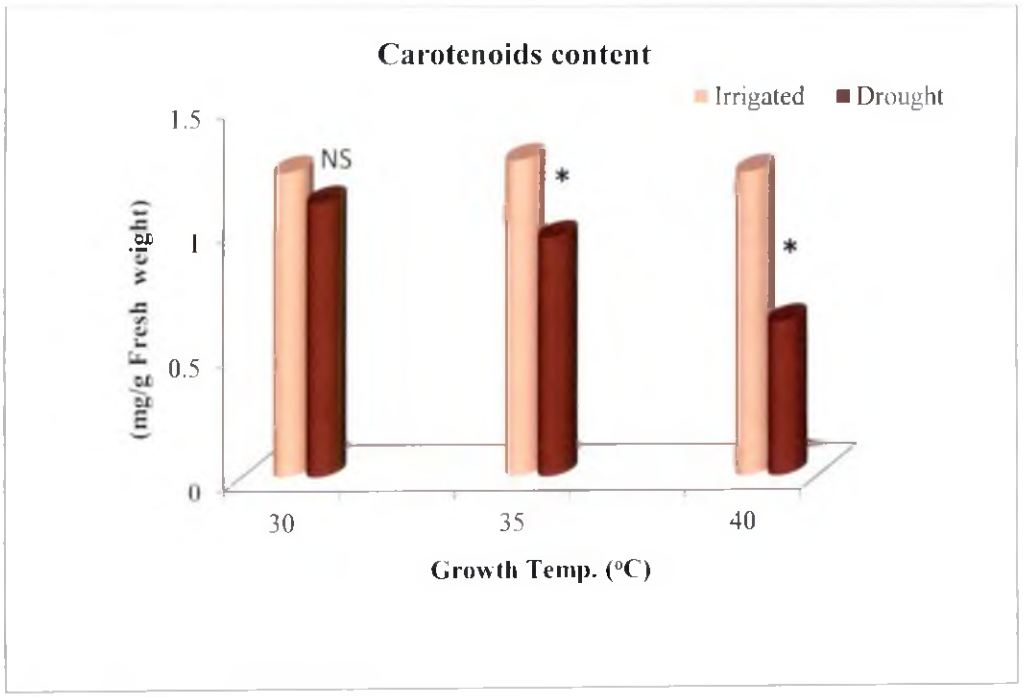


Fig.3.10. Leaf carotenoids content of irrigated and drought imposed young plants of *Hevea* grown at different temperature (° C) under growth chamber conditions.

*Values are the means ± standard error of ten replicates. * indicates the values are significantly different at 5% level.*

3.4.3. 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. High temperature (35^0C and 40^0C) grown plants recorded a drastic reduction in water potential than ambient temperature (30^0C) (Fig. 3.11).

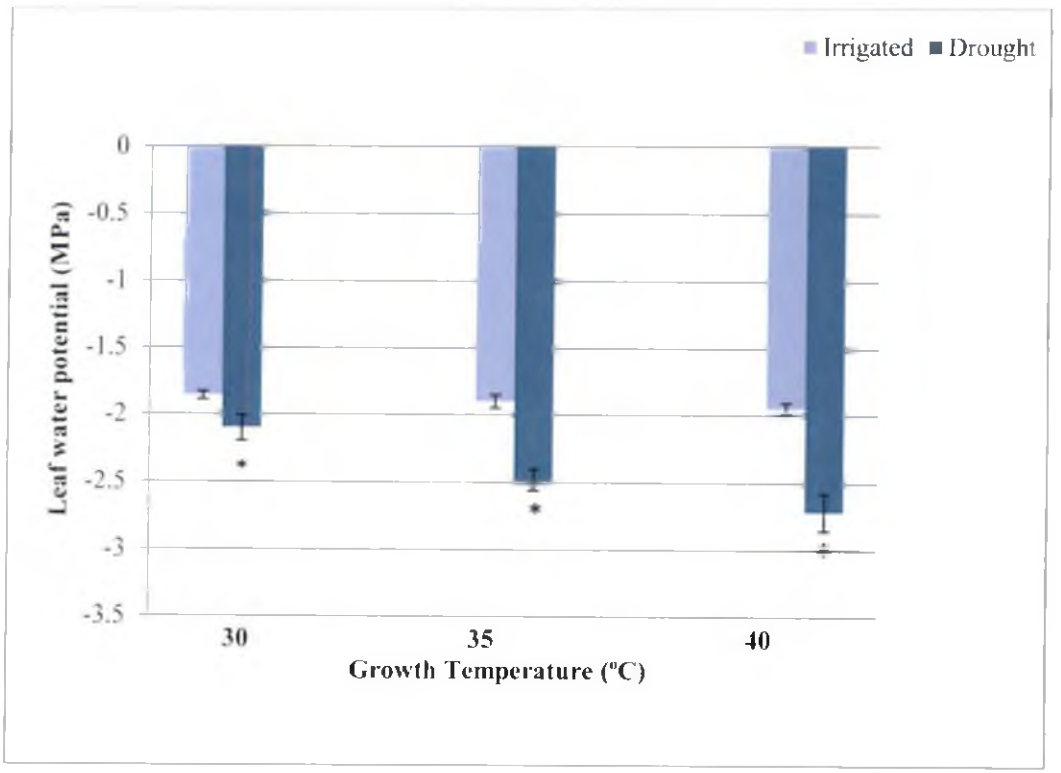


Fig 3.11. Leaf water potential (MPa) of irrigated and drought imposed young plants of *Hevea* at different temperature (° C) regimes. Drought was imposed by withholding irrigation for 5 days under growth chamber conditions.

*Values are the means ± standard error of ten replicates. * indicates the values are significantly different at 5% level.*

3.4.4. Photosystem II Activity

Among the irrigated plants there was no much reduction in ϕPS II activity when plants were grown under 30^0C and 35^0C. When the plant growth temperature increased to 40^0C there was a significant reduction in ϕPS II even in

irrigated plants. After drought imposition the magnitude of reduction of ϕPS II was small at 30^0C and very high at 40^0C (Fig 3.12).

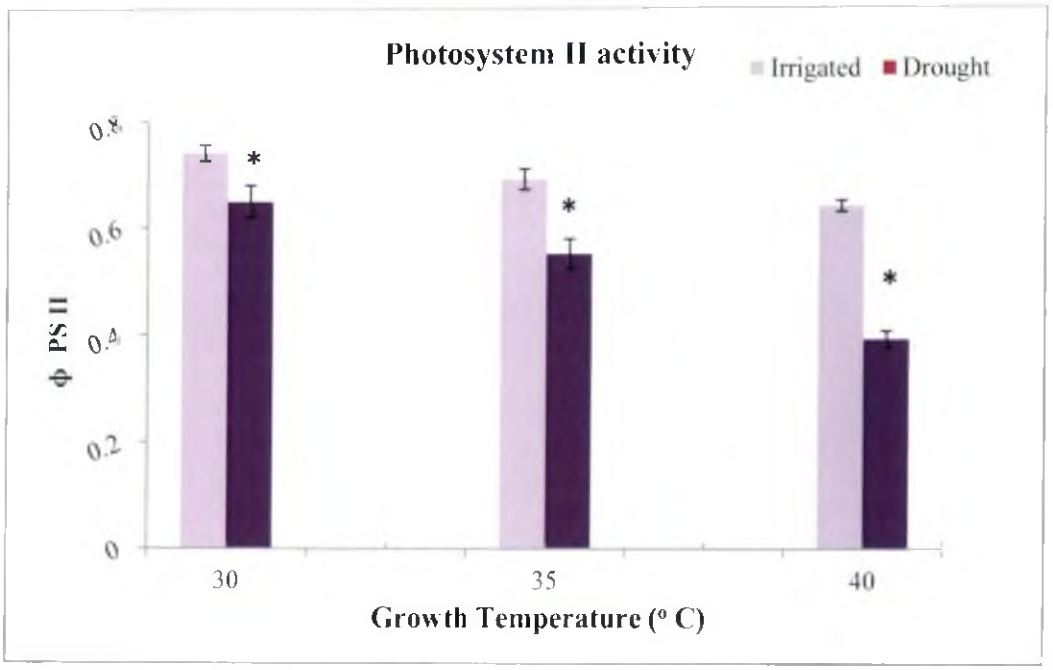


Fig 3.12. Effective quantum yield of PS II (ϕPS II) in irrigated and drought imposed young plants of *Hevea* grown at different temperature (° C) under growth chamber conditions.

*Values are the means ± standard error of ten replicates. * indicates the values are significantly different at 5% level.*

3.5. DISCUSSION

Climate-resilient smart rubber clones need to be developed for expansion of rubber cultivation to regions where environmental stresses are a limiting factor. When plants are exposed to abiotic stresses such as drought and chilling one of the first physiological processes that is inhibited is photosynthesis (Baker, 1996). Drought with concomitant occurrence of high solar light intensity strongly affects photosynthetic events such as stomatal control of gas exchange, photosystem II (PS II) photochemical activities and carboxylation reaction in young rubber plants grown under unfavourable climatic conditions prevailing mostly in non-traditional rubber growing areas (Devakumar *et. al.*, 1998; Jacob *et. al.*, 1999; Chen *et. al.*, 2010). A decrease in net CO2 assimilation is usually observed under water deficit stress. This inhibition may also result from events such as inhibition of electron transport activity, limited generation of reducing power and limitation in overall metabolic activity. Therefore, measurement of

photosynthesis (P_N) can be used as an indicator of stress effect. The physiological process of the response to water stress depends upon the severity and the duration of stress, growth stage at which stress is imposed and the genotype of the plant (Jaleel *et. al.,* 2009).

There was no significant difference in ψ_L among the irrigated plants when comparing ten different clones. However, after exposure to drought condition there was significant difference among the clones. A clone which is able to tolerate water deficit stress manages comparatively better water relationship within the plant. The concept of water potential (ψ_w) defines the thermodynamic or energy status of water within the plant (tissues and cells) and along the soil-plant-atmosphere continuum (Taiz and Zeiger, 1991; Kozlowski and Pallardy, 1997). The value of ψ_w is always negative or near to zero (in the case of pure water). The gradient of potential is the driving force of water flow from less negative towards the more negative values across cell membranes, tissues and in the whole soil-plant-atmosphere continuum. Comparing seasons, Chandrashekar *et. al.,* (1990) suggested anisohydric behaviour in *Hevea* trees under drought condition when leaf ψ_w decreased from -1.3 MPa in wet season to -1.8 MPa in dry season. The difference could be related to difference of evaporative demand and magnitude of transpiration. Additionally, lower values of $\psi_{predawn}$ have been noticed in seasonal drought but no threshold for water stress or transpiration decline assessed (Chandrashekar, 1997; Gururaja Rao *et. al.,* 1990). Wheat genotypes under drought stress showed significant differences in water potential as observed by Singh *et. al.,* (1990). In the present study clones such as RRIM 600, RRII 430 and RRII 208 maintained better leaf water potential than other clones during water deficit periods. These clones are recently proven to be drought tolerant in field condition also (Annamalainathan *et. al.,* 2010; Sumesh *et. al* ., 2011).

In the present study ten elite rubber clones were used to screen their potential against drought stress based on physiological response during their early growth period. Elite high yielding modern clones namely RRII 105, PB 260 and RRII 414 were found relatively drought susceptible as observed from severe decline of PS II activities, photosynthetic oxygen evolution rate, leaf

water potential and net photosynthesis under moderate moisture deficit condition. In contrast, clones such as RRIM 600, RRII 430 and RRII 208 could tolerate moisture stress by maintaining relatively better and stable photosynthetic activities under similar level of stress conditions. The response of photosynthetic parameters to soil moisture deficit stress was genotypic specific in these modern *Hevea* clones. The most commonly used protocol for measuring the photosynthetic efficiency of photosystem II under a stress condition is effective quantum yield of PS II, a crucial factor to analyze the potentials of PS II activity in light exposed plants. It is an indication of the amount of energy utilized in photochemistry by photosystem II under steady-state photosynthetic lighting conditions. The present result demonstrates differential response of photosystem II activity to drought associated water deficit and high temperature stresses in young plants of different clones. The decline in PSII efficiency is probably a regulatory mechanism serving a photo protective role. Increased levels of energy dissipation which decrease ΦPS II may help to protect PSII from over excitation and photo damage (Schindler and Lichtenthaler, 1994).

It has been shown that the oxygen evolving complex (OEC) is the most susceptible component in photosynthetic apparatus to water and high temperature stresses in many plants (Berry and Bjorkman, 1980). The degree of susceptibility of RRII 105, a popular high yielding rubber clone in India, to drought condition has been well documented in many previous studies (Alam *et. al.*, 2005; Annamalainathan *et. al.*, 2006). Interestingly, a modern high yielding clone namely, RRII 414 is also shown to be drought susceptible in terms of severe inhibition of various photosynthetic parameters under stressful growth conditions.

The magnitude of drought induced damage to the photosynthetic apparatus was comparatively small in a few clones like RRIM 600, RRII 430 and RRII 208. The clones, such as RRIM 600 and RRII 430 have already been proved to be relatively drought tolerant in terms of photosynthetic performances under soil moisture deficit conditions in previous studies also (Annamalainathan *et. al.*, 2010). Gas exchange and fluorescence studies revealed that clone RRII 430 was more likely to endure drought stress better than the other modern RRII 400 series clones. In terms of water use efficiency (WUE) studies in many

clones RRIM 600 and RRII 430 were found physiologically better adapted and can withstand water stress for a relatively longer period of time (Annamalainathan *et. al.*, 2010; Sumesh *et. al*., 2011).

In the study conducted with RRII 105 under growth chamber conditions drought stress coupled with high temperature resulted in photo oxidation of chlorophyll and carotenoids pigment (Fig 3.9; Fig 3.10). 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 photo protection 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). 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). Carotenoids also play a major role in dissipation of excess electrons as non-photochemical quenching (NPQ) through xanthophyll cycle (Demmig-Adams and Adams, 1992). 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). When compared to the irrigated plants, there was a decline (more negative) in leaf water potential in drought imposed plants under all temperature regimes (Fig 3.11). Generally high temperature influences the water loss through elevated transpiration rate, there by more negative tissue water potential. Certain genotypes maintain better leaf water potential under drought periods.

The photosystem II (PS II) activity in chloroplast is known to be a sensitive photochemical reaction influenced by environmental parameters. In the present study the PS II activity was found more sensitive to water deficit with concomitant occurrence of high temperature (Fig 3.12). The cumulative effects

of these environmental stresses were obviously seen with young *Hevea* plants. Photosynthetic carbon reduction and carbon oxidation cycles are the main electron sink for PS II activity during mild drought (Cornic and Fresneau, 2002). Within PS II 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). Photosynthesis sensitivity to heat occurs mainly due to damage of photosystem II components located in the thylakoid membranes of the chloroplast and membrane properties (Al-Khatib and Paulsen, 1999).

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. When the growth temperature increased, there was a drastic reduction of chlorophylls, carotenoids and photosystem II activity. The degree of susceptibility of this clone seems to be higher under the growth chamber conditions and it indicates that this clone is comparatively drought susceptible as observed from many studies conducted in traditional as well as non-traditional drought prone areas of India (Sreelatha *et. al.*, 2007; Annamalainathan *et. al.*, 2010; Thomas *et. al.*, 2011).

Chapter IV

Identification and Purification of a Low Molecular Weight (LMW) Chloroplast Stress Protein from Young Rubber Plants

4.1. Introduction

Higher plants have evolved a variety of strategies to acclimatize to various kinds of environmental factors including biotic and abiotic stresses. Reduction in crop growth and productivity is caused largely due to abiotic stress. Drought and salinity are the major abiotic factors which cause membrane disorganization, metabolic toxicity, decreased photosynthetic activity, closure of stomata, generation of reactive oxygen species (ROS) and altered nutrient acquisition (Hasegawa *et. al.,* 2000). Drought is the most significant environmental stress affecting the world agricultural production (Tuberosa and Salvi, 2006; Cattivelli *et. al.,* 2008). The degree of yield loss due to drought which can occur at any stage of plant growth depends on the intensity, onset time and duration of stress (Hu and Xiong, 2014). Plants have evolved several mechanisms to cope up with drought stress such as drought escape via a short life cycle or developmental plasticity, drought avoidance via reduced water loss and enhanced water uptake, drought tolerance via antioxidant capacity, osmotic adjustment and cellular level desiccation tolerance (Zhang, 2007).

Though the responses of plants to drought are relatively well known, the performance when multiple stresses occur in a more complex environment is fragmentary. Plants have to respond simultaneously to multiple stresses in the field such as drought, excessive heat that occurs in the field. Such kinds of investigations are usually not predictable from single factor studies (Zhou *et. al.,* 2007). Response and adaptation of plants to water deficit occurs at both cellular and molecular levels by accumulation of proteins and osmolytes specifically involved in stress tolerance. These kind of stresses induces or represses an assortment of genes with diverse functions (Shinozaki *et. al.,* 2003; Bartels and Sunkars, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005). The differential expression of genes leading to changes in transcript and protein patterns occurs in plants after drought sensing as a result of accumulation of abscisic acid (ABA) which serves as a signalling molecule (Shinozaki *et. al.,* 2003, Shinozaki and Yamaguchi-Shinozaki 2007; Hirayama and Shinozki, 2010; Fulda *et. al.,* 2011) and an adaptive response to various environmental stresses. ABA also induces gene expression, reduces water loss via transpiration and induces stomatal closure under abiotic stress condition (Chandler and Robertson, 1994).

Stress response and tolerance at the cellular level will be the function attributed to most of their gene products. Drought-specific genes are grouped into three major categories based on expression studies such as genes with membrane and protein protection functions, genes involved in signal transduction pathways (STPs) and transcriptional control, and genes assisting with water and ion uptake and transport (Vierling, 1991; Ingram and Bartels, 1996; Smirnoff, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). Metabolic changes related to protein turnover are usually induced by drought (Bray, 1997). Proteins that play role in protection function involve enzymes required for the biosynthesis of various osmoprotectants, late embryogenesis abundant (LEA) proteins, chaperones, antifreeze proteins and detoxification enzymes whereas regulatory proteins include transcription factors and protein kinases (Seki *et. al.,* 2003; Shinozaki and Yamaguchi-Shinozaki, 2000). Dehydrins (DHNs) or group 2 LEA are a group of heat-stable plant proteins with chaperone like functions produced during late embryogenesis and believed to play a protective role during cellular dehydration (Close, 1996; Campbell and Close, 1997). Dehydrins are also referred as RAB proteins (Responsive to ABA) because the expression of many DHNs increases by the phytohormone ABA (Hanin *et. al.,* 2011).

Heat Shock Proteins (HSPs) specified as molecular chaperones for protein molecules (Schoffl *et. al.,* 1998) increase their expression when cells are exposed to high temperature or to other stresses (Lindquist, 1986). HSPs functions in cell vary from protein-protein interactions, folding, assembly, intracellular localization, degradation or prevention of unwanted protein aggregation and reactivation of damaged proteins (Vierling, 1991; Parsell and Lindquist, 1993). Playing an essential role in protein maintenance heat shock proteins are present in cells under perfectly normal conditions (Demirevska *et. al.,* 2008). Induction of HSPs occurs when a cell undergoes various types of environmental stresses like cold, heat and oxygen deficiency (Feder and Hofmann, 1999; Kregel, 2002). Under drought stress cellular ROS needs to be maintained at non toxic levels and so as to prevent stress injury. Degradation of ROS is achieved by antioxidants and these include enzymes such as catalase, superoxide dismutase (SOD), glutathione reductase and ascorbate peroxidase

(APX) whereas nonenzymes includes ascorbate, glutathione, anthocyanins and carotenoids (Wang *et. al.*, 2003).

Regulatory proteins induced under drought incidence have a major role in signal transduction pathways (STPs). Drought stress signals are received by specific receptor molecules which vary in identity, structure, perception, signal relay mechanism and location within the cell and messages are conveyed to the appropriate downstream components before transcriptional activation of genes occurs. Secondary messengers involved in plant stress STPs modify these signals prior to conveying them from receptor molecules to the activators of the appropriate gene expression pathway (Xiong and Ishitani, 2006). Many other molecules are involved in stress STPs which functions in the recruitment and assembly of signaling complexes, targeting of signaling molecules and regulation of signaling molecule life span (Xiong and Ishitani, 2006). The major molecules involved in stress STPs include receptor molecules/osmosensors, phospholipid-cleaving enzymes (PLEs), reactive oxygen species (ROS) such as nitric oxide (NO), hydrogen peroxide (H_2O_2), mitogen-activated protein kinases (MAPK), and Ca2+ sensors (Jewell *et. al.*, 2010).

Adverse environmental conditions such as drought, extremes of temperature, high light intensity etc. are increasingly becoming limiting factors for cultivation of rubber especially in non-traditional areas. Even in the present climate warming situations establishment of young rubber plants in the field become a constraint in traditional regions also (Jessy *et. al.*, 2010). Drought tolerance is an extremely complex multi-gene controlled physiological process that is emerging as an important selection criterion in *Hevea* crop improvement programmes (Jacob *et. al.*, 1999). Tolerance to drought, high light etc. can be evaluated in rubber plants using various techniques at leaf level to whole plant level. There is no single vital parameter that can be used to identify the most tolerant *Hevea* genotypes for abiotic stresses like drought. Moreover, any single trait/mechanism is unlikely to contribute consistently to the relative success and tolerance of crop plants against any abiotic factor. However, the magnitude and degree of adaptability to drought stress can be estimated in plants by measuring various tolerance traits by *in-vivo* or *in-vitro*.

One of the most important heat sensitive physiological processes in plants is photosynthesis (Crafts-Brandner and Salvucci, 2002). In chloroplast, photochemical reactions in thylakoid lamellae and carbon metabolism of the stroma are considered as the primary sites of injury at high temperatures (Wang *et. al.*, 2009; Marchand *et. al.*, 2005). Altered structural organization of thylakoids, swelling of grana and loss of grana stacking under heat stress shows the susceptible nature of thylakoid membrane (Rodriguez *et. al.*, 2005; Ashraf and Hafeez, 2004). As a sensor of environmental changes by both coordinating the expression of nuclear-encoded plastid–localised proteins chloroplasts are considered to be a key element in plant stress response (Tamburino *et. al.*, 2017).

In the present study we evaluated young plants of elite *Hevea* clones for stress responsive protein changes in the photosynthetic apparatus and implications of such proteins in acclimation and adaptation of rubber plants to drought. Those proteins responsive to drought stress showing differential expression in tolerant/susceptible clones were purified by electro-elution and the homogeneity of the purified protein was further tested by 1-D and 2-D gel electrophoresis.

4.2. Materials and Methods

4.2.1. Identification of a Low Molecular Weight (LMW) Protein in Response to Drought

4.2.1.1. Collection of Leaf Sample for Chloroplast Isolation

Mature leaves were collected from one-year-old polybag plants on the tenth day of withdrawl of irrigation and from control (with saturated level of irrigation). In the case of plants grown under growth chamber, leaf samples were collected on the fifth day of drought induction. Leaves were stored in deep freezer (-80°C) until used for biochemical analysis. Chloroplasts were isolated by the method of Reeves and Hall (1973).

4.2.1.2. Isolation of Chloroplasts

The leaf bits were homogenised with liquid nitrogen in a mortar and pestle into powder. During grinding a pinch of crystalline PVP was added. The

powdery samples (3 g) were extracted with 5 ml of ice cold grinding buffer consisting of 20 mM TrisHCl (pH 7.8),10 mM NaCl, 5 mM $MgCl_2$, 350 mM Mannitol, 2 mM Ascorbate and filtered through four layered cheese cloth and centrifuged at 800g for 2 min at 4°C. The pellet represented unbroken cells and tissue was removed and the supernatant was spun at 3500g for 5 min at 4°C and the resulting pellet was suspended in 1 ml of grinding buffer (pH 7.8) as chloroplast suspension.

4.2.1.3. Chloroplast Protein Extraction

The chloroplast suspension (1 ml) was made upto 10 ml using 100% acetone and refrigerated for 1 hr inside freezer followed by centrifugation in order to remove pigments and lipids. The pellet was later suspended in 10% TCA (1 ml) for 5 min to precipitate the protein followed by centrifugation and any trace amount of acidity left behind by TCA was removed by three washing with 80% ice cold acetone with centrifugations in between. Finally the pellet was suspended in 3 ml of 100% ice cold di-ethyl-ether to remove fat content followed by another round of centrifugation. All the above centrifugations were carried out at 8000g for 10 min and all procedures were carried out at 4°C. The final pellet obtained was air dried and was used for further analysis.

4.2.1.4. Isolation of Total Leaf Protein

Total leaf protein was extracted by the method of Nelson *et. al.,* (1984). The leaves were homogenised with liquid nitrogen in a mortar and pestle. Leaf samples were extracted with 3 ml/gm tissue of 18% sucrose (w/v), 0.01 M $MgCl_2$, 0.1 M Tris-HCl (pH 7.8), 40 mM β-mercaptoethanol. The homogenate was filtered through two layered cheese cloth and centrifuged at 10,000g for 15 min and the supernatant containing total soluble proteins was collected. For insoluble proteins the pellet was re-extracted with 0.05 volume (relative to original homogenate) of 2% (w/v) SDS, 6% (w/v) sucrose and 40 mM β-mercaptoethanol and centrifuged at 15,600g for 15 min to remove remaining membrane fraction. Both the supernatants were mixed and total protein concentration was determined by the method of Lowry *et. al.,* (1951).

4.2.1.5. Protein Estimation

The chloroplast protein was further solubilized in small volume of 10% SDS and the protein content was determined by the method of Lowry *et. al.*, (1951). In this method, the blue colour developed by the reduction of the phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein and the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured at 660nm.

The following reagents were used in this method:
Reagent A: 2% Na_2CO_3 in 0.1 N NaOH.
Reagent B: 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% sodium potassium tartrate.
Reagent C: 50 ml of Reagent A + 1 ml of Reagent B prior to use (alkaline copper reagent).
Reagent D: Folin-Ciocalteau (1:1) with distilled water.

A series of bovine serum albumin (BSA) standards (40 µg, 80 µg, 120 µg, 160 µg and 200 µg) and unknown samples were prepared in dist. water. 5ml of alkaline copper reagent (Solution C) was added to each tube, mixed well and allowed to stand for 10 min. To this 0.5 ml of reagent D was added. After incubation in dark for 30 min at room temperature the absorbance of the solution was read at 660 nm in a UV-Visible spectrophotometer. Total protein content in the unknown sample was calculated from the calibration curve.

4.2.1.6. Sodium dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Isolated proteins were loaded into a polyacrylamide gel containing an anioinic denaturing agent (SDS) which linearize proteins and impart a negative charge to all linearized protein. The proteins were separated in the gel on the basis of their molecular mass using electrophoresis (Laemmli, 1970). Separation was carried out in the Broviga electrophoresis device (M/S Broviga, Chennai).

Glass plate sizes are 18x16x0.1 cm and comb were having twelve wells for sample loading. For resolving gels, 10% polyacrylamide gel was prepared by mixing 12.05 ml of water, 10 ml of 30% acrylamide mixture, 7.5 ml of 1.5M Tris-HCl (pH 8.8), 150 µl of freshly prepared 10% ammonium per sulphate, 300 µl of 10% SDS and 10 µl of TEMED to final volume of 30 ml. The mixture was poured continuously into the thin gap between two glass plates and allowed to polymerize for 25-30 minutes. Stacking gels, 4% gel was prepared by mixing 5.5 ml of water, 1.35 ml of 30% acrylamide mixture, 3 ml of 0.5 M Tris-HCl (pH6.8), 50 µl of freshly prepared 10% ammonium per sulphate, 100 µl of 10% SDS and 5 µl of TEMED to final volume of 10 ml. The mixture was poured onto the top of polymerized resolving/separating gel. The comb was placed on the gel without any air bubbles before polymerization. After polymerization of stacking gel, the comb was carefully removed from the top of gel. The gel unit was shifted to a buffer tank and the reservoirs were filled with 1X Tris-Glycine running buffer prepared by mixing 25 mM Tris-HCl, 192 mM Glycine and 0.1% SDS in distilled water until it reached the filling line level. Sample buffer was prepared for 10 ml by mixing 2.5 ml of 0.5M Tris-HCl (pH 6.8), 2.5 ml of β-mercaptoethanol, 2.5 ml of glycerol, 1.25 ml of 1% bromophenol blue and 1.25 ml of dist. water. The 100 µg chloroplast protein samples were mixed with sample buffer. The samples were diluted with sample buffer with the ratio of 1:1 (v/v) and was boiled at 100°C for 3 min, cooled to room temperature and was centrifuged at 10,000 rpm for 2 min to remove un-dissolved materials if any. The supernatant was loaded in the gel along with standard molecular weight protein marker (M/S Genei, Bangalore). Electrophoresis was conducted at constant voltage of 60V till the dye front crossed the stacking gel followed by constant voltage of 120V until the blue dye reached the bottom of the gel. Once electrophoresis was completed, the gel was taken out carefully by separating the glass plates and after carefully removing the stacking layer the separating layer was washed with dist. water and transferred to staining solution.

4.2.1.7. Staining and Destaining

After electrophoresis, chloroplast proteins were visualized by soaking the gel in staining solution containing 0.5 g of coomassie brilliant blue R-250 (CBB)

in 80 ml of methanol,100 ml of dist. water and 20 ml of glacial acetic acid for overnight. The gel was then washed with dist. water and destained using destaining solution containing ethanol, dist. water and glacial acetic acid in 40:50:10 ratios till the background is clear and appearance of sharp bands. The electrophorograms were photographed and analysed using gel documentation system.

4.2.2. Electro-elution of protein from SDS polyacrylamide gels.

Chloroplast protein isolated from control and drought exposed plants was loaded onto a 10 % preparative SDS polyacrylamide gel with a reference well holding the standard molecular weight protein marker. After electrophoresis staining and destaining were performed as mentioned. The gel was placed on a glass plate on a white paper and using a clean scalpel the desired protein band was cut off from the gel. The gel slices were washed with 5 ml elution buffer prepared by mixing 25 mM Tris-HCl, 192 mM Glycine and 0.1% SDS in distilled water and was then placed on a piece of parafilm and cut into smaller pieces to fit in the electro-eluter glass tubes.

4.2.2.1. Protein Elution

Isolation of desired proteins induced by water deficit stress was carried out using the Bio-Rad model 422 electro-eluter apparatus which can be used to electro-elute six samples simultaneously. Contamination of the samples was prevented by wearing gloves throughout the procedure. Membrane caps of molecular weight cut off 12-15 kDa (clear caps) were used which was soaked in elution buffer for 1 hr at 60°C. Pre-treatment of membrane caps was not done when reused. Onto each glass tube a frit was placed in a way that it is flush with the bottom of the frosted end. The glass tubes with the frit inside were pushed into the electro-eluter module so that the open end is even with the top of the grommet. Empty grommet holes if any were closed with stoppers. Pre-wetted membrane caps were placed onto the bottom of silicon adaptors and the adaptor was filled with elution buffer and air bubbles were removed by pipetting the buffer up and down. The silicon adaptor with the membrane cap was then slide down onto the bottom of the glass tube with the frit. The tubes were then filled with elution buffer and the pieces of the gel slice were placed inside tubes up to a

height of 1 cm. The electro-eluter module was placed inside the buffer chamber and the lower chamber was filled with 600 ml elution buffer so that the level of buffer is above silicone adaptors and upper chamber with 100 ml. A stir bar was used to prevent bubbles from sticking to the bottom of dialysis membrane. Elution was carried out overnight at 10 mA. Once elution was completed the electo-eluter module was removed and the lower buffer was replaced with fresh buffer without SDS and elution was continued for another 1 hr to remove SDS from the collected sample which concentrates in the lower buffer during the run. After completion of elution the module was removed from the tank and the upper buffer chamber was drained first. The first glass tube was then removed and the buffer above the frit inside the tube was pipetted out. The silicone adaptor together with the membrane was carefully removed from the bottom of the glass tube. The solution in the membrane cap was pipetted to a microfuge tube. The membrane cap was carefully rinsed with another 100-200 µl fresh elution buffer without SDS and collected together. Procedure was repeated with other tubes. All eluted proteins were then spun at 10,000g for 2 min, supernatant discarded and pellet was retained as pure protein. To remove colour of dye content the protein was washed with 0.5 ml of 100% acetone followed by refrigeration for 1 hr and centrifuged at 10,000g for 2 min. To increase sample recovery for antibody raising the desired protein band were sliced and used from nearly 30-35 SDS polyacrylamide gels and electro-elution was repeated.

4.2.3. Two-Dimensional Gel Electrophoresis (2-D Electrophoresis) of eluted protein.

Two-dimensional gel electrophoresis (2-DE) is a powerful technique for high-resolution profiling of low abundance proteins. In 2-DE proteins are sorted according to two independent properties in two discrete steps: first-dimension, isoelectric focusing (IEF), where proteins are separated linearly according to their isoelectric point (pI); second-dimension, SDS-PAGE, where proteins are separated according to their molecular weights (Mr, relative molecular weight). A small fraction of the drought induced stress protein purified by repeated electro-elution from 10% SDS polyacrylamide gels was subjected to 2-D electrophoresis using O'Farrell method (1975) to confirm the homogeneity.

4.2.3.1. Sample preparation and loading

The eluted protein pellet was cleaned up using 2-D Clean-up Kit (GE Healthcare, USA) following the manufacturer's protocol. The procedure works by quantitatively precipitating proteins while leaving behind in solution interfering substances such as detergents, salts, lipids, phenolics and nucleic acids. The pellet was resuspended with the lysis buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 13 mM DTT, 1% PMSF, 1% IPG buffer of pH 3–10, and vortexed for thirty seconds with 30-min intervals during the two hour incubation at room temperature. After centrifugation for 15000 rpm for 15 minutes at 20°C, protein concentration was determined using 2-D Quant Kit (GE Healthcare, USA) following the manufacturer's protocol. BSA was used as the standard. Concentration of unknown sample was calculated from the calibration curve.

4.2.3.2. 1st Dimension- Isoelectric Focusing (IEF)

Immobilized non-linear pH gradient IPG strips were used for separation of proteins by isoelectric focusing in the first dimension. Rehydration buffer (GE Healthcare, USA) was used to make the volume up to 150 µl of the sample containing about 50 µg of eluted stress protein and was subsequently loaded onto an IPG strip holder with 11 cm, pH 3–10, non-linear gradient IPG strips (GE Healthcare), and rehydrated for sixteen hours at room temperature. To prevent from drying the strip was covered with dry strip cover-fluid (GE Healthcare). After rehydration IEF was performed on the Ettan IPGphor3 isoelectric focusing system (GE Healthcare, USA) under the following conditions:

250 V for 20 min (Linear); 1000 V for 2.5 h (Linear) and a gradient to 10,000 V up to 40,000 V hrs.

4.2.3.3. Equilibration

After IEF, these strips were equilibrated with 10 ml equilibration buffer I, containing 375 mM Tris, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 130 mM DTT, 0.002% bromophenol blue for 15 min, followed by 15 min incubation in the equilibration Buffer II containing 375 mM Tris, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 135 mM iodoacetamide, 0.002% bromophenol blue on a rocker.

4.2.3.4. 2nd Dimension Electrophoresis (SDS-PAGE)

The IPG strips after equilibration was then soaked in running buffer and placed on top of a 1.5 mm thick 15% SDS polyacrylamide gel and run in the Midi system (Bio-Rad). Electrophoresis was carried out at 40 V for 2 h and 80 V 10 h at a constant temperature of 20 °C. After electrophoresis, for visualizing the resolved spots, the gels were silver stained.

4.2.3.5. Silver Staining of 2-D gel.

In two dimensional gel electrophoresis, protein spots were visualized by silver staining of the gel. In this technique the polyacrylamide gel is impregnated with soluble silver ion (Ag+) and then developed by treatment with a reductant. Binding of silver ions (Ag^+) to the macromolecules in the gel followed by reduction to free metallic silver (Ag^0), which is insoluble, and visible, allows protein or nucleic acid containing bands to be seen. Metallic silver deposited initially promotes further deposition in an autocatalytic process, resulting in exceptionally high sensitivity.

After electrophoresis the gel was removed from the cassette and placed into a tray containing fixing solution (50% methanol and 5% glacial acetic acid) for 1 hr which will restrict protein movement from gel matrix and also will remove interfering ions and detergent from the gel. The fixative solution was then discarded and washed with dist. water twice for 1 min each. The gel was then washed with washing solution containing 20% ethanol for 20 min. Washing solution was changed twice to remove any detergent remaining in gel as well as fixation acid from the gel. After discarding the washing solution, the gel was washed with dist.water twice for 1 min each and the gel was then incubated with constant shaking for 1 min in sensitizing solution containing 0.02% sodium thiosulphate which will increase the sensitivity and contrast of the staining. After discarding the sensitizing solution, the gel was rinsed thrice with dist. water for 1 min each. The gel was later stained with chilled staining solution containing 0.1% silver nitrate added slowly through the corner of the tray for 20 min with constant shaking to allow the silver ions to bind to proteins. The staining solution was discarded and gel was rinsed gently with dist. water twice for 1 min each. For visualization of the protein spot, the gel was rinsed shortly with freshly

prepared developing solution containing 2% sodium carbonate and 0.08% formaldehyde. After the desirable intensity of the protein spot was obtained, the reaction was stopped by adding 5% glacial acetic acid to the gel. In all steps sufficient volume of solutions were used to fully immerse the gel.

4.3 Results

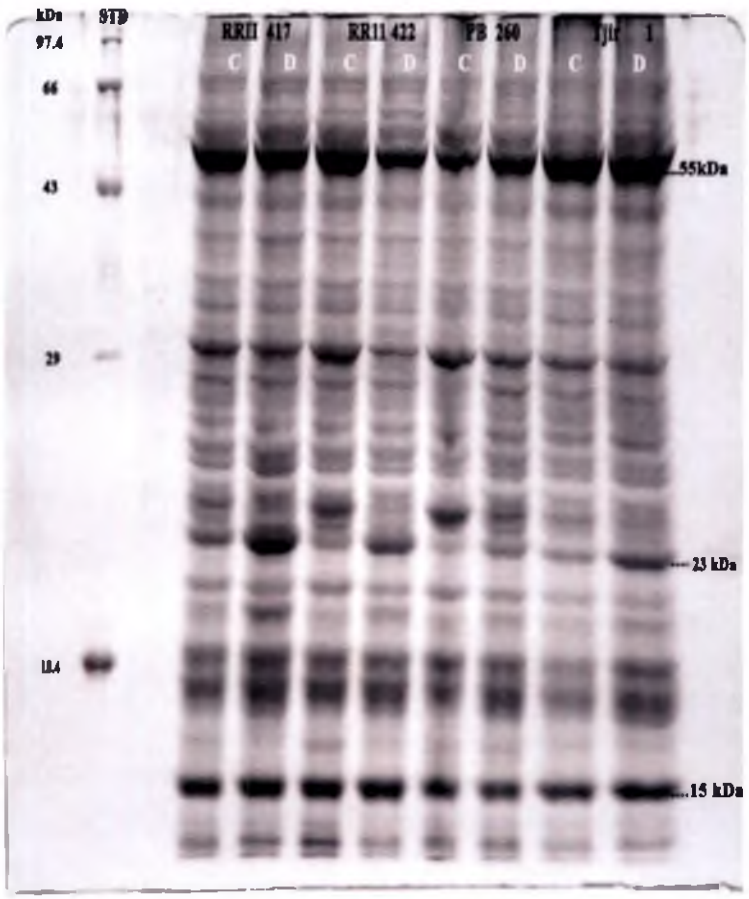
4.3.1. Identification of Low Molecular Weight Stress Protein in Chloroplast

4.3.1.1. Collection of leaf sample and isolation of chloroplast protein.

After assessing various physiological parameters as described in the previous chapter mature leaves were collected from control and drought induced plants and immediately wrapped in aluminium foil with proper marking, kept in ice and carried to lab in an ice-box and chloroplast isolation was carried out. Chloroplasts were isolated by the process of differential centrifugation as chloroplast suspension and purification of chloroplast protein was carried out from this chloroplast suspension.

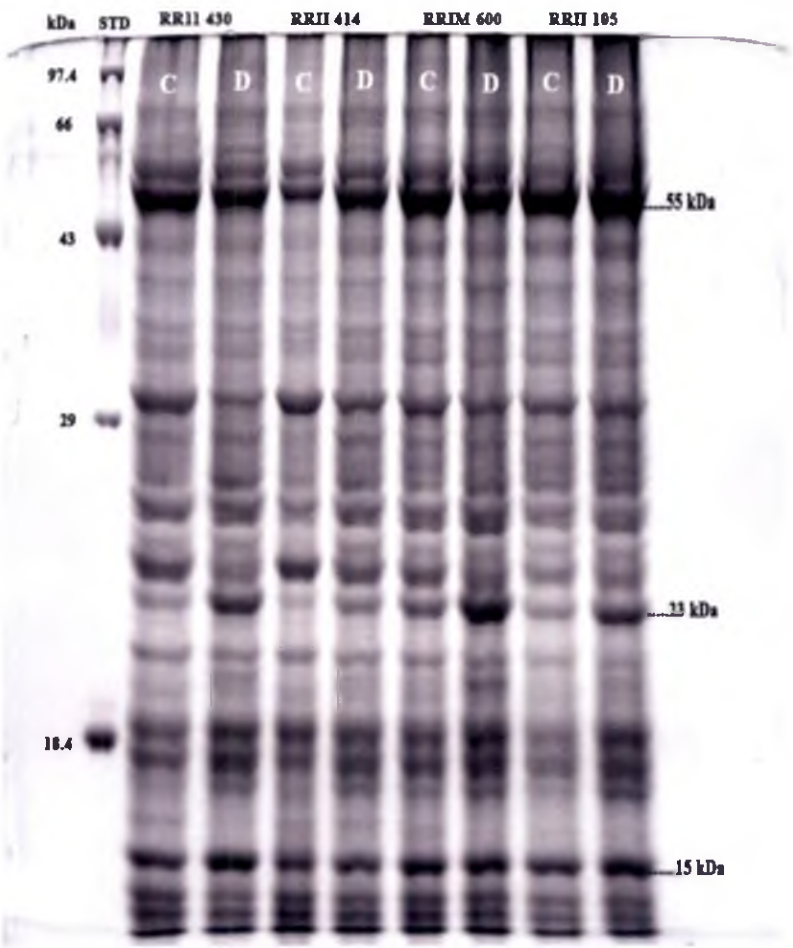
4.3.1.2. Profiling of chloroplast protein from stress induced and control plants of *Hevea*

The chloroplast protein profile in all the clones studied from drought induced and irrigated control plants, was obtained by coomassie staining of SDS polyacrylamide gels and a protein of molecular weight approximately 23 kDa was observed to be over expressing consistently in drought imposed young plants of various *Hevea* clones. The water deficit stress imposed plants accumulated significantly higher level of this stress protein in comparison to the control plants. The chloroplast protein of 100 µg when resolved on 10% SDS gel the abundance of this stress protein was very prominent in relatively drought tolerant clones when compared to their respective irrigated counterparts. In the remaining clones the expression level was within medium to optimum range. The chloroplast protein isolated from clones RRII 429 and RRII 208 after 5 days of drought imposition showed that the expression level of the protein on 5^{th} day was found to be as prominent as on 10^{th} day of drought imposition (Fig 4.1. A, B & C).



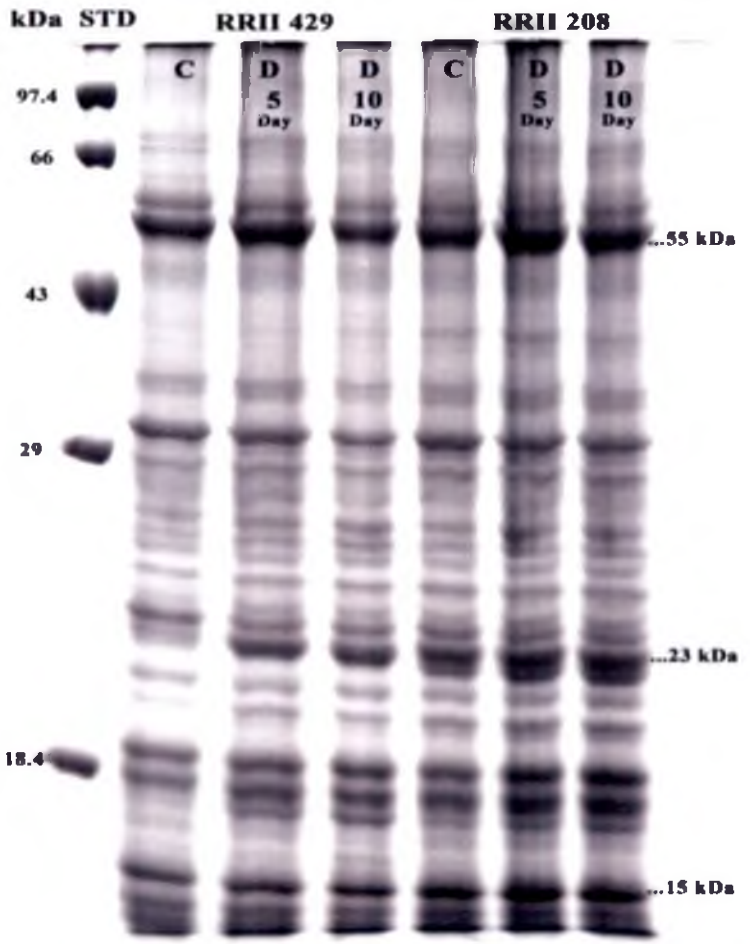
(A)

Fig 4.1.(A). Chloroplast protein profile of clones RRII 417, RRII 422, PB 260 and Tjir 1. The plants were grown with (C) or without (D) irrigation for 10 days during summer season. The stress protein 23 kDa is indicated on the right side along with RuBisCO larger subunit (55 kDa) and smaller subunit (15 kDa). The molecular weight markers (STD) are indicated in the left side.



(B)

Fig 4.1.(B). Chloroplast protein profile of clones RRII 430, RRII 414, RRIM 600 and RRII 105. The plants were grown with (C) or without (D) irrigation for 10 days during summer season. The stress protein 23 kDa is indicated on the right side along with RuBisCO larger subunit (55 kDa) and smaller subunit (15 kDa). The molecular weight markers (STD) are indicated in the left side.



(C)

Fig 4.1.(C). Chloroplast protein profile of clones RRII 429 and RRII 208. The plants were grown with (C) or without (D) irrigation for 10 days during summer season. The stress protein 23 kDa is indicated on the right side along with RuBisCO larger subunit (55 kDa) and smaller subunit (15 kDa). The expression of the stress protein was checked on 5^{th} day and indicated as (D 5day). The molecular weight markers (STD) are indicated in the left side.

4.3.1.3. Profiling of total leaf protein from stress induced and control plants of *Hevea*

Total leaf protein profile obtained from two clones RRII 105 and RRIM 600 after exposure with 10 days drought stress and irrigated control was analysed by coomassie staining of SDS polyacrylamide gels. The magnitude of expression of the stress protein was not as prominent as obtained when chloroplast protein was resolved on SDS gel from the same clones (Fig 4.2).

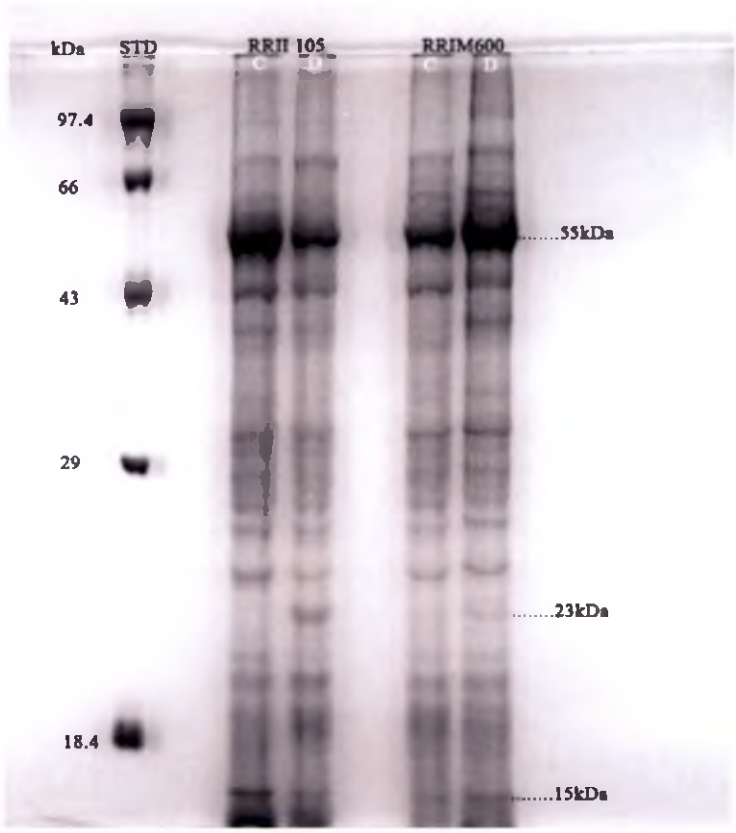


Fig 4.2. Total leaf protein profile from young plants of *Hevea* (clone RRII 105 and RRIM 600). The plants were grown with (C) or without (D) irrigation for 10 days during summer season. The stress protein 23 kDa is indicated on the right side along with RuBisCO larger subunit (55 kDa) and smaller subunit (15 kDa). The molecular weight markers (STD) are indicated in the left side.

4.3.1.4 Profiling of chloroplast protein from water deficit stress induced plants of *Hevea* (RRII 105) grown under growth chamber conditions

The chloroplast protein profile of clone RRII 105 grown under growth chamber condition for 5 days failed to show prominent accumulation of 23 kDa stress protein under drought condition at all temperature regimes (30, 35 and 40^{0}C). However, the large (55 kDa) and small (15 kDa) subunits of RuBisCO were shown to be degraded after exposure to high temperature. Even the level of rubisco was lesser in irrigated control plants grown at all temperature when compared to irrigated plants of the same clone under nursery conditions (Fig. 4.3). Chloroplast proteins isolated from irrigated plants of clone RRII 105 grown outside was also resolved for comparison.

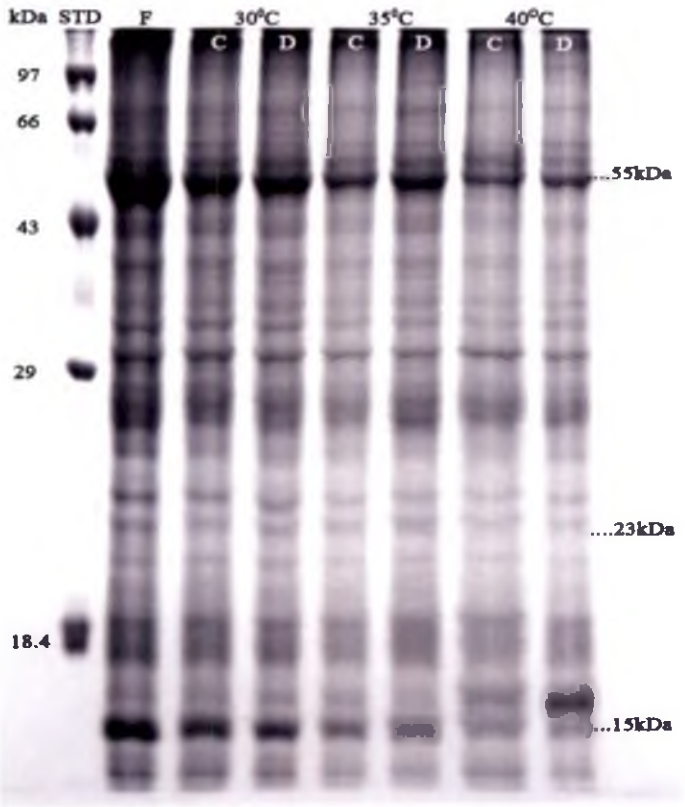


Fig 4.3. Chloroplast protein profile of young plants of *Hevea* (clone RRII 105) grown under growth chamber conditions. The plants were grown with (C) or without (D) irrigation for 5 days under different temperature regimes (30, 35 and 40^{0}C). Irrigated plants grown outside are represented as 'F'. The RuBisCO large (55 kDa) and small (15 kDa) subunits are indicated on the right side. The molecular weight markers (STD) are indicated in the left side.

4.3.2 Purification of stress induced protein from SDS polyacrylamide gels

4.3.2.1. Electro-elution of stress induced protein.

The stress induced LMW protein resolved on 10% SDS gels was further purified by electro-elution (Fig 4.4) and the protein after purification was again loaded onto 10% gel and confirmed that the molecular weight of protein was around 23 kDa (Fig 4.5).

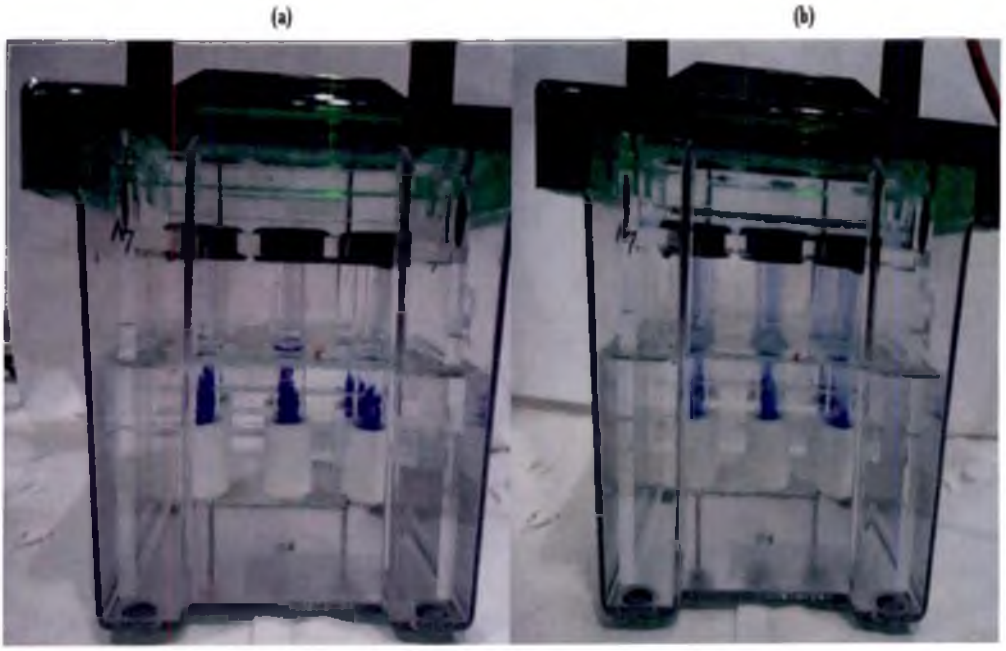


Fig 4.4. The apparatus showing the purification of stress protein by electro-elution (model 422 electo-eluter of Biorad) (a) represents beginning of electro-elution and (b) represents towards completion of electro-elution.

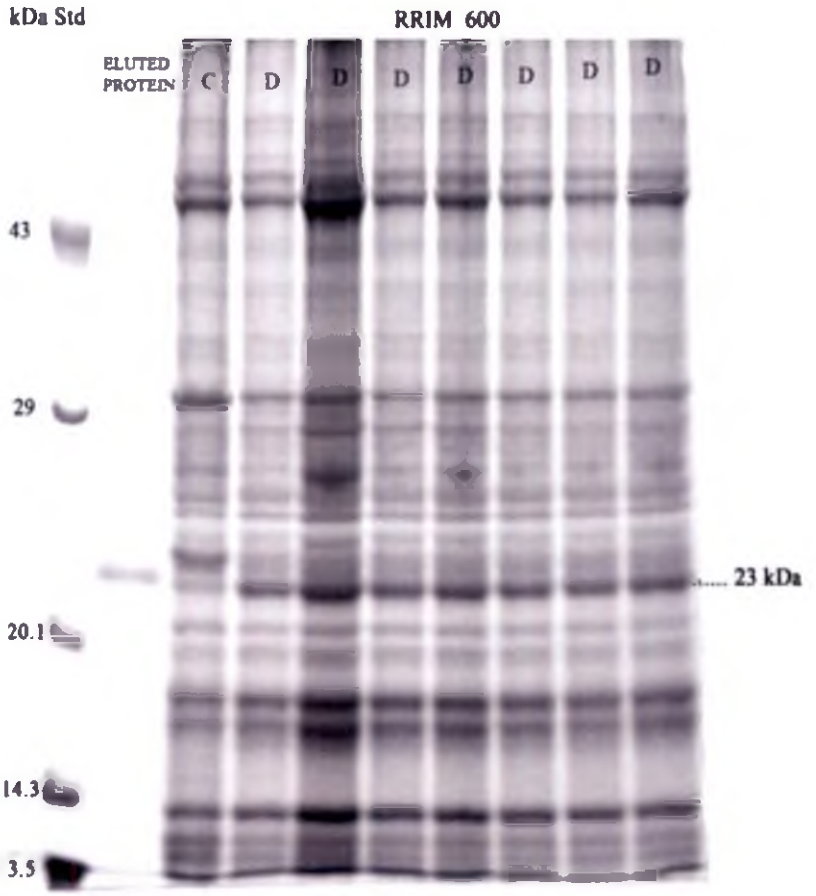


Fig 4.5. SDS PAGE showing the eluted and purified stress protein (lane 2) and the 23 kDa protein expressed in 50 µg of 10 days drought induced plants of RRIM 600 (lanes 4-10). The protein profile of irrigated control plants is shown in lane 3. The molecular weight markers (STD) are indicated on the first lane.

4.3.2.2. Two Dimensional Gel Electrophoresis (2-DE) of purified protein.

Purified stress protein when resolved under 2-DE was seen as a spot with pI value near 8 (Fig 4.6).

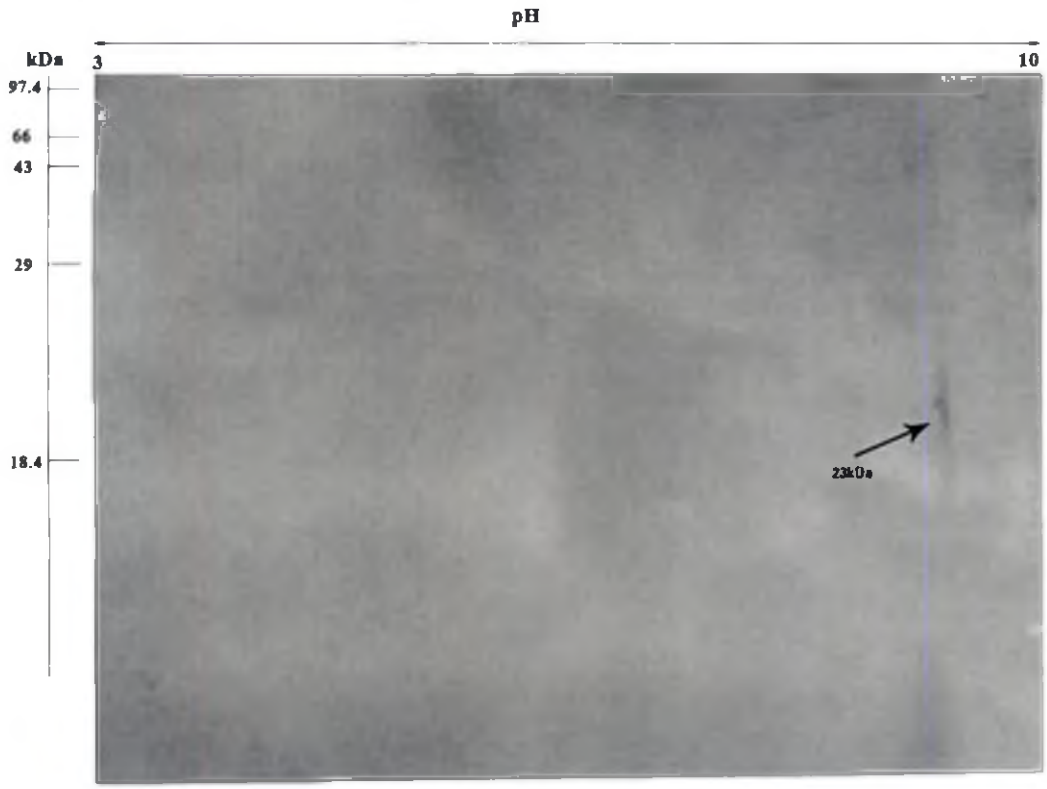


Fig 4.6. 2D gel profile of electro-eluted 23 kDa stress protein indicated on the right side. The molecular weight markers (STD) are indicated on the left side.

From the clone RRIM 600 which showed the maximum expression of this sHSP approximately 1 mg of stress protein was purified by repeated electro-elution (Fig. 4.5). The purified protein was subjected to 2-D electrophoresis and the purified protein was visualized as a single spot with pI value near 8 (Fig 4.6).

4.4. Discussion

Stress responses and resistance to drought are complex biological processes in plants which need to be analysed using physiological and genomics approaches. Plants grown in field conditions have to face diverse stress factors which often activate similar cell signalling pathways and cellular responses, such as accumulation of compatible solutes, up regulation of the antioxidant machinery and production of stress related proteins. Mechanisms are activated in plants at morphological, physiological, biochemical and molecular levels to cope with the extreme climatic stresses. Profound changes in gene expression which result in changes in composition of plant transcriptome, proteome and metabolome are the responses of plants to stress (Perez-Alfocea *et. al.,* 2011). Proteins being the direct effectors of plant stress response, the relationship between protein abundance and plant stress acclimation can be understood by proteomics studies (Perez-Clemente *et. al.,* 2013).

Chloroplasts being central organelles where photosynthetic reactions take place, changes in activities and protein pools are expected in response to drought like abiotic stresses. In view of this the present study was designed towards identification of drought induced proteins, if any, in the photosynthetic apparatus of *Hevea* plants. Further objective was to relate such proteins with the functions of photosynthetic apparatus and developing as a marker for identifying tolerant *Hevea* genotypes. In the present study chloroplast proteins isolated from young plants of ten *Hevea* clones showed prominent abundance of a low molecular weight protein of around 23 kDa in size. It was found over-expressing in drought exposed plants while a very low to medium level accumulation was seen in irrigated plants. It is a striking common observation in many plants that low to moderate level of stress proteins occur in cells that have not been stressed but accumulate to very high levels in stressed cells (Young and Elliott, 2002). The relative abundance of this protein in drought tolerant clones RRIM 600, RRII 430 and RRII 429 was very prominent than relatively drought susceptible clones such as PB 260, RRII 414 and Tjir 1 whereas in other clones expression was with medium to optimum range. However there was an exception to this general observation in clone RRII 105 which was graded as drought susceptible through the studies of physiological parameters, but had a relatively fair degree

of accumulation of stress protein under water deficit condition (Fig. 4.1.B). However, the degree of drought susceptibility of this popular clone in India is highly debatable so far. Earlier workers reported that this clone was relatively drought tolerant in traditional rubber growing areas (Gururaja Rao *et. al.,* 1990). On the other hand, recent reports indicated that this clone is comparatively drought and high light susceptible (Sreelatha *et. al.,* 2007; Annamalainathan *et. al.,* 2010; Thomas *et. al.,* 2011).

Common features of high temperature, high light and water deficit conditions induced proteins are known as 'stress protein', (Vierling, 1991). In an earlier study this 23 kDa stress protein was reported as small heat shock protein (sHSP) and was observed to be consistently over expressing in chloroplast thylakoid membrane of rubber plants experiencing drought and high solar light. A total of six different peptides from the induced stress protein successfully matched several sHSP from tobacco, petunia and tomato (Annamalinathan *et. al.,* 2006). Other than heat shock like proteins, an array of regulatory and functional proteins like many transcription factors, proteins involved in signal transduction, LEA and other protein chaperones, proteases and ROS scavenging enzymes etc. are also induced under drought situations (Todaka *et. al.,* 2015). In studies with different plant species, stress proteins of similar kind were ascribed to associate with chloroplast functions related to photosynthetic activities, including PS II electron transport and oxygen evolution activity in the PS II (Barua *et. al.,* 2003). Heckathorn *et. al.,* (2004) has observed protection of thylakoid membranes and PS II electron transport by similar 23 kDa sHSP in chloroplasts. In chloroplast, stress proteins have been implicated in protecting this organelle from photoinhibitory and oxidative stress by preventing protein aggregation and stabilizing thylakoid membrane (Torok *et. al.,* 2001). Similar low molecular weight (LMW) stress proteins also protect photosynthetic electron transport from inhibitory effects of heavy metals (Kumar *et. al.,* 2015). Many stress proteins seem to function as molecular chaperones by regulating protein folding, while others play a role in regulating the function of receptors (Vierling, 1991; Heckathorn *et. al.,* 2004).

When compared with chloroplast protein profile, total leaf protein showed only a small fraction of the stress protein got separated. However,

considering total protein extraction is a rapid protocol it is felt that the method can be exploited for further studies in relation to Western blot technique etc. for the development of protein marker. In the case of an experiment to study the interactive effect of drought and high temperature under growth chamber conditions the clone RRII 105 failed to induce the stress protein significantly when compared to their respective control at all temperature regimes (Fig. 4.3.) which elucidates the complexity of drought acclimation when plants are grown under field condition where the effect of multiple stresses induces or suppress the expression of specific proteins.

In the present study various steps were tried for purification of the stress protein. Triton X-100 a non ionic detergent in phosphate buffer saline was not able to dissolve the chloroplast protein completely. Ammonium sulphate precipitation also didn't prove to be successful. In 1.0 N NaOH, the protein got solubilised but with major change in pH. As an alternative proteomic approach combining protein separation on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electro-elution of the stress protein has been found successful and hence this method was adopted for purification of the stress protein. Successful studies have been reported combining SDS PAGE followed by in-gel digestion and shotgun analysis (LC-MS/MS) to be more adapted than other approaches to membrane proteins (Wu *et. al.,* 2003; Le Naour *et. al.,* 2006; Speers and Wu, 2007; Lu *et. al.,* 2008; Savas *et. al.,* 2011) and the same technique was applied for the identification of this sHSP by Annamalinathan *et. al.,* (2006). Further non-redundant protein data base (MSDB) available through the Mascot search engine was used to match the polypeptides with several small heat shock proteins (sHSPs) of other reported species and this protein was reported as heat shock type protein associated with chloroplast thylakoid membrane in *Hevea* (Annamalainathan *et. al.,* 2006).

Chapter V

Developing a chloroplast LMW protein marker for screening drought tolerance in young Hevea plants

5.1. Introduction

Plants require an adequate supply of water which is vital for cell expansion and all other stages of development. A deeper understanding of drought tolerance is very much essential in the increasing oscillations of climatic conditions. Multiple mechanisms at the morphological, physiological, cell and molecular levels that contribute for reduced drought stress have evolved in plants. Proteins which play a key role in stress signalling, transcription regulation, protection of macromolecules, cellular detoxification, and an array of other cellular processes are the primary factors which aids in drought tolerance (Tester and Langridge, 2010). Molecular events that remain hidden during DNA or mRNA analysis can be better understood through proteomic methods since proteins are the translated portion of the genome and they are the structural or functional units of the cellular metabolism.

Survey of the expression of all proteins in cells/tissues/organisms at a given time and condition is represented by the term "proteome" (PROTEins expressed by genOME) (Wilkins *et. al.,* 1996). Compared to genome based technology proteomics have several advantages because rather than genetic code or mRNA abundance it directly focuses on functional molecules. In the field of crop abiotic stress-tolerance research proteomics is applied for the comparative analysis of different proteomes. Comparison of proteomes of non-stressed plants (control) and corresponding proteomes under stress condition are very informative and give insights into the plant reactions to environmental stimuli. Comparison of proteomes from two different genotypes or plant species with contrasting level of tolerance to a given stress factor can also be determined with this method. Identification of protein expression profile, post-translational modifications (PTMs) and protein-protein interactions under stress and non-stress conditions is better understood through proteomics study (Hashiguchi *et. al.,* 2010; Nam *et. al.,* 2012; Mertins *et.al,* 2013; Ghosh and Xu, 2014). Crop productivity can be improved if breeders have knowledge on response of plants towards abiotic stress at the molecular level. In the recent past, gene expression analyses have paved the way for understanding of plant responses to abiotic stress. Translational and post-translational modifications that would significantly change the abundance as well as activity of proteins can be detected through

proteomics techniques. However, various studies have proved that changes in the transcript level do not correlate with changes in protein abundance (Piques *et. al.,* 2009; Böhmer and Schroeder, 2011).

The recent concepts of "omics" involve developments of technologies which serves to identify key proteins or metabolites in plant science research covering proteomics, metabolomics and genomics for stress tolerance in plants and also the genes involved in the regulation of such biomolecules (Ahmad *et. al.,* 2013; Emon, 2016). Proteins that are responsive to abiotic stresses would show differential expression and the role of protein accumulation under stress conditions and its association with stress tolerance can be elucidated through proteomic approach (Witzel *et. al.,* 2009; Hossain *et. al.,* 2012; Perez-Clemente *et. al.,* 2013). Identification of possible candidate genes that can be used for the genetic enhancement of plants against stresses is an added advantage that is made possible through plant stress proteomics study (Cushman and Bohnert, 2000; Rodziewicz *et. al.,* 2014; Barkla *et. al.,* 2016). Proteomics field largely depends on the basic instrumentation, affinity enrichment and depletion, quantification techniques, peptide and protein identification by mass spectrometry, structural remodelling, statistics, and data mining (Praveen *et. al.,* 2015). The most widely used proteomics methods, two-dimensional electrophoresis (2-DE) and mass spectroscopy (MS) help to catalog and identify proteins in different proteome states or environments (Eldakak *et. al.,* 2013).

In response to stresses different signalling pathways are reported to be activated resulting in complex regulatory network involving transcription factors, antioxidants, hormones, osmolyte synthesis, ROS, kinase cascades and ion homeostasis (Suzuki *et. al.,* 2014; Yin *et. al.,* 2015). However, in different organs of the plant the cell's response to abiotic stress varies. Hence enhancement of crop stress tolerance requires better understanding of cellular mechanisms that regulate stress response and signal transduction in various organelles. Understanding of those mechanism can be attained through organ–specific combined with subcellular proteomic studies from leaf to root (Komatsu and Hossain, 2013; Yin *et. al.,* 2015). Genetic and molecular mechanisms underlying plant stress response was unveiled through advances in proteomic technologies. Since proteins are the key players in majority of cellular events,

proteomics as a technique is advantaged over other "omics" tools. Proteomics can also detect translational and post- translational regulations in addition to its ability in complementing transcriptome level changes there by providing new insights into abiotic stress responses of plants (Salekdeh *et. al.,* 2002).

Proteomics have to be dealt with certain technical difficulties though it addresses complex biological questions. One of the critical factors is good sample quality which is much challenging to obtain from plant tissues. In plant tissues enriched level of protease and oxidative enzymes makes it difficult to extract stable protein mixtures. Secondary metabolites produced in plants add up to this difficulty since it interferes with subsequent protein fractionation and downstream analysis. Cell wall which is difficult to fragment is another hindrance. To a certain extent trichloroacetic acid (TCA)-acetone precipitation and phenol extraction help to overcome the above challenges (Isaacson *et. al.,* 2006). Low protein content in plant cells poses another limitation. Considering the heterogeneity between species, optimization of certain specific experimental conditions is essential for protein related studies (Ghosh and Xu, 2014).

An indirect selection process where a trait of interest is selected based on a marker linked to a trait of interest rather than on the trait itself is known as marker assisted selection (MAS). The majority of MAS in the present era utilizes DNA based genomic markers. However, markers could be divided into four categories: Morphological or phenotypic markers, cytological markers, DNA markers and biochemical markers. Morphological markers are based on the traditional botanical description of visible characters. Cytological markers are based upon the variations in banding patterns of the chromosome and are used for chromosome characterization, detection of mutation and for studying taxonomical relationships which can provide additional information apart from morphological markers. Differences in DNA sequences across genotypes have led to the advent of DNA-based markers. These markers are the variations observed in a particular portion of the DNA among the individuals of a species. Biochemical/physiological markers, one among the popular tool in plant genetics are based on the expression of proteins and secondary metabolites (Jangpromma *et. al.,* 2010; Kadirvel *et. al.,* 2015).

As a powerful technique proteomics is employed in separating complex protein mixtures. Any change in protein profile in response to environmental factors can be determined with this method. Hence proteins that are responsive to drought stress may show differential expression in different clones/varieties. A few investigations were done to obtain such proteins and by establishing its functional relevance for screening drought tolerant *Hevea* clones in line with other physiological markers. In the present study accumulation of chloroplast stress protein of molecular weight 23 kDa was identified by western blotting technique using polyclonal antibody raised against stress protein in rabbit. The stress protein abundance in chloroplast was quantified and further expression level related with photosynthetic activities of ten different clones. Total leaf protein also was isolated from two different clones and subjected to western blot analysis in order to develop a quick method for screening rubber clones. The amino acid sequence of the stress protein was already elucidated by combining SDS PAGE followed by in-gel digestion and shotgun analysis LC-MS/MS and mass spectrometry and was reported as a HSP type protein associated with chloroplast thylakoid membrane in *Hevea* (Annamalainathan *et. al.,* 2006). The feasibility of using the relative expression level of this HSP type protein along with specific physiological activities to screen rubber clones for drought stress tolerance was also attempted. The 23 kDa stress protein that showed consistent over expression in drought induced clones was identified as small heat shock protein in *Hevea* (sHSP23.8). Further the expression analysis of *HbsHSP23.8* gene was quantified in four clones of *Hevea* with varying level of drought tolerance to check the role of *HbsHSP23.8* in drought response.

5.2. Materials and Methods

5.2.1. Development of chloroplast stress protein as a marker.

5.2.1.1. Raising of polyclonal antibody against stress protein in rabbit

Chloroplast stress protein (molecular mass of 23 kDa) purified by repeated electro-elution as described in chapter 4 was used for raising antibodies in rabbit. Pre-immune serum was collected first from rabbit and this served as the negative control. First immunization was given using 500 µg of stress protein emulsified in Freund's complete adjuvant. After 15 days, the rabbit was boosted

five times with 200 µg stress protein each in incomplete Freund's adjuvant at 10 days interval. The serum was obtained 10 days after the last bleeding (GeNei, Bangalore). The project report of custom polyclonal antibody service by GeNei, Bangalore is represented in Appendices.

5.2.1.2. Western blotting analysis of chloroplast stress protein

Chloroplast proteins (30 µg) were electrophoretically separated using 10% SDS-polyacrylamide gels as described in chapter 4. Chloroplast protein profile was transferred from the gel onto a nitrocellulose membrane (NC) following Towbin's (1979) method of transfer. The NC membrane, filter pad and filter paper were first made wet in ice cold transfer buffer (Towbins buffer) containing Trizma base 25 mM, Glycine 192 mM and 20% methanol. 10X Tris-buffered saline (TBS) containing 24.2 g Trizma Base and 80 g NaCl with pH adjusted to 7.6 served as the stock for preparing TBS-Tween for washing membrane and antibody dilution. 1X TBS-Tween (TBST) was prepared by adding Tween 20 to TBS to a final concentration of 0.1%. The transfer stack was first prepared without any air-bubbles by rolling a glass-rod over the entire set-up and was then inserted into Mini Trans-Blot apparatus (Bio-Rad) containing ice cold transfer buffer. Electro-blotting was performed overnight at 40V in 4°C. After transfer the NC membrane was carefully removed and rinsed with double dist. water. The transfer of protein was ascertained by staining the NC membrane with reversible stain Ponceau-S (0.2% in 1% acetic acid). The membrane was then washed with TBST (1X) to remove the stain. The membrane was blocked for 1hr at room temperature (RT) with 3% skimmed milk powder and subsequently probed with polyclonal primary antibody to the stress protein at a dilution of 1:10,000 for 1hr at RT. The membrane was washed three times with TBST and then incubated in horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (GeNei, Bangalore) at a dilution of 1:3000 for 1hr at RT. The blot was developed finally using 1X tetramethylbenzidine (TMB) / hydrogen peroxide (H_2O_2) for localization (GeNei, Bangalore). The magnitude of expression was quantified from the relative band intensity using a Gene Genius bio-imaging system, Syngene, USA. For consistent values the blotting experiment was repeated thrice.

5.2.1.3. Western blotting analysis of total leaf protein

Total leaf protein isolated as mentioned in chapter 4 after resolving on 10% SDS PAGE (30 µg) was subjected to Western blotting as per the procedure described in 5.2.1.2.

5.2.1.4. Screening of *Hevea* germplasm accessions

30 µg of chloroplast protein from six germplasm accessions viz. RO 3261, AC 612, RO 3157, RO 3184 (drought tolerant), RO 3242 and MT 1619 (drought susceptible) and two check clones *viz.*,RRII 105 (drought susceptible), RRIM 600 (drought tolerant) was resolved on 10% SDS PAGE and subjected to western blotting as per the procedure described in 5.2.1.2. The induction, of drought stress and chloroplast protein isolation was performed as described in chapter 3 and 4.

5.2.2. In silico studies of LMW protein from *Hevea brasiliensis*

The amino acid sequence of the LMW protein sHSP23.8 that was reported by Annamalainathan *et. al.,* (2006) was analysed for physico-chemical properties. The full length amino acid sequence of sHSP23.8 was obtained from nucleotide sequence of the gene (NCBI GenBank ID: KT376983) by translation using the ExPASy (Expert Protein Analysis System) tool (http://web.expasy.org/translate/). Physico chemical properties like molecular weight, isoelectric point (pI), aliphatic index, instability index, amino acid property and Grand Average of hydropathicity (GRAVY) were obtained for the protein from the ExPASy tool ProtParam (http://web.expasy.org/protparam/). The subcellular localization of the protein was also predicted using the predict protein server (https://www.predictprotein.org/).

5.2.3 Expression analysis of *HbsHSP23.8* gene in *Hevea* with varying level of drought tolerance.

5.2.3.1 Primer Designing

Quantitative PCR analysis was carried out to study the expression level of *HbsHSP23.8* gene in four clones of *Hevea* viz., RRII 105, RRII 414, RRII 430 and RRIM 600. The imposition of drought and physiological measurements to

assess the impact of drought stress were performed as mentioned in Chapter 3. The house keeping gene, GAPDH, was used as the internal control for normalization. Primers were designed (amplicon size of 100-200bp) from the *H. brasiliensiss* HSP23.8 (GenBank ID.KT 376983) gene deposited in the NCBI GenBank accessions using the Primer Express Software and got synthesized by Eurofins and the details are given in Table 5.1.

5.2.3.2. Total RNA Isolation

Total RNA was extracted from the leaf samples using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. Leaf tissues were ground to fine powder in liquid nitrogen using mortar and pestle. 100 mg powdered tissue was then mixed with 500 µl of lysis solution: β-mercaptoethanol mixture and vortexed vigorously for 30 sec. The samples were then incubated at 56°C for 5 min and centrifuged. The lysate supernatant was transferred to a filtration column and centrifuged. 500 µl of binding solution was mixed with the clarified filtrate. The mixture was then transferred to a binding column and centrifuged at 12000 rpm for 1 min to facilitate binding of RNA. 500 µl of wash solution I was added to the binding column and centrifuged at 12000 rpm for 1 min followed by washing with wash solution 2 and centrifugation at 12000 rpm for 1 min. The flow through was discarded and the column was transferred to fresh tube. The RNA was eluted with 70 µl of sterilized DEPC water. The quality of RNA was confirmed by resolving on 1.4 % denatured agarose gel and quantified spectrophotometrically using Nanodrop ND 1000 (USA).

5.2.3.3. cDNA preparation

cDNA synthesis was carried out using Superscript™ III first strand synthesis system (Invitrogen). 03 µg of total RNA was combined with 01µl of oligo dT primer (50 µM) and 01 µl of 10 mM dNTP mix and made up to 10 µl by adding sterilized DEPC treated water. The mixture was kept at 65°C for 05 minutes and immediately chilled on ice for a minute. The cDNA synthesis system mix (10 µl) was prepared by combining 02 µl of 10X RT buffer, 04 µl of $MgCl_2$ (25 mM), 02 µl 0.1M DTT, 01 µl of RNase OUT (40U/µl) and 01 µl of Superscript III reverse transcriptase (200 U/µl). This reaction mix was added to

10 µl of prepared RNA-primer mixture and incubated at 50°C for 50 minutes for cDNA synthesis. After that, reaction was terminated by incubating at 85°C for 5 minutes followed by chilling on ice. RNA was removed by adding 01 µl of RNase H and kept the tube at 37°C for 20 minutes. The cDNA was quantified and stored at -20°C for further use.

5.2.3.4. Quantitative real time PCR analysis

Real time PCR was performed using Light Cycler 480II, Roche Real Time PCR System. The reaction consisted of 01 µl of 1:10time's diluted cDNA, 125 nM of each forward and reverse primers and 10 µl of Lightcycler 480 SYBR Green I Master (Roche Diagnostics Gmbh, Germany) in a 20 µl reaction volume. The reaction conditions included 95°C for 07min, followed by 40 cycles of 95°C for 20 s and 60°C for 30 s. This was followed by a melt curve analysis (95°C for 20 s, 60°C for 01 min and 95°C for 05 min). Reaction efficiency of the target gene and the endogenous control was calculated based on the formula, $E=10^{(-1/slope)}-1$ and the slope values of the primers were between -3.2 and -3.5. Three biological replications for each treatment were included in the qPCR analysis. No template controls (NTC) were run to assay for false positive signals and GAPDH was used as the endogenous control. Relative Quantification (RQ) values were used to study the fold change in the expression rate of the gene using Light Cycler 480 Software; release 1.5.0.

5.2.3.5. Data analysis

The relative changes in gene expression from qPCR experiments were analyzed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and the data are presented as the fold change. Statistical analysis was performed with the relative quantification data using ANOVA. The difference between groups was assessed by means of the 2-tailed Student *t* test. P-value <0.05 was considered to be statistically significant.

Table 5.1: List of genes and the corresponding primers used for qPCR analysis

Sl no	Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
1	*HbsHSP23.8*	GATGTGGTCGACTCATTTTCTCCA	CTTTGACGTCCTGTTTGCTTAGCC
2	GAPDH	GCCTGTGATAGTCTTCGGTGTTAG	GCAGCCTTATCCTTGTCAGTGAAC

5.3. Results

5.3.1. Detection of drought induced stress protein by western blotting analysis

5.3.1.1. Development of antibody against stress protein in rabbit and western blotting

Polyclonal antibody for the purified stress protein was successfully raised in rabbit by GeNei, Bangalore. The purity of the antigen was confirmed by SDS PAGE before immunization and titre analysis of the antibody raised was finally fixed as 1:10,000 by Direct ELISA and Western Blot. The same dilution was fixed for our study after checking different dilutions by western blotting.

The relative abundance of stress protein in western blot profile was checked in two popular clones viz., RRII 105 and RRIM 600. The irrigated control plants showed very low abundance of this protein in these clones whereas the drought and high light exposed plants showed very prominent expression (Fig 5.1). The expression and accumulation of chloroplast stress protein was consistently very high in drought exposed plants confirming further that the protein is associated with drought responses in young plants of natural rubber.

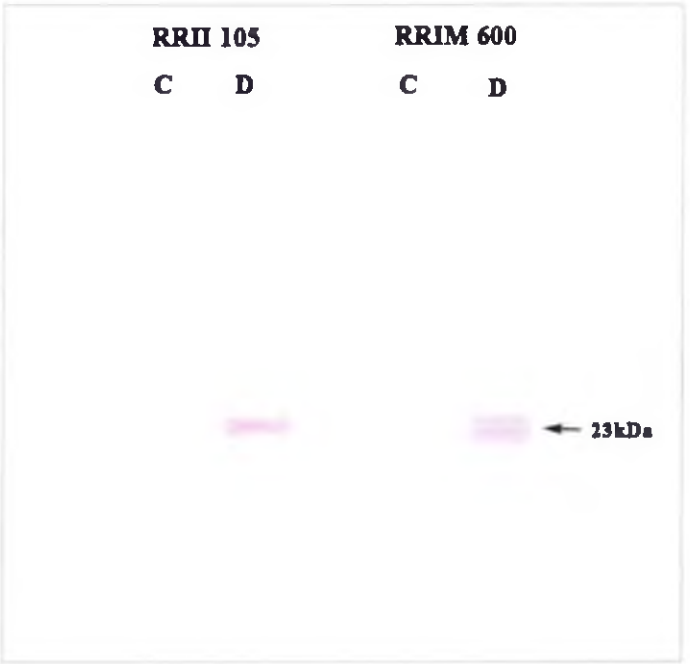


Fig 5.1. Western blot profile of 23 kDa chloroplast stress protein isolated from two popular *Hevea* clones (RRII 105 and RRIM 600)

5.3.1.2. Validation of chloroplast stress protein as a marker for drought tolerance

In order to validate the chloroplast stress protein's association with drought response, ten different rubber clones were tested for relative expression level of stress protein after drought exposure for ten days. The antibody raised against the stress protein was used to detect and quantify the protein in Western blots. In the irrigated control plants the accumulation of this protein was very less to obscure. On the contrary the water deficit stress imposed plants accumulated significantly higher level of the stress protein (Fig 5.2). The relative abundance of this protein was very prominent in relatively drought tolerant clones such as RRIM 600, RRII 430 and RRII 429 than relatively drought susceptible clones such as PB 260, RRII 414 and Tjir 1. In the remaining clones the expression level was with medium to optimum range. The drought tolerant clones recorded around 71-110% over expression of this stress protein upon exposure to drought compared to their respective irrigated counterparts whereas the susceptible clones had a relative abundance of only 8-30% over their control plants (Table 5.2). Interestingly, a well-known drought tolerant rubber clone

RRIM 600 recorded around 110% higher abundance than the control irrigated plants. In clone RRII 105, which was graded as drought susceptible in the present study had a relatively fair degree (around 30% over control plants) of accumulation of stress protein under water deficit condition. There was significant relationship existed between the abundance of this protein and relative drought tolerance traits (R^2=0.58) among the clones studied (Fig. 5.3 and 5.4).

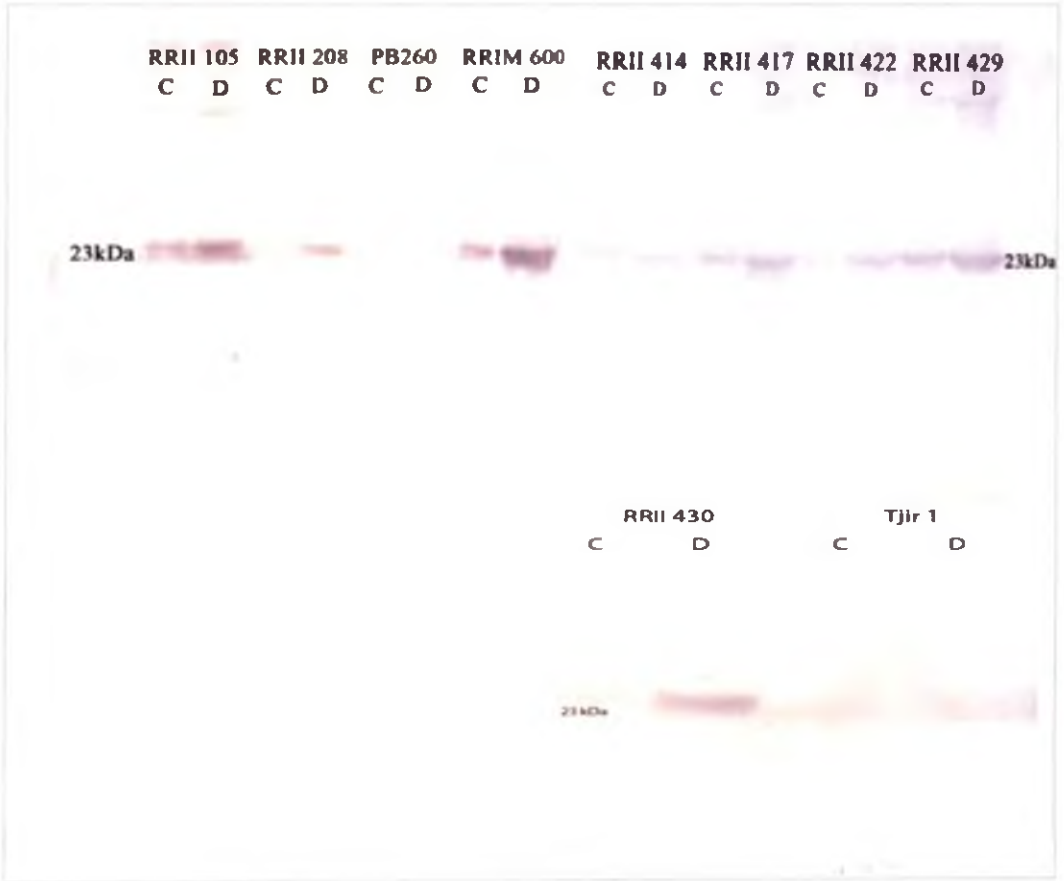
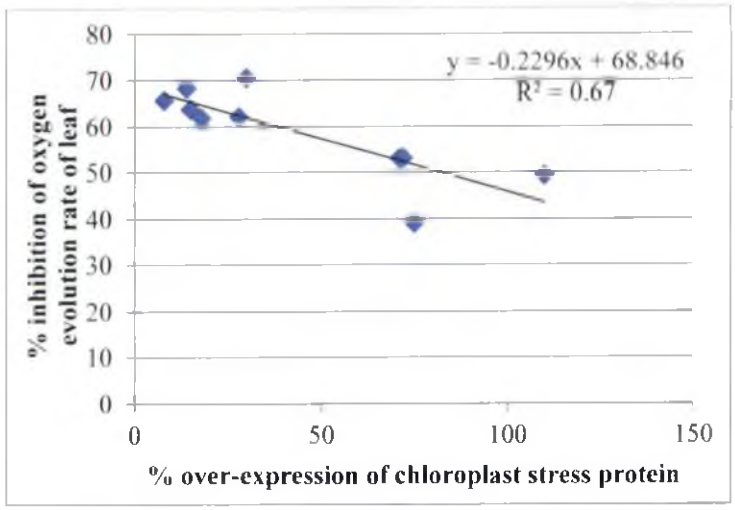


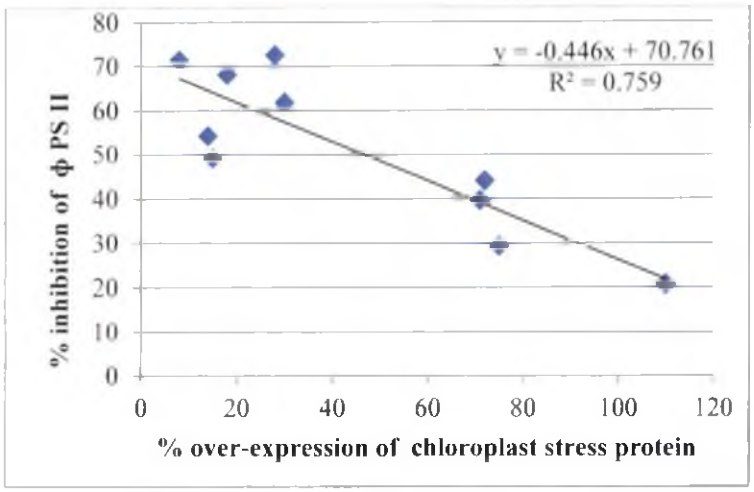
Fig 5.2. Western blot profile of 23 kDa chloroplast stress protein extracted from ten different *Hevea* clones. One set of plants was irrigated in polybags to the level of field saturation (C-control) and another set was maintained without irrigation (D-drought) for ten days. Chloroplast protein of 30 µg was loaded uniformly in each lane. The stress protein was detected by incubating the chloroplast protein profile in NC membrane with a polyclonal antibody raised against this protein.

Table 5.2. The relative abundance of 23 kDa chloroplast stress protein in different clones of *Hevea* after exposure to 10 days of soil moisture deficit stress by withholding irrigation. The relative abundance was calculated based on keeping the value for respective control (irrigated) plant as 0%.

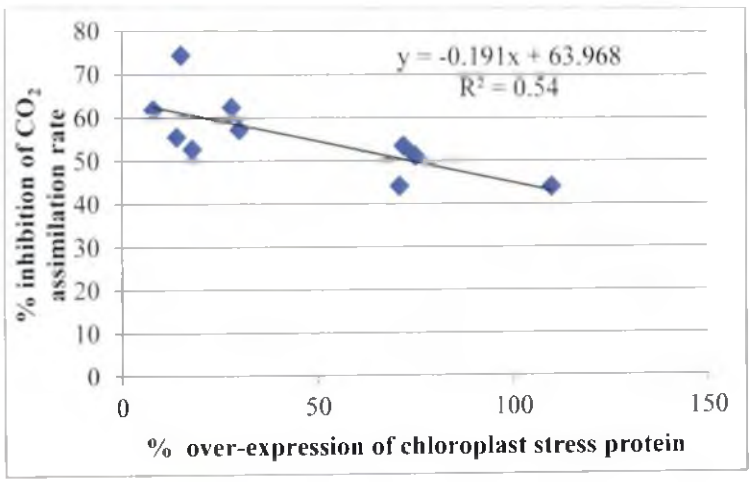
S. No.	Clone Name	% increase of protein abundance in drought samples over the respective irrigated control
1	RRIM 600	110
2	RRII 430	75
3	RRII 429	72
4	RRII 208	71
5	RRII 105	30
6	RRII 417	28
7	RRII 422	18
8	Tjir 1	15
9	RRII 414	14
10	PB 260	8



(a)



(b)



(c)

Fig. 5.3. Relationship between stress protein abundance and percentage inhibition of (a) oxygen evolution rate of leaf (b) ϕPSII and (c) CO_2 assimilation rate

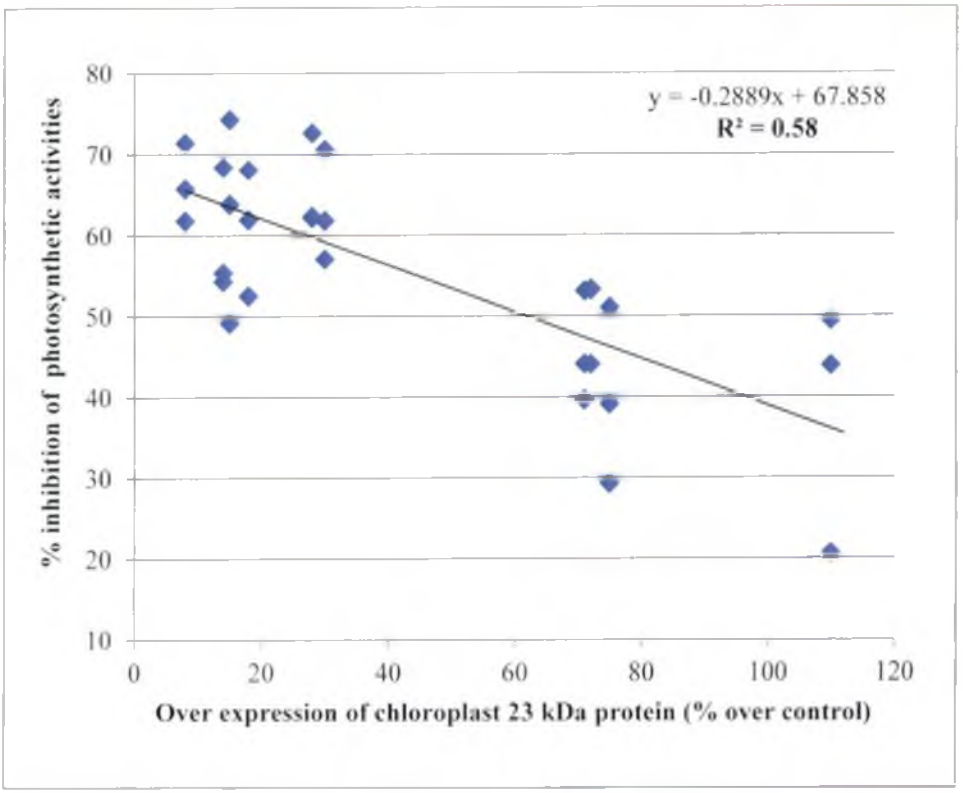


Fig. 5.4. Relationship between stress protein abundance and percentage inhibition of photosynthetic activities on an average

5.3.1.3. Western blot analysis of total leaf protein.

Total leaf protein was isolated from the clones RRII 105 and RRIM 600 after 10 days drought imposition. 30 µg of total leaf protein was resolved on 10% SDS PAGE and subjected to western blotting. The blotting was effective and showed prominent expression of the 23 kDa stress protein (Fig. 5.5). This method was found rapid when compared to chloroplast proteins preparation. However, the protein resolution was not refined enough when the question of accuracy comes.

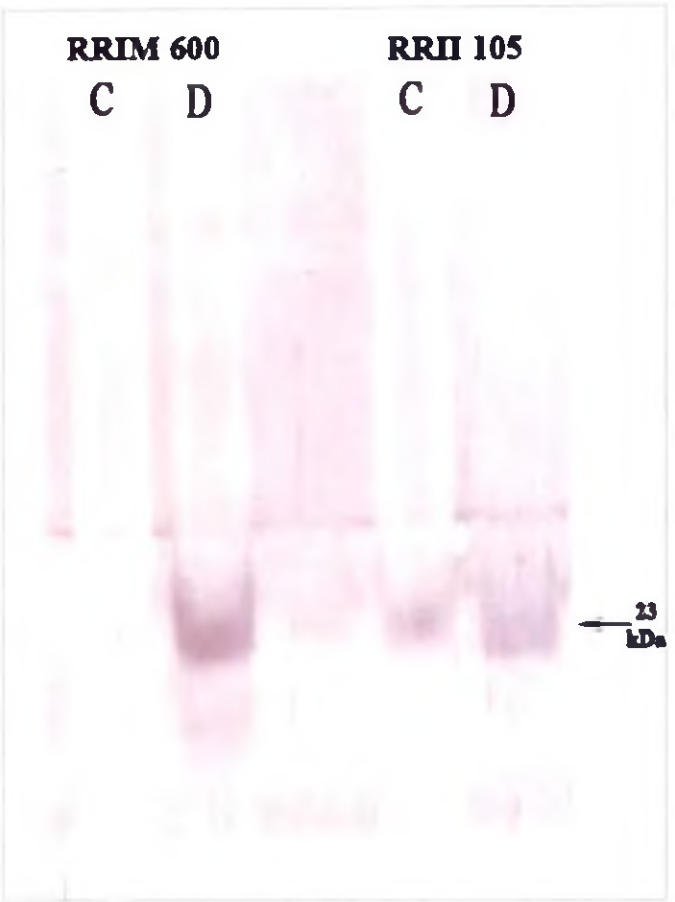


Fig 5.5. Western blot profile of 23 kDa stress protein (from total leaf protein) extracted from *Hevea* clones RRIM 600 and RRII 105. One set of plants was irrigated in polybags to the level of field saturation (C-control) and another set was maintained without irrigation (D-drought) for ten days. Total leaf protein of 30µg was loaded uniformly in each lane. The stress protein was detected by incubating the total leaf protein profile in NC membrane with a polyclonal antibody raised against this protein.

5.3.1.4. Validation of stress protein in *Hevea* germplasm accessions

In order to ascertain further the association of stress protein with drought tolerance traits, western blotting analyses was carried out with 30 µg of chloroplast protein resolved on 10% SDS-PAGE from four relatively drought tolerant (RO 3261, AC 612, RO 3157 and RO 3184) and two susceptible (RO 3242 and MT 1619) germplasm accessions along with check clones RRIM 600 and RRII 105 after imposing drought for 10 days. The relative expression of the protein in relatively drought tolerant germplasm accessions RO 3261, AC 612, RO 3157 and RO 3184 was very prominent than susceptible clones RO 3242 and MT 1619 in comparison with check clones (Fig. 5.6).

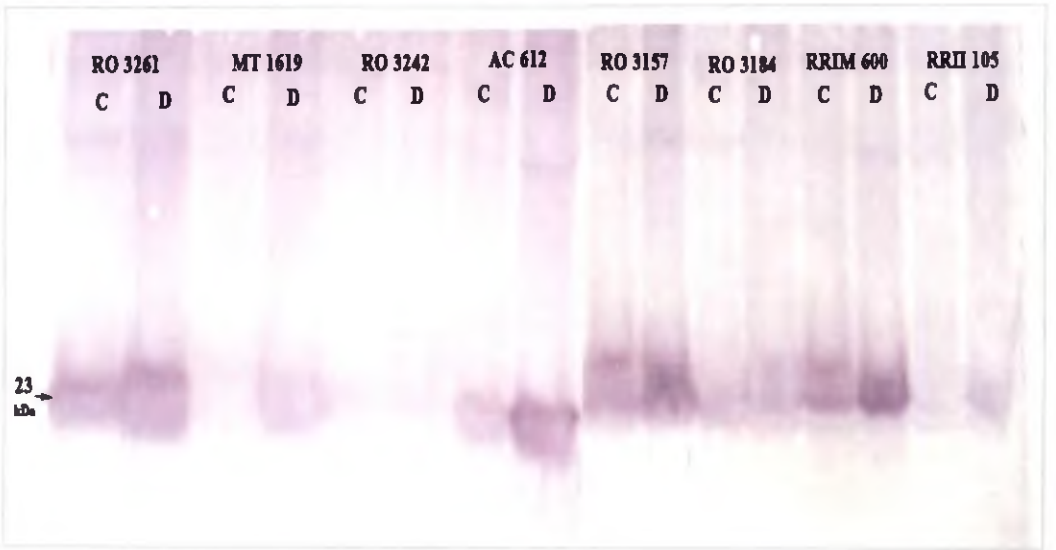


Fig 5.6. Western blot profiles of 23 kDa chloroplast stress protein extracted from six *Hevea* germplasm accessions and check clones RRIM 600 and RRII 105. One set of plants was irrigated in polybags to the level of field saturation (C-control) and another set was maintained without irrigation (D-drought) for ten days. Chloroplast protein of 30 µg was loaded uniformly in each lane. The stress protein was detected by incubating the total leaf protein profile in NC membrane with a polyclonal antibody raised against this protein.

5.3.2. In silico studies to find out the physico-chemical properties of the stress protein.

In silico studies of *Hevea* small heat shock protein (sHSP23.8) reported by Annamalianathan *et. al.*, 2006 were carried out. Amino acid sequence of sHSP23.8 (214 aa) protein (Fig. 5.8) was obtained from nucleotide sequences of sHSP23.8 gene (Fig. 5.7) by translation using ExPASy tool. Physico-chemical properties were obtained for the protein by the ExPASy tool ProtParam as shown in table 5.3. The physico-chemical properties showed that the sHSP protein had a molecular weight of 23.83 kDa. The theoretical pI value was 7.88 and the total number of negatively charged and positively charged residues were 32 and 33 respectively. The instability value and aliphatic index for sHSP was computed as 56.56 and 77.29. The grand average of hydropathicity (GRAVY) was -0.621 and the subcellular localization for the protein was carried out using PredictProtein software and identified that sHSP is located in chloroplast of eukaryote (Fig 5.9).

ATGGCATCACTGATTGCTTTAAGGAAAGCAACCGCTTCTGCTCTCTTC
TCCAAGCTCATCAATCCTGTCCGCTCTGCCTCTGTGGCACCTTCTGTTT
CTCGCTCCTTCAGCACTGAAACCCAGGTCACCAACTTTGGCGGCGAC
GATAGTGGCAACGTCGACGTTAATAGGCGCTCCTCTGATCGCAGCGT
CTCTCGCCGCCGAGATACTTCTCGCAGTTTCTTCCCAGATGTGGTCGA
CTCATTTTCTCCAACGAGGACTCTGAGCCAGGTGTGGAACCTAATGG
ACCAGTTAATGGAGTACCCGTTGGGCGTGGGAGCCGGAGGTGGCGTT
GGTGCGAGGCGAGGGTGGGACGTGAAGGAGGACGAGGAAGCTCTGT
ATCTAAGGATGGACATGCCAGGGCTAAGCAAACAGGACGTCAAAGT
GGGCGTGGAGCAGAACACACTGGTGATAAAAGGGGAAGGCCCAAAA
GAAAACGAAGAAGAAGAGAGTGGAAGAAGGTACTCAAGCAGACTGG
AGTTGCCTCGAAATCTGTACAAGCTCGATGAGATTAAGGGTGAAATG
AAGAACGGTGTTTTGAAGGTGGTGGTACCAAAGGTGAAAGAACAAG
AGAGAAAGGATGTCCATGAGGTTCAGATTCAGTGA

The start codon ATG is marked in blue colour and stop codon TGA is marked in red colour.

Fig.5.7. Open Reading Frame (ORF) of small heat shock protein 23.8 (sHSP 23.8) from *H.brasiliensis* (GenBank ID: KT376983).

```
        10         20         30         40         50         60
MASLIALRKA TASALFSKLI NPVRSASVAP SVSRSFSTET QVTNFGGDDS GNVDVNRRSS

        70         80         90        100        110        120
DRSVSRRRDT SRSFFPDVVD SFSPTRTLSQ VWNLMDQLME YPLGVGAGGG VGARRGWDVK

       130        140        150        160        170        180
EDEEALYLRM DMPGLSKQDV KVGVEQNTLV IKGEGPKENE EEESGRRYSS RLELPRNLYK

       190        200        210
LDEIKGEMKN GVLKVVVPKV KEQERKDVHE VQIQ
```

Fig. 5.8. Predicted amino acid sequence of small heat shock protein 23.8 (sHSP23.8) from *H. brasiliensis* (Using ExPASy Translation tool)

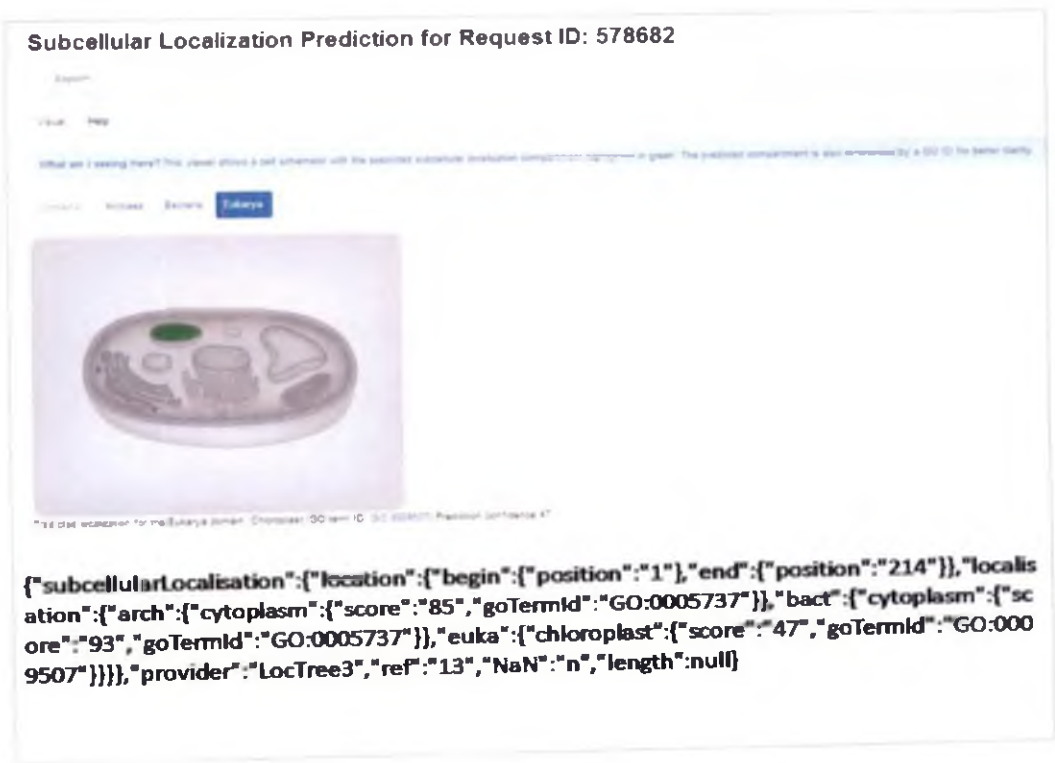


Fig. 5.9. Subcellular localization of small heat shock protein 23.8 (sHSP23.8) from *H. brasiliensis* (Using PredictProtein tool)

Table.5.3. Physico-chemical properties of the predicted sequence of small heat shock protein 23.8 (sHSP23.8) from *H.brasiliensis*

Number of aminoacids	214
Molecular weight	23.83 kDa
pI	7.88
Total number of negatively charged residues	32
Total number of positively charged residues	33
Instability index	56.56
Aliphatic index	77.29
Grand average of hydropathicity (GRAVY)	-0.621

5.3.3. Expression analysis of gene encoding *HbsHSP23.8* in *Hevea* clones with varying level of drought tolerance

Total RNA with good quality was isolated from the leaf tissues of four different clones of *Hevea viz,* RRII 105, RRII 414, RRII 430 and RRIM 600 (Fig 5.10). The plants were already exposed to drought stress by withholding irrigation for 10 days and the impact of stress was assessed by crucial physiological parameters like leaf water potential, PS II activities and measurement of P_N. The transcript of the LMW protein *HbsHSP23.8* in these four clones were tested for their association with drought stress tolerance by quantifying its expression level by qPCR analysis and the results are given in Fig 5.11. From the study it was found that *HbsHSP23.8* got significantly upregulated in drought exposed plants over their irrigated control. In case of relatively drought tolerant clones RRIM 600 and RRII 430, the magnitude of expression (8 fold increase) was significantly higher than other two drought susceptible clones. On the other hand in RRII 105 the expression level was slightly higher (4 fold) than RRII 414 (3fold) which showed optimum range of expression.

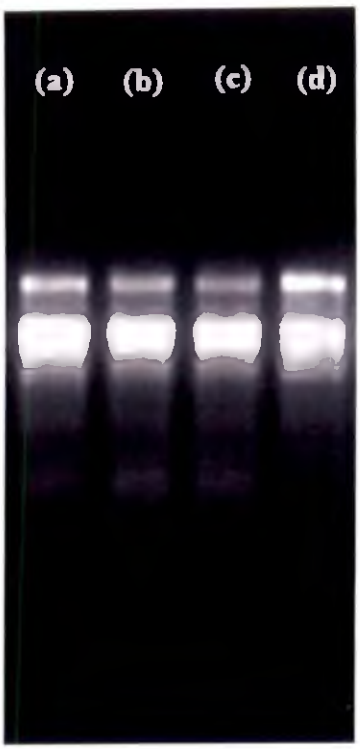


Fig 5.10. Gel photograph showing total RNA isolated from the leaf tissues of drought induced clones of (a) RRIM 600 (b) RRII 430 (c) RRII 105 and (d) RRII 414

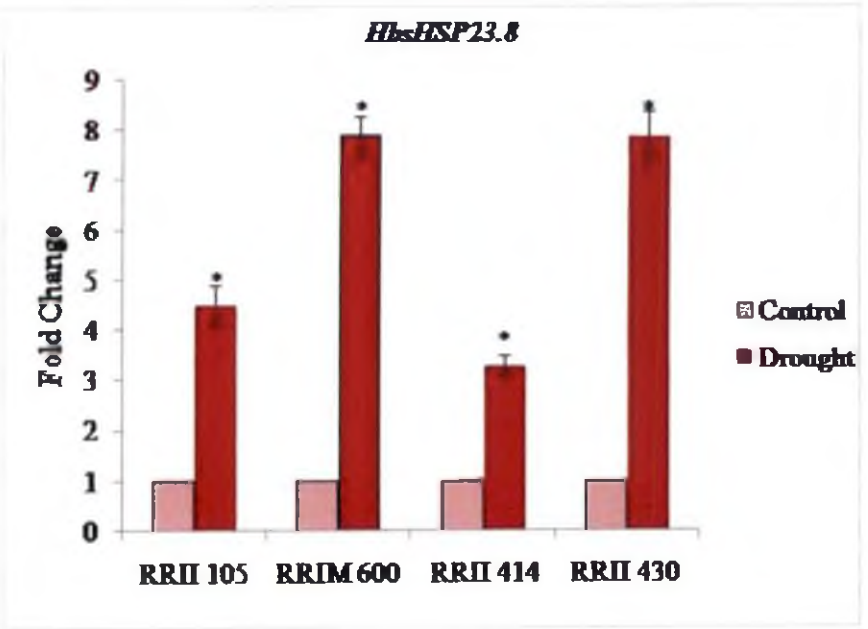


Fig 5.11: Expression analysis of *HbsHSP23.8* gene in four clones of *Hevea* under drought condition. Y axis indicated fold change in the expression of *HbsHSP23.8* mRNA in drought samples over their respective irrigated plants (Control). The mRNAs were quantified by means of real-time polymerase chain reaction and normalized with the use of GAPDH mRNA. Values are expressed as mean ± SD (n=3/group). *P<0.05 Drought vs Control.

5.4. Discussion

One of the prevalent abiotic stresses that vastly affect the physiological and metabolic functions of a growing plant is drought, as a result of prolonged water deficit in the soil. Selection of stress-tolerant cultivars for breeding programs is therefore necessary to screen plants suitable for such drought prone areas. Extensive investigations have been done in model plants, crops and woody plants to understand the proteomic aspects of plant drought response. Development of biochemical markers relating to drought tolerance have become a popular tool in plant genetics like other molecular markers. Due to feasible extraction processes, proteins or secondary metabolites from leaves are often used as markers (Jangpromma *et. al.,* 2010). More than 2200 drought-responsive proteins have been identified in leaves from 25 plant species mainly involved in signalling, transcription, stress and defence, protein synthesis, folding and degradation, photosynthesis and photorespiration, carbohydrate and energy metabolism, membrane and transport, cell structure and cell cycle, nitrogen assimilation and amino acid metabolism, as well as fatty acid metabolism (Wang *et. al.,* 2016). In the present study drought responsive protein marker in *Hevea* has been investigated.

The 23 kDa chloroplast stress protein with prominent expression in drought imposed young plants of *Hevea* reported as HSP type (sHSP) associated with chloroplast thylakoid membrane by Annamalinathan *et. al.,* 2006 was purified by repeated electro-elution from the drought tolerant clone RRIM 600. This protein was found associated with thylakoid membrane and induced under combined abiotic stress conditions like water deficit, high light and high temperature. The purified protein was used towards raising antibody against this protein. Polyclonal antibody was raised in rabbit by utilizing the service of GeNei, Bangalore and used to detect and quantify the protein in western blots. Among the ten elite clones studied the relative abundance of this protein in relatively drought tolerant clones was very prominent than relatively drought susceptible clones. Clone RRIM 600 recorded the maximum abundance of the stress protein in comparison to the control irrigated plants. However, clone RRII 105 which was graded as drought susceptible in the present study had a

relatively fair degree of accumulation of stress protein under water deficit condition. R^2value of 0.58 (Fig. 5.4) shows that there exists significant relationship between the abundance of this protein and relative drought tolerance traits among the clones studied. The western blot analysis of ten clones clearly demonstrated that most of the drought tolerant clones accumulated this protein in a higher extent than drought susceptible clones (Fig. 5.2). Those clones susceptible in terms of high magnitude of inhibition of PSII activity, photosynthetic oxygen evolution and net photosynthesis rate under soil moisture deficit stress had very low level of this protein. Antibody raised against p18 protein (18 kDa) isolated from sugarcane leaves was used in a similar way for screening drought tolerance in sugarcane cultivars (Jangpromma *et. al.,* 2010). Therefore, the quantitative analysis of this protein along with measurement of crucial photosynthetic parameters like PSII, oxygen evolution activities and CO_2 assimilation rates may be used as screening tools for the identification of relatively drought tolerant *Hevea* clones in young immature stage of plants.

In total leaf protein preparations also prominent expression of 23.8 kDa protein was observed. It was found that total protein extraction method was less time consuming and effective and could be employed to screen large number of plants, by western blotting. Attempts were also made to ascertain the association of stress protein with drought tolerance by western blotting using the drought tolerant germplasm accessions selected by Thomas *et. al.,*(2015). The results clearly demonstrated that the stress protein expression was prominent in RO 3261, AC 612, RO 3157 and RO 3184 along with tolerant check clone (RRIM 600). In susceptible germplasm accessions (RO3242 and MT 1619) the expression of stress protein was very less in MT 1619 and RO 3242 (Fig 5.6). These results once again confirmed the association of the stress protein with drought tolerance and also having very good conformity with the susceptibility/tolerance nature of germplasm accessions which have already been evaluated using physiological and biochemical parameters (Thomas *et. al.,* 2015). In earlier studies the stress protein was identified and reported as sHSP by LC/MSMS (Annamalainathan *et. al.,* 2006). Since the nature of the protein was already elucidated as a sHSP in earlier studies and further antibody was raised against the protein preparations.

ExPASy tool that was used to carry out the in silico studies of proteins was provided as a service to the life science community by a multidisciplinary team at the Swiss Institute of Bioinformatics (SIB). ExPASy provides access to a variety of databases and analytical tools dedicated to proteins and proteomics (Gasteiger *et. al.,* 2003). Detailed study of sHSP protein was carried out using ExPASy tool. From this data it was found that sHSP protein was less stable as the Instability index was above 40. A protein with an instability index of less than 40 is predicted to be stable while above 40 is unstable. The GRAVY value of a protein is a measure of its hydrophobicity or hydrophilicity and is based on hydropathy values which ranges from -2 to +2 for most proteins, with the positively rated proteins being more hydrophobic. GRAVY value is calculated as the sum of hydropathy values of all the aminoacids, divided by the number of residues in the sequence (Kyte and Doolittle, 1982). In this study the GRAVY value of the protein was found negative, with a value of -0.621 predicting the protein to be hydrophilic in nature (Table 5.3).

Later to predict the sub-cellular localization of the protein the software PredictProtein was used. PredictProtein, an internet service for sequence analysis that has been predicting the protein structure and function which went online in 1992 at the European Molecular Biology Laboratory (EMBL), and belonged to a group of five pioneering internet sites for molecular biology. Queried with a protein sequence the service incorporates various analysis methods which include sub-cellular localization prediction of proteins which is one aspect of protein function (Rost and Liu, 2003; Yachdav *et. al.,* 2014). Using the software it was predicted that sHSP protein is located inside the chloroplast. Earlier studies by Heckathorn *et. al.,* (2004) have reported the role of sHSP that is involved in the protection of PSII in chloroplasts of plants that experience abiotic stresses including that of metal toxicity. In *Hevea* the role of sHSP in protection of thylakoid membrane of chloroplast from oxidative stress leading to survival during stress or facilitating recovery from stress of this organelle have also been reported (Annamalinathan *et. al.,* 2010). The sub-cellular localization of the protein hence was further reaffirmed through the results obtained via PredictProtein services (Fig. 5.9).

In order to determine the association of *HbsHSP23.8* gene to drought tolerance/susceptibility of *Hevea* quantitative expression analyses were carried out by qPCR (Fig.5.11). The results showed that *HbsHSP23.8* was found significantly upregulated in relatively drought tolerant clones RRIM 600 and RRII 430 than other two drought susceptible clones. In case of RRII 105 the expression level was slightly higher than RRII 414 which showed optimum range of expression. Recent studies using RT-PCR analyses have established the involvement of *HbsHSP23.8* gene in enhancing rubber plant's tolerance to environmental stresses including drought (Jun *et. al.*, 2015). Significant level of up-regulation of *HbsHSP23.8* in tolerant clones of *Hevea* in the present study indicates its strong association with drought tolerance.

In the present study transcript of the LMW protein *HbsHSP23.8* from *H. brasiliensis* was validated for their association with drought stress tolerance. This was made possible by quantifying its expression in four different clones of which two were graded as drought tolerant (RRIM 600 and RRII 430) and remaining two as drought susceptible (RRII 105 and RRII 414). The data obtained from physiological and proteomics studies also supported the fact in the case of tolerant clones. RRIM 600 and RRII 430 were graded as abiotic stress tolerant clones in previous studies conducted in *Hevea* hence suitable for drought and cold prone regions (Priyadarshan *et. al.*, 2000; Sumesh *et. al.*, 2011; Mydin, 2014). From the gene expression analysis data, *HbsHSP23.8* was identified to be strongly associated with drought responses in clones RRIM 600 and RRII 430 (up-regulation with a tune of 7-8 folds) whereas in RRII 105 and RRII 414 the up-regulation was 4 and 3.3 folds, respectively under drought.

Chapter VI

General Conclusion

6.1. Summary and Conclusions

Hevea brasiliensis is the major commercial source of natural rubber (NR) and India is one of the leading producers and consumers of natural rubber. Through extension of cultivation to the non-traditional regions, efforts are being made to increase the NR production in order to narrow down the demand-supply gap. Drought is probably the largest factor which limits the agricultural productivity in general and is the most important factor that prevents the expansion of cultivation of *H. brasiliensis* which affects the performance of the crop severely in non-traditional regions. The extent and rate of progress in improving drought tolerant traits in *Hevea* clones without compromising on the productivity through conventional breeding is limited owing to its multigenic nature and complex mechanisms involved. A practical approach could be searching for candidate genes conferring drought tolerance through screening of large collection of wild accessions and identification of tolerant genotypes. Hence, this study was conducted to identify potential drought responsive low molecular (LMW) weight proteins from *H. brasiliensis* which could be eventually used as a marker for selection of clones for abiotic stress tolerance.

Small heat shock proteins and other stress induced proteins in plants constitute a diverse and abundant group. Majority of sHSPs are highly induced upon heat stress, and such expression often confers with increased thermal tolerance by protecting proteins from irreversible denaturation. The composition and expression of such proteins have recently been studied in several plants which play a pivotal role in abiotic stress responses and tolerance. Various studies conducted on these stress induced proteins indicated the possibility of employing as potential markers towards developing stress tolerant plants.

In this context, the present study was undertaken to identify and characterize the role of low molecular weight stress protein from *H. brasiliensis* clones with particular reference to drought stress. Physiological responses of young plants belonging to ten clones of *H. brasiliensis* were initially analyzed under soil moisture deficit condition. The relative drought tolerance potential of these clones were evaluated using key physiological parameters such as leaf water potential, leaf photosynthetic oxygen evolution rate, quantum yield of

PS II and CO_2 assimilation rates. *Hevea* clones *viz.*, RRIM 600, RRII 208 and RRII 430 were found relatively drought tolerant even as they recorded comparatively less decline in photosynthesis and PS II activity under soil moisture deficit stress. On the other hand, clones PB 260, RRII 105, RRII 414 and RRII 417 were relatively more drought susceptible in terms of severe inhibition of various photosynthetic activities under moisture stress. A growth chamber study was also conducted to understand the interactive effects of drought and high temperature stress on photosynthetic apparatus in a popular clone RRII 105, grown under different temperature regimes. The impact of drought and high temperature stresses in this clone was analysed by measuring photosynthetic pigments, leaf water potential and photosystem II activity. It was noticed that when drought was imposed at ambient temperature (30°C) there was no much reduction in photosynthetic pigments and photosystem II activity. When the growth temperature was increased to 35°C and 40°C there was a drastic reduction of chlorophyll, carotenoids and photosystem II activity. However, the leaf water potential was found declining in drought imposed plants under all temperature regimes. These results once again confirmed the degree of drought susceptibility of the popular clone RRII 105.

The clones which were graded as drought tolerant and susceptible based on physiological responses were further evaluated for stress responsive protein abundance in photosynthetic apparatus and implications of such proteins in acclimation and adaptation for drought. The protein responsive to drought stress showing differential expression in tolerant/susceptible clones was purified by electro-elution and the homogeneity of the purified protein was further tested by 1-D and 2-D gel electrophoresis. The chloroplast protein profile of clone RRII 105 grown under different temperature regimes inside the growth chamber were also analysed to identify the stress responsive protein. In order to develop a rapid and easy protocol for stress protein identification, total leaf protein extraction method was also attempted. The results showed prominent abundance of a low molecular weight protein with a molecular mass of 23.8 kDa which was found over-expressing in drought exposed plants while a very low to medium level accumulation was seen in irrigated plants. The relative abundance of this protein in drought tolerant clones RRIM 600, RRII 430 and RRII 429 was very

prominent than drought susceptible clones such as PB 260, RRII 414 and Tjir 1 whereas in other clones expression was with medium to optimum range. However, there was an exception to this general observation in clone RRII 105, which was graded as drought susceptible from the physiological parameters studied, but had a relatively fair degree of accumulation of stress protein under water deficit condition. The amino acid sequence of the stress protein of molecular weight 23.8 kDa was reported earlier as a small chloroplast heat shock protein. Total leaf protein isolated from clones RRII 105 and RRIM 600 when resolved on SDS-PAGE, only a small fraction of the stress protein was observed. However, considering total protein extraction is a rapid protocol, it is felt that the method can be exploited for further studies to screen a large sample through western blot analysis. In an experiment to study the interactive effect of drought and high temperature under growth chamber conditions, the clone RRII 105 failed to induce the stress protein significantly compared with their respective control at all temperature regimes which elucidates the complexity of drought acclimation when plants are grown under field condition where the effect of multiple stresses induces or suppress the expression of specific proteins. The purified protein when analysed by 1-D and 2-D gel electrophoresis resulted in a single band of approximately 23 kDa in 1-D and as a single spot at pI near to 08 in 2-DE and thereby confirming its homogeneity. Stress protein purified by repeated electro-elution was later used for raising polyclonal antibody in rabbit.

Western blot analysis using the antibody raised against 23.8 kDa chloroplast stress protein was validated in known drought tolerant and susceptible clones as well as in germplasm accessions, the results substantiated with tolerance/susceptibility traits of clones evaluated based on physiological parameters. The study also confirmed that there was significant relationship existed between the abundance of the stress protein and relative drought tolerance traits (R^2=0.58) among the clones studied. Western blotting using total leaf protein was found rapid when compared to chloroplast proteins preparation. However, the protein resolution was not refined enough when the question of accuracy comes. In order to confirm the role of *HbsHSP23.8* gene in drought stress tolerance gene expression analysis was carried out in four elite clones of *Hevea* using the gene specific primers. The results of RT-PCR also substantiated

the physiological and proteomics results as the gene *HbsHSP23.8* was found to have a stronger association with drought tolerance. From this study it was confirmed that the relative expression level of the stress protein sHSP23.8 together with other crucial physiological parameters such as photosynthetic activity can be used as potential screening tools for selection of drought tolerant clones, wild accessions, ortets, pipelines etc. of rubber plants at a young stage.

List of Publications

Journals

1. **S. Pramod,** K. Annamalainathan, Smitha. M. Xavier, K.V. Sumesh and A. Thulaseedharan. (2017). Expression analysis of genes encoding sHSP and Rab1 in *Hevea brasiliensis*. ***International Journal of Biotechnology and Biochemistry,*** 13(4): 391-402.

2. K. Annamalainathan, **S. Pramod,** K.V. Sumesh and James Jacob. (2017). A 23 kDa chloroplast protein as marker for drought tolerance in *Hevea brasiliensis*. ***Rubber Science,*** 30(2): 128-139.

Seminars

1. **S. Pramod**, K. Annamalainathan, K.V. Sumesh, A. Thulaseedharan and James Jacob. (2017). Chloroplast stress protein as marker for screening drought tolerance in rubber plants (*Hevea brasiliensis*). **National Seminar,** 27th Swadeshi Science Congress, Focal Theme: Science and Technology for Societal Development, 7-9, November 2017, Amrita Viswa Vidyapeetham, Amrita University, Kollam.

2. Annamalainathan, K., **Pramod, S.,** Sumesh, K.V. and James Jacob. (2016). Identification of a 23 kDa chloroplast stress protein as a marker for drought tolerance in *Hevea brasiliensis*. **National conference** of Plant Physiology, Challenges in Crop Physiology Research: from Molecular to Whole Plant, 8-10, December 2016, University of Agricultural Sciences, Bengaluru.

Appendices

GeNei™

SERVICE REPORT

Name of Scientist:	Dr. K.Annamalainathan
Address:	Rubber Institute, Kottayam
Name of Service:	Custom Polyclonal Antibody Service in Rabbit
Catalogue Number:	640501200011730
Service Number:	10652
Number of Samples:	1no. (23kD Chloroplast Protein)
Order Received on:	17th Oct. 2011
Service Completed on:	01st Mar. 2012
Deliverables:	1.Service Report 2. Pre- Immune serum -2ml 3. Polyclonal Antiserum -25ml

Executed by: 1/3/12

Reviewed by: 1/3/12

Approved by: 1/3/2012

PROJECT REPORT

Name Of Scientist:	Service: Custom Polyclonal	Service No.:
DR. K.ANNAMALAINATHAN	Antibody Service	SER11/10801

Anti serum: **Lot # 146512**
Pre immune: Lot# 146

Job Completed:

- Antigen purity check on SDS PAGE
- Estimation of protein content by Absorbance method
- Screening of pre-immune serum from different rabbits.
- Immunization
- Titer analysis by Direct ELISA and western Blot.

IMMUNIZATION DETAILS:

Day 0	Pre-immune serum
Day 1	Immunize 500µg with FCA
Day 15	Booster 1 200µg with IFA
Day 25	Booster 2 200µg with IFA
Day35	Booster 3 200µg with IFA
Day45	Booster 4 200µg with IFA
Day55	Booster 5 200µg with IFA
Day65	**Bleed –I**
Day75	**Bleed –II**

Direct ELISA (dELISA):
Microtiter plates were coated with antigen at 10µg/ml concentration (0.5µg/100µl) in 0.05M carbonate-bicarbonate buffer pH 9.6. 100µl of diluted antiserum was allowed to bind for 30 minutes at room temperature. Wells washed and incubated with Goat anti rabbit IgG – HRP 1:15000 diluted, for 30 minutes at room temperature. Concentration of bound antibody was estimated using OPD/H_2O_2 having 1mg/ml concentration in citrate phosphate buffer pH 5. The dilution that corresponds to reading of 1.0 to 1.2 at 490 nm in ELISA reader is quoted as titer.

ELISA titer

At Dilution **1:10,000** **Reading:** **1.361**

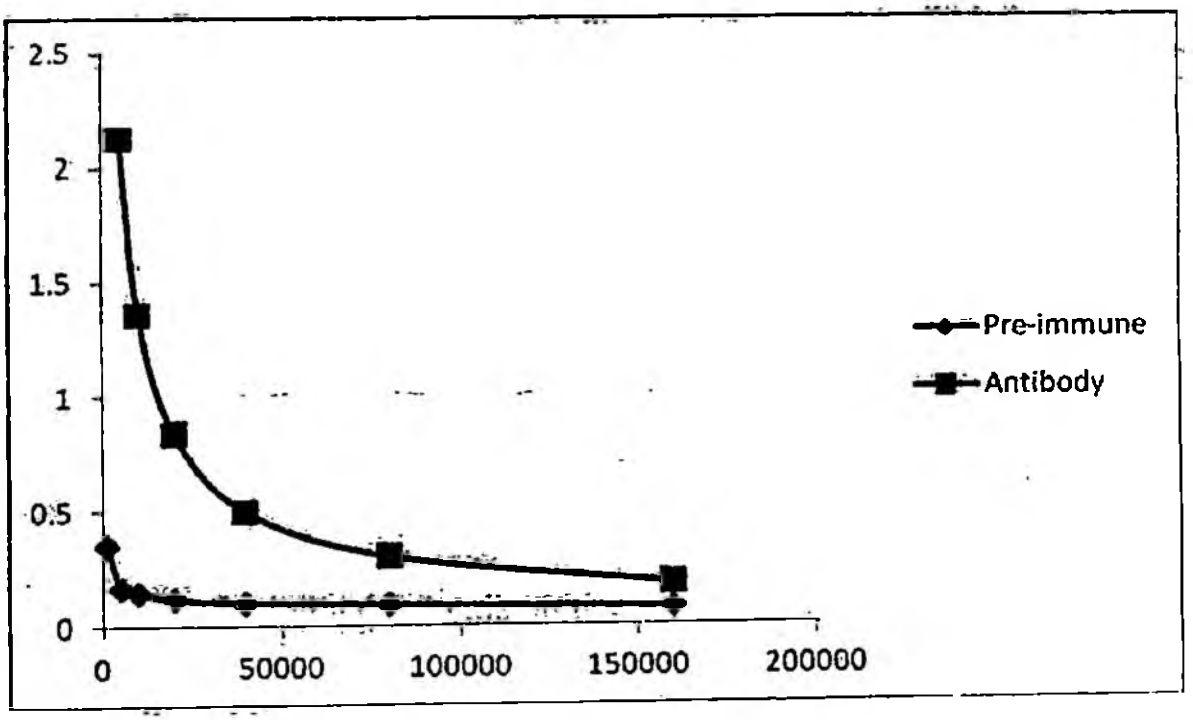
01040908

PROJECT REPORT

Name Of Scientist: DR. K.ANNAMALAINATHAN	Service: Custom Polyclonal Antibody Service	Service No.: SER11/10801

Direct ELISA Readings at Absorbance 450 nm		
Dilution	**Pre- immune Serum**	**Polyclonal Antiserum**
1000	0.355	over
5000	0.168	2.130
10000	**0.146**	**1.361**
20000	0.116	0.838
40000	0.102	0.499
80000	0.093	0.304
160000	0.078	0.186

Direct ELISA Titre of the Polyclonal antibody for 14.5 kD (Syn.)



PROJECT REPORT

Name Of Scientist:	Service: Custom Polyclonal Antibody Service	Service No.:
DR. K.ANNAMALAINATHAN		SER11/10801

Western Blotting:
Antigen is electrophoreses under denatured condition on SDS-PAGE. The antigen was transferred to nitrocellulose membrane, blocked with 3% skimmed milk and incubated with diluted primary antibody. Membrane washed and incubated with 1:3000 diluted **Goat anti Rabbit IgG –HRP and** finally Blot was developed with TMB/H2O2 (SFE-3).

Western Blot Titre:
At 1:10000

1µg

Note:

Working dilution will depend on assay condition. Due to differences in assay Systems, these titers may not reflect the user's actual working dilution.

Store: -20°C

- For continuous use, keep the product refrigerated. For extended use, the product can be stored frozen in smaller aliquots.
- Repeated freeze-thaw cycles not recommended.

References

Acquaah, G. (2007). *Principles of plant genetics and breeding,* Blackwell, Oxford, UK, 740 p.

Ahamad, B. (1999). Effect of rootstock on growth and water use efficiency of *Hevea* during water stress. *Journal of Rubber Research,* 2: 99-119.

Ahmad, F., Rafii, M., Ismail, M.R., Juraimi, A.S., Rahim, H.A., Asfaliza, R., Latif, M.A. (2013). Waterlogging tolerance of crops: breeding, mechanism of tolerance, molecular approaches, and future prospects. *Biomed Research International,* 2013: 963525.

Ahn, Y.J., Zimmerman, J.L. (2006). Introduction of the carrot HSP17.7 into potato (*Solanum tuberosum* L.) enhances cellular membrane stability and tuberization in vitro. *Plant, Cell & Environment,* 29: 95-104.

Akhtar, I., Nazir, N. (2013). Effect of waterlogging and drought stress in plants. *International Journal of Water Resources and Environmental Sciences,* 2(2): 34-40.

Alam, B., Annamalainathan, K., Singh, M. and Jacob, J. (2005). Light quintessentially modulates the impact of drought stress on photosynthesis in leaves of field grown *Hevea brasiliensis*. In: *Proceedings of International Natural Rubber Conference,* Kochi, India, pp. 239-245.

Al-Khatib, K. and Paulsen, G.M. (1999). High-temperature effects on photosynthetic processes in temperate and tropical cereals. *Crop Sci*ence, 39: 119-125.

Allen, S.P., Polazzi, J.O., Gierse, J.K., Easton, A.M. (1992). Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in *Escherichia coli* . *Journal of Bacteriology*, 174: 6938-6947.

Almoguera, C., Jordano, J. (1992). Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNAs. *Plant Molecular Biology,* 19: 781-792.

Al-Whaibi. (2011). Plant heat-shock proteins: A mini review. *Journal of King Saud University,* 23: 139-150.

Annamalainathan, K., George, G., Joy, S., Thomas, S., James Jacob. (2010). Drought-induced changes in photosynthesis and chloroplast proteins in young plants of *Hevea brasiliensis*. *Natural Rubber Research*, 23(1&2): 55-63.

Annamalainathan, K., Heazlewood, J.L. and Jacob, J. (2006). Studies on chloroplastic factors responsible for drought tolerance in rubber plants (*Hevea brasiliensis*). *Journal of Plantation Crops,* 34(3): 643-649.

Annamalainathan, K., Satheesh, P.R. and Jacob, J. (2015). Role of Plantation Crops in Climate Change Mitigation: Special Reference to Natural Rubber. In: *Agroforestry,* (Eds. Dhyani, S.K., Newaj, R., Alam, B., Dev, I.). Biotech Books, pp. 229-246.

Annamma, Y., Marattukalam, J.G., Premakumari, D., Saraswathyamma C.K., Licy, J., Panikkar A.O.N. (1990). Promising Rubber planting materials with special reference to Indian clones. *Proceedings of Planters Conference,* Kottayam, India. pp. 62-70.

Apel, K., Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology,* 55: 373-399.

Arbona, V., Manzi, M., de Ollas, C., Gómez-Cadenas, A. (2013). Metabolomics as a tool to investigate abiotic stress tolerance in plants. *International Journal of Molecular Sciences,* 14: 4885-4911.

Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology,* 24(1): 1-15.

Arrigo, A.P., Suhan, J.P., Welch, W.J. (1988). Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein. *Molecular and Cellular Biology,* 8: 5059-5071.

Arrigo, A.P., Welch, W.J. (1987). Characterization and purification of the small 28,000-dalton mammalian heat shock protein. *Journal of Biological Chemistry,* 262: 15359-15369.

Asada, K. (1999). The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology,* 50: 601-639.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology,* 141: 391-396.

Ashoub, A., Beckhaus, T., Berberich, T., Karas, M., Brüggemann, W. (2013). Comparative analysis of barley leaf proteome as affected by drought stress. *Planta,* 237: 771-781.

Ashraf, M., Hafeez, M . (2004). Thermotolerance of pearl millet and maize at early growth stages: Growth and nutrient relations. *Biologia Plantarum,* 48: 81-86.

Ashraf, M., Harris, P.J.C. (2013). Photosynthesis under stressful environments: An overview. *Photosynthetica,* 51(2): 163-190.

Athar, H.R. and Ashraf, M. (2009). Strategies for crop improvement against salinity and drought stress: An Overview. In: *Salinity and water stress, improving crop efficiency,* (Eds. Ashraf, M., Ozturk, M., Athar, H.R.). Springer, Netherlands. pp. 1-16.

Augustine, S.M. (2016). Function of Heat-Shock Proteins in Drought Tolerance Regulation of Plants. In: *Drought Stress Tolerance in Plants,* Volume I, Physiology and Biochemistry, (Eds. Hossain, M.A., Wani, S.H., Bhattacharjee, S., Burritt, D.J., Tran, L.P.). Springer, Switzerland. pp. 163-185.

Ayutthaya, S.I.N., Do, F.C., Pannangpetch, K., Junjittakarn, J., Maeght, J.L., Rocheteau, A., Cochard, H. (2011). Water loss regulation in mature *Hevea brasiliensis*: effects of intermittent drought in the rainy season and hydraulic regulation. *Tree physiology,* 31: 751-762.

Backhaus, R. A. (1985). Rubber formation in plant—a mini review. *Israel Journal of Botany,* 34: 283-293.

Baker, N.R. (1996). Environmental constraints on photosynthesis. In: *Photosynthesis and the Environment*, (Ed. Baker, N.R.). Kluwer Academic Publishers, London, pp. 469-476.

Barkla, B.J., Vera-Estrella, R. and Raymond, C. (2016). Single-cell-type quantitative proteomic and ionomic analysis of epidermal bladder cells from the halophyte model plant *Mesembryanthemum crystallinum* to identify salt-responsive proteins. *BMC Plant Biololgy,* 16: 110.

Barnett, T., Altschuler, M., McDaniel, C.N., Mascarenhas, J.P. (1980). Heat shock induced proteins in plant cells. *Developmental Genetics,* 1: 331-340.

Bartels, D., Sunkars, R. (2005). Drought and salt tolerance in plants. *Critical Reviews in Plant Sciences,* 24: 23-58.

Barua, D., Downs, C.A., Heckathorn, S.A. (2003). Variation in chloroplast small heat-shock protein function is a major determinant of variation in thermotolerance of photosynthesis electron transport among ecotypes of *Chenopodium album. Functional Plant Biology,* 30(10): 1071-1079.

Basha, E., Jones, C., Wysocki, V., Vierling, E. (2010). Mechanistic differences between two conserved classes of small heat shock proteins found in the plant cytosol. *Journal of Biological Chemistry,* 285: 11489-11497.

Basha, E., O'Neill, H., Vierling, E. (2012). Small heat shock proteins and alpha-crystallins: dynamic proteins with flexible functions. *Trends in Biochemical Sciences,* 37: 106-117.

Bastiah, A. (1999). Effect of rootstock on growth and water use efficiency of *Hevea* during water stress. *Journal of Rubber Research*, 2(2): 99-119.

Battaglia, M., Olvera-Carrillo, Y., Garciarrubio, A., Campos, F., Covarrubias, A.A. (2008). The enigmatic LEA proteins and other hydrophilins. *Plant Physiology,* 148: 6-24.

Behlke, J., Lutsch, G., Gaestel, M., Bielka, H. (1991). Supramolecular structure of the recombinant murine small heat shock protein hsp25. *FEBS Letters,* 288: 119-122.

Bernacchia, G., Furini, A. (2004). Biochemical and molecular responses to water stress in resurrection plants. *Physiologia Plantarum,* 121: 175-181.

Berry, J.A., Bjorkman, O. (1980). Photosynthetic response and adaptation to temperature in higher plants. *Annual Review of Plant Physiology,* 31: 491-543.

Bhargava, S., Sawant, K. (2013). Drought stress adaptation: metabolic adjustment and regulation of gene expression. *Plant Breeding,* 132: 21-32.

Böhmer, M. and Schroeder, J.I. (2011). Quantitative transcriptomic analysis of abscisic-induced and reactive oxygen species-dependent expression changes and proteomic profiling in *Arabidopsis* suspension cells. *Plant Journal,* 67: 105-118.

Booth, R.J., Harris, D.P., Loe, J.M., Watson, J.D. (1988). Antigenic proteins of *Mycobacterium leprae.* complete sequence of the gene for the 18-kDa protein. *Journal of Immunology,* 140: 597-601.

Boston, R.S, Viitanen, P.V., Vierling, E. (1996). Molecular chaperones and protein folding in plants, *Plant Molecular Biology,* 32: 191-222.

Bradshaw, M.J. (1977). *Earth, the living Planet,* Hodder and Stoughton, London. 302 p.

Bray, E.A. (1997). Plant responses to water deficit. *Trends in Plant Science,* 2: 48-54.

Bray, E.A. (2007). Plant response to water deficit stress. *Encyclopedia of Life Sciences,* doi:10.1002/9780470015902.a0001298.pub2.

Bray, E.A., Bailey-Serres, J., Weretilnyk, E. (2000). Responses to abiotic stresses. In: *Biochemistry and molecular biology of plants,* (Eds. Buchanan, B.B., Gruissem, W., Jones, R.L). American Society of Plant Physiologists, Rockville. pp. 1158-1203.

Brestic, M., Zivcak, M., Kalaji, H.M., Carpentier, R., Allakhverdiev, S.I. (2012). Photosystem II thermostability in situ: environmentally induced acclimation and genotype-specific reactions in *Triticum aestivum* L. *Plant physiology and Biochemistry,* 57: 93-105.

Campbell, S.A., Close, T.J. (1997). Dehydrins: genes, proteins, and associations with phenotypic traits. *New Phytologist,* 137: 61-74.

Cardinal, A. B. B., Goncalves, P. D. and Martins, A. L. M. (2007). Stock-scion interactions on growth and rubber yield of *Hevea brasiliensis. Scientia Agricola (Piracicara, Braz.),* 64(3): 235-240.

Cattivelli, L., Rizza, F., Badeck, F.W., Mazzucotelli, E., Mastrangelo, A.M., Francia, E., Mare, C., Tondelli, A., Stanca, A.M. (2008). Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Research,* 105: 1-14.

Chandler, P.M., Robertson, M. (1994). Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology,* 45: 113-141.

Chandrashekar, T.R., Jana, M.K., Thomas, J., Vijaykumar, K.R., Sethuraj, M.R. (1990). Seasonal changes in physiological characteristics and yield in newly opened trees of *Hevea brasiliensis* in North Konkan. *Indian Journal of Natural Rubber Research,* 3: 88-97.

Chandrashekar, T.R., Mydin, K.K., Alice, J., Varghese, Y.A. and Saraswathyamma, C.K. (1997). Intraclonal variability for yield in rubber (*Hevea brasiliensis*). *Indian Journal of Natural Rubber Research,* 10(1&2): 43-47.

Chang, Z., Primm, T.P., Jakana, J., Lee, I.H., Chiu, W., Gilbert, H.F., Quiocho, F.A. (1996). *Mycobacterium tuberculosis* 16-kDa antigen (Hsp16.3) functions as an oligomeric structure *in vitro* to suppress thermal aggregation. *Journal of Biological Chemistry,* 271: 7218-7223.

Chantuma, A., Kunarasiri, A., Chantuma, P. (2012). Rubber new planting in Thailand: towards the world affected on climate change. *Rubber Thai Journal,* 1: 40-47.

Chauhan, H., Khurana, N., Nijhavan, A., Khurana, J.P., Khurana, P. (2012). The wheat chloroplastic small heat shock protein (sHSP26) is involved in seed maturation and germination and imparts tolerance to heat stress. *Plant, Cell & Environment,* 35: 1912-1931.

Chen, J.W., Zhang, Q., Li, X.S. and Cao, K.F. (2010). Gas exchange and hydraulics in seedlings of *Hevea brasiliensis* during water stress and recovery. *Tree Physiology,* 30(7): 876-885.

Chernyad'ev, I.I. (2005). Effect of water stress on the photosynthetic apparatus of plants and the protective role of cytokinins: a review. *Applied Biochemistry and Microbiology,* 41: 115-128.

Close, T.J. (1996). Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum,* 97: 795-803.

Coca, M.A., Almoguera, C., Jordano, J. (1994). Expression of sunflower low-molecular-weight heat shock proteins during embryogenesis and persistence after germination: localization and possible functional implications. *Plant Molecular Biology,* 25: 479-492.

Collier, N.C., Heuser, J., Levy, M.A., Schlesinger, M.J. (1988). Ultrastructural and biochemical analysis of the stress granule in chicken embryo fibroblasts. *Journal of Cell Biology,* 106: 1131-1139.

Cornic, G. and Fresneau, C. (2002). Photosynthetic carbon reduction and carbon oxidation cycles are the main electron sinks for photosystem II activity during a mild drought. *Annals* of *Botany,* 89: 887-894.

Cornic, G., Massacci, A. (1996). Leaf photosynthesis under drought stress, In: *Photosynthesis and the Environment,* (Ed. Baker N.R.). Kluwer Academic Publishers, The Netherlands. pp. 347-366.

Cornish, K., Siler, D.J., Grosjean, O.K., Godman, N. (1993). Fundamental similarities in rubber particle architecture and function in three evolutionarily divergent plant species. *Journal of Natural Rubber Research,* 8: 275-285.

Couee, I., Sulmon, C., Gouesbet, G., El Amrani, A. (2006). Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany,* 57: 449-459.

Crafts-Brandner, S.J., Salvucci, M.E. (2002). Sensitivity of photosynthesis in a C4 plant, maize, to heat stress. *Plant Physiology,* 129: 1773-1780.

Cushman, J.C. and Bohnert, H.J. (2000). Genomic approaches to plant stress tolerance. *Current Opinion in Plant Biology,* 3: 117-124.

de Barros, J.C.M., de Castro, A .M .G., Miranda, J., Schenkel. C.S. (1983). Development and present status of rubber cultivation in Brazil. *Proceedings of the RRIM Planters' Conference,* Kuala Lumpur. pp. 18-30.

de Jong, W.W., Leunissen, J.A., Voorter, C.E. (1993). Evolution of the α-crystallin/small heat-shock protein family. *Molecular Biology and Evolution,* 10: 103-126.

Degani, C., Ei Bastri, R. and Gazit, S. (1990). Enzyme polymorphism in mango, *Journal of American Society for Horticultural Science*, 115: 844-847.

Demirevska, K., Simova-Stoilova1, L., Vassileva1, V., Vaseva, I., Grigorova1, B., Feller, U. (2008). Drought-induced leaf protein alterations in sensitive and tolerant wheat varieties. *General and Applied Plant Physiology,* 34: 79-102.

Demmig-Adams, B. and Adams III, W.W. (1992). Photoprotection and other response of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology,* 43: 599-626.

DeRocher, A.E., Helm, K.W., Lauzon L.M. and Vierling, E. (1991). Expression of a conserved family of cytoplasmic low molecular weight heat shock proteins during heat stress and recovery. *Plant Physiology,* 96: 1038-1047.

Devakumar, A., Gururaja Rao, G., Rajagopal, R., Sanjeeva Rao, P., George, M., Vijayakumar, K., Sethuraj, M. (1988). Studies on soil-plant-atmosphere system in Hevea: II. Seasonal effects on water relations and yield. *Indian Journal of Natural Rubber Research,* 1: 45-60.

Devakumar, A.S., Prakash, P., Sathik, M. B. M. and Jacob, J. (1999). Drought alters the canopy architecture and micro-climate of *Hevea brasiliensis* trees. *Trees,* 13:161-167.

Devakumar, A.S., Sathik, M.B.M., Jacob, J., Annamalainathan, K., Prakash, P.G. and Vijayakumar, K.R. (1998). Effects of atmospheric and soil drought on growth and development of *Hevea brasiliensis. Journal of Rubber Research,* 1(3): 190-198.

Dijkman, M.J. (1951). *Hevea*: *Thirty years of research in the Far East.* University of Miami press, Coral Gables, Florida. 329 p.

Ding, Y., Tao, Y., Zhu, C. (2013). Emerging roles of MicroRNAs in the mediation of drought stress response in plants. *Journal of Experimental Botany,* 64: 3077-3086.

Dobson, C.M., Sali, A., Karplus, M. (1998). Protein folding: a perspective from theory and experiment. *Angewandte Chemie International Edition,* 37: 868-893.

Dutta, P., Bandopadhyay, P., Bera, A.K. (2016). Identification of Leaf Based Physiological Markers for Drought Susceptibility during Early Seedling Development of Mungbean. *American Journal of Plant Sciences,* 7: 1921-1936.

Ehrnsperger, M., Graber, S., Gaestel, M., Buchner, J. (1997). Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. *EMBO Journal,* 16: 221-229.

Eldakak, M., Milad, S.I.M., Nawar, A.I. and Rohila, J.S. (2013). Proteomics: a biotechnology tool for crop improvement. *Frontiers in plant science,* 4: 35.

Emon, J.M.V. (2016). The omics revolution in agricultural research. *Journal of Agricultural and Food Chemistry,* 64: 36-44.

Errea, P. (1998). Implications of phenolic compounds in graft incompatibility in fruit tree species, *Scientia Horticulturae*, 74: 195-205.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., Basra, S.M.A. (2009). Plant drought stress: effects, mechanisms and management. *Agronomy for Sustainable Development,* 29: 185-212.

Feder, M.E., Hofmann, G.E. (1999). Heat shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology,* 61: 243-282.

Frank, H.A. and Cogdell, R.J. (1993). Photochemistry and functions of carotenoids in Photosynthesis. In: *carotenoid in photosynthesis,* (Eds. Young, A.J. and Britton, G.). Chapman & Hall, London, pp. 252-326.

Fu. X. (2015). Insights into How Small Heat Shock Proteins Bind a Great Diversity of Substrate Proteins: A Super-Transformer Model. In: *The big book on small heat shock proteins,* (Eds. Tanguay, R.M., Hightower, L.E.). Springer, Switzerland, pp. 101-117.

Fulda, S., Mikkat, S., Stegmann, H., Horn, R. (2011). Physiology and proteomics of drought stress acclimation in sunflower (*Helianthus annuus* L.). *Plant Biology,* 13: 632-642.

Galau, G., Hughes, D.W., Dure, L. III. (1986). Abscisic acid induction of cloned cotton late embryogenesis abundant (Lea) mRNAs. *Plant Molecular Biology,* 7: 155-170.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D. and Bairoch, A. (2003). ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research,* 31: 3784-3788.

George, K.T., Haridasan, V. and Sreekumar, B. (1988). Role of Government and Structural Changes in Rubber Plantation Industry in India. *Economic and Political Weekly,* 23(48): M158-166.

Ghosh, D. and Xu, J. (2014). Abiotic stress responses in plant roots: a proteomics perspective. *Frontiers in Plant Science, 5*: 6.

Giorno, F., Wolters-Arts, M., Grillo, S., Scharf, K.D., Vriezen, W.H., Mariani, C. (2010). Developmental and heat stress-regulated expression of HsfA2 and small heat shock proteins in tomato anthers. *Journal of Experimental Botany,* 61: 453-462.

Golldack, D., Li, C., Mohan, H., Probst, N. (2014). Tolerance to drought and salt stress in plants: unraveling the signaling networks. *Frontiers in Plant Science,* 5: 151.

Goloubinoff, P., Mogk, A., Ben Zvi, A.P., Tomoyasu, T. and Bukau, B. (1999). Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proceedings of the National Academy of Sciences USA,* 96: 13732-13737.

Goyal, K., Walton, L.J., Tunnacliffe, A. (2005). LEA proteins prevent protein aggregation due to water stress. *The Biochemical Journal,* 388: 151-157.

Guo, S.J., Zhou, H.Y., Zhang, X.S., Li, X.G., Meng, Q.W. (2007). Overexpression of CaHSP26 in transgenic tobacco alleviates photoinhibition of PSII and PSI during chilling stress under low irradiance. *Journal of Plant Physiology,* 164: 126-136.

Gururaja Rao, G., Devakumar, A., Rajagopal, R., Annamma, Y., Vijayakumar, K., Sethuraj, M. (1988). Clonal variations in leaf epicuticular waxes and reflectance: possible role in drought tolerance in *Hevea. Indian Journal of Natural Rubber Research,* 1: 84-87.

Gururaja Rao, G., Sanjeeva Rao, P., Devakumar, A. S., Vijayakumar, K. R. and Sethuraj, M. R. (1990). Influence of soil, plant and meteorological factors on water relations and yield of *Hevea brasiliensis. International Journal of Biometeorology,*34: 175-180.

Hajra, N.G. and Potty, S.N. (1986). Rubber cultivation in north eastern region. *Rubber Board Bulletin,* 22(1): 5-10.

Hakeem, K.R., Chandna, R., Ahmad, P., Ozturk, M., Iqbal, M. (2012). Relevance of proteomic investigations in plant stress physiology. *OMICS,* 16(11): 621-635.

Hamilton, E.W. III. and Heckathorn, S.A. (2001). Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. *Plant Physiology,* 126: 1266-1274.

Hamilton, E.W., Heckathorn, S.A., Downs, C.A., Schwarz, T.E., Coleman, J.S., Hallberg, R.L. (1996). Heat shock proteins are produced by field-grown naturally occurring plants in the summer in the temperate northeast. *US Bulletin of the Ecological Society of America,* 77 (Suppl. Part 2): 180.

Han, C., Yang, P.F., Sakata, K., Komatsu, S. (2014). Quantitative proteomics reveals the role of protein phosphorylation in rice embryos during early stages of germination. *Journal of Proteome Research,* 13: 1766-1782.

Hanin, M., Brini, F., Ebel, C.,Toda, Y., Takeda, S. and Masmoudi, K. (2011). Plant dehydrins and stress tolerance: Versatile proteins for complex mechanisms. *Plant Signalling and Behaviour,* 6: 1503-1509.

Hao, P., Zhu, J., Gu, A., Lv, D., Ge, P., Chen, G., Li, X., Yan, Y. (2015). An integrative proteome analysis of different seedling organs in tolerant and sensitive wheat cultivars under drought stress and recovery. *Proteomics,* 15: 1544-1563.

Haridasan, V. and Nair, V.K.G. (1980). History of rubber plantation industry in India. In: *Handbook of Natural Rubber Production in India*, (Ed. by Pillay, P.N.R.). Rubber Research Institute of India (RRII), Kottayam, Kerala, India, pp. 3-12.

Hartl, F.U. (1996). Molecular chaperones in cellular protein folding. *Nature,* 381: 571-580.

Hartmann, H. and Kester, D.E. (1976). *Plant propagation: Principles and practices.* Prentice-Hall international Inc., New Jersey.

Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000). Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology,* 51: 463-499.

Hashiguchi, A., Ahsan, N., and Komatsu, S. (2010). Proteomics application of crops in the context of climatic changes. *Food Research International,* 43: 1803-1813.

Haslbeck, M., Miess, A., Stromer, T., Walter, S., Buchner, J. (2005). Disassembling protein aggregates in the yeast cytosol. The cooperation of Hsp26 with Ssa1 and Hsp104. *Journal of Biological Chemistry,* 280: 23861-23868.

Haslbeck, M., Weinkauf, S., Buchner, J. (2015). Regulation of the chaperone function of small Hsps. In: *The big book on small heat shock proteins,* (Eds. Tanguay, R.M., Hightower, L.E.). Springer, Switzerland, pp. 155-178.

Havaux, M. (1992). Stress tolerance of photosystem II in vivo: Antagonistic effects of water, heat and photoinhibition stresses. *Plant Physiology,* 100: 424-432.

Heckathorn, S.A., Muller, J.K., Laquidice, S., Zhu, B., Barrett, T., Blair, B., Dong, Y. (2004). Chloroplast small heat shock proteins protect photosynthesis during heavy metal stress. *American Journal of Botany,* 91 (9): 1312-1318.

Helm, K.W., Schmeits, J., Vierling, E. (1995). An endomembrane-localized small heat-shock protein from *Arabidopsis thaliana. Plant Physiology,* 107: 287-288.

Heywood, V. H. (1978). In: *Flowering Plants of the World,* Oxford University Press, Oxford. 335 p.

Hirayama, T., Shinozaki, K. (2010). Research on plant abiotic stress responses in the post-genome era: past, present and future. *The Plant Journal,* 61: 1041-1052.

Hoekstra, F.A., Golovina, E.A., Buitink, J. (2001). Mechanisms of plant desiccation tolerance. *Trends in Plant Science,* 6: 431-438.

Horwitz, J. (1992). α-crystallin can function as a molecular chaperone. *Proceedings of the National Academy of Sciences USA,* 89: 10449-10453.

Hossain, Z., Nouri, M.Z. and Komatsu, S. (2012). Plant cell organelle proteomics in response to abiotic stress. *Journal of Proteome Research,* 11: 37-48.

Hsieh, M. H., Chen, J.T., Jinn, T. L., Chen, Y. M. and Lin, C. Y. (1992). A class of soybean low molecular weight heat shock proteins: immunological study and quantitation. *Plant Physiology,* 99: 1279-1284.

Hu, H., and Xiong, L. (2014). Genetic engineering and breeding of drought-resistant crops. *Annual Review of Plant Biology,* 65: 715-741.

Hu, X.L., Yang, Y.F., Gong, F.P., Zhang, D.Y., Zhang, L., Wu, L.J., Li, C.H., Wang, W. (2015). Protein sHSP26 improves chloroplast performance under heat stress by interacting with specific chloroplast proteins in maize (*Zea mays*). *Journal of Proteomics,* 115: 81-92.

Huang, Z.D., Pan, Y.Q. (1992). Rubber Cultivation under Climatic Stresses in China. In: *Natural Rubber: Biology, Cultivation and Technology,* (Eds. Sethuraj, M.R. and Mathew, N.M). Elsevier Science Publishers, Amsterdam, Netherlands. pp. 220-238.

Iba K. (2002). Acclimation response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. *Annual Review of Plant Biology,* 53: 225-245.

Imle, E.P. (1978). *Hevea* rubber: past and future. *Economic Botany,* 32: 264-277.

Ingolia, T.D., Craig, E.A. (1982). Four small *Drosophila* heat shock proteins are related to each other and to mammalian α-crystallin. *Proceedings of the National Academy of Sciences USA,* 79: 2360-2364.

Ingram, J., Bartels, D. (1996). The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology,* 47: 377-403.

Isaacson, T., Damasceno, C.M., Saravanan, R.S., He, Y., Catalá, C., Saladié, M., Rose, J.K. (2006). Sample extraction techniques for enhanced proteomic analysis of plant tissues. *Nature Protocols,* 1: 769-774.

Jacob, J. (2005). The science, politics and economics of global climate change: Implications for the carbon sink projects. *Current Science,* 89(3): 464-474.

Jacob, J. and Nataraja, K.N. (1998). Excess photosynthetic electrons: A matter of life and death for green leaves of *Hevea* experiencing stress. *Thirteenth Symposium on Plantation Crops,* Coimbatore, India. p. 29.

Jacob, J., Annamalainathan, K., Alam, B., Sathik, M.B.M., Thapliyal, A.P. and Devakumar, A.S. (1999). Physiological constraints for cultivation of *Hevea brasiliensis* in certain unfavorable agro-climatic regions of India. *Indian Journal of Natural Rubber Research,* 12: 1-16.

Jacob, J., Thulaseedharan, A., Kala, R.G., Narayanan, C., Mydin, K.K. and Idicula, S.P. (2016). *Biology of Hevea brasiliensis (Rubber): Series of Crop Specific Biology Documents,* Prepared by Ministry of Environment, Forest and climate Change (Government of India) and Rubber Research Institute of India, Rubber Board, Kottayam under UNEP/GEF supported Phase II Capacity Building Project on Biosafety, 30p.

Jakob, U., Gaestel, M., Engel, K., Buchner, J. (1993). Small heat shock proteins are molecular chaperones. *Journal of Biological Chemistry,* 268: 1517-1520.

Jaleel, C.A., Manivannan, P., Wahid, A., Farooq, M., Somasundaram, R., Panneerselvam, R. (2009). Drought stress in plants: A review on morphological characteristics and pigments composition. *International Journal of Agriculture and Biology,* 11: 100-105.

Jangpromma, N., Kitthaisong, S., Lomthaisong, K., Daduang, S., Jaisil, P. and Thammasirirak, S. (2010). A proteomics analysis of drought stress-responsive proteins as biomarker for drought-tolerant sugarcane cultivars. *American Journal of Biochemistry and Biotechnology,* 6: 89-102.

Jessy, M.D., Krishnakumar, R., Annamalainathan, K. and James Jacob. (2010). Climate uncertainties and early establishment of young rubber plantation in traditional rubber growing regions of India. *International workshop on climate change and rubber cultivation: R&D priorities,* Kottayam, India, July-2010, pp: 25-27.

Jessy, M.D., Prasannakumari, P., Jacob, J. (2014). Agromanagement techniques to mitigate drought in young rubber plantations. *Rubber Science,* 27(1): 54-60.

Jewell, M.C., Campbell, B.C., Godwin, I.D. (2010). Transgenic plants for abiotic stress resistance. In: *Transgenic Crop Plants,* (Eds. Kole, C. *et al.*,). Springer, Berlin. pp. 67-132.

Jiang, C., Xu, J., Zhang, H., Zhang, X., Shi, J., Li, M., Ming, F. (2009). A cytosolic class I small heat shock protein, RcHSP17.8, of *Rosa chinensis* confers resistance to a variety of stresses to *Escherichia coli*, yeast and *Arabidopsis thaliana. Plant, Cell & Environment,* 32: 1046-1059.

Joe, M.K., Park, S.M., Lee, Y.S., Hwang, D.S., Hong, C.B. (2000). High temperature stress resistance of *Escherichia coli* induced by a tobacco class I low molecular weight heat-shock protein. *Molecules and Cells,* 10: 519-524.

Jones, K.P. and Allen P.W. (1992). Historical development of the world rubber industry. In: *Natural Rubber: Biology, Cultivation and Technology,* (Eds. Sethuraj, M.R. and Mathew, N.M.). Elsevier Science Publishers, Amsterdam, Netherlands. pp. 1-22.

Jun, L.D., Hui-Na, G., Zhi, D., Hui, L., Jiang-Shu, C., Da, J., Li-Qiong, X., Zhi-Hui, X. (2015). Cloning, Bioinformatics and Expression Analysis of *sHSP23.8* Gene in *Hevea brasiliensis*. *Plant Physiology Journal,* 51(11): 1955-1962.

Kadirvel, P., Senthilvel, S., Geethanjali, S., Sujatha, M. and Varaprasad, K.S. (2015). Genetic markers, trait mapping and marker-assisted selection in plant breeding. In: *Plant Biology and Biotechnology,* (Eds. Bahadur, B., Rajam, M.V., Sahijram, L., Krishnamurthy, K.V.). Springer, India. pp. 65-88.

Key, J.L., Lin, C.Y., Chen, Y.M. (1981). Heat shock proteins of higher plants. *Proceedings of the National Academy of Sciences USA,* 78: 3526-3530.

Keyvan S. (2010). The effects of drought stress on yield, relative water content, proline, soluble carbohydrates and chlorophyll of bread wheat cultivars. *Journal of Animal and Plant Sciences,* 8: 1051-1060.

Kim, D.H., Xu, Z.Y., Hwang, I. (2013). AtHSP17.8 overexpression in transgenic lettuce gives rise to dehydration and salt stress resistance phenotypes through modulation of ABA-mediated signaling. *Plant Cell Reports,* 32: 1953-1963.

Kim, K.H., Alam, I., Kim, Y.G., Sharmin, S.A., Lee, K.W., Lee, S.H., Lee, B.H. (2012). Overexpression of a chloroplast-localized small heat shock protein OsHSP26 confers enhanced tolerance against oxidative and heat stresses in tall fescue. *Biotechnology Letters,* 34: 371-377.

Kirschner, M., Winkelhaus, S., Thierfelder, J.M., Nover, L. (2000). Transient expression and heat-stress induced co-aggregation of endogenous and heterologous small heat-stress proteins in tobacco protoplasts. *Plant Journal,* 24: 397-411.

Kmiecik, P., Leonardelli, M., Teige, M. (2016). Novel connections in plant organellar signalling link different stress responses and signalling pathways. *Journal of Experimental Botany,* 67: 3793-3807.

Komatsu, S., and Hossain, Z. (2013). Organ-specific proteome analysis for identification of abiotic stress response mechanism in crop. *Frontiers in Plant Science,* 4:71.

Koninkx, J.F. (1976). Protein synthesis in salivary glands of *Drosophila hydei* after experimental induction. *Biochemical Journal,* 58: 623-628.

Kotak, S., Larkindale, J., Lee, U., von Koskull-Doring, P., Vierling, E., Scharf, K.D. (2007). Complexity of the heat stress response in plants. *Current Opinion in Plant Biology,* 10: 310-316.

Kozlowski, T.T. and Pallardy, S.G. (1997). *Growth Control in Woody Plants.* Academic Press, San Diego, London. 641p.

Krause, G.H., Weis, E. (1991). Chlorophyll fluorescence and photosynthesis: The basics. *Annual Review of Plant Physiology and Plant Molecular Biology,* 42: 313-349.

Kregel, K.C. (2002). Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *Journal of Applied Physiology,* 92: 2177-2186.

Krishna, P., Gloor, G. (2001). The Hsp90 family of proteins in *Arabidopsis thaliana*. *Cell Stress Chaperones,* 6: 238-246.

Krishnakumar, R., Asokan, M.P. and Sethuraj, M.R. (1992). Polymorphic isozyme expression caused by stock scion interaction in *Hevea brasiliensis,* clone RRII 105. *Indian Journal of Natural Rubber Research,* 5(1&2): 161-171.

Kumar, R., Lavania, D., Singh, A.K., Negi, M., Siddiqui, M.H., Al-Whaibi, M.H., Grover, A. (2015). Identification and characterization of a small heat shock protein 17.9-CII gene from faba bean (*Vicia faba* L.). *Acta Physiologiae Plantarum,* 37: 190.

Kuruvilla, L., Sathik, M.B.M., Thomas, M., Luke, L.P., Sumesh, K.V. and Annamalainathan, K. (2016). Expression of miRNAs of *Hevea brasiliensis* under drought stress is altered in clones with varying levels of drought tolerance. *Indian Journal of Biotechnology,* 15: 153-160.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology,* 157: 105-132.

Labudda, M., Safiul Azam, F.M. (2014). Glutathione-dependent responses of plants to drought: a review. *Acta Societatis Botanicorum Poloniae,* 83: 3-12.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227: 680-685.

Langridge, P. and Chalmers, K. (2004). The Principle: Identification and Application of Molecular Markers. In: *Biotechnology in Agriculture and Forestry, Molecular Marker Systems in Plant Breeding and crop Improvement,* (Eds. Lorz, H. and Wenzel, G.). Springer, Berlin, Hidelberg, pp.129-149.

Le Naour, F., Andre, M., Boucheix, C., Rubinstein, E. (2006). Membrane microdomains and proteomics: Lessons from tetraspanin microdomains and comparison with lipid rafts. Review. *Proteomics*, 6: 6447-6454.

Lee, B.Y., Hefta, S.A., Brennan, P.J. (1992). Characterization of the major membrane protein of virulent *Mycobacterium tuberculosis. Infection and Immunity,* 60: 2066-2074.

Lee, G.J., Roseman, A.M., Saibil, H.R., Vierling, E. (1997). A small heat shock protein stably binds heat denatured model substrates and can maintain a substrate in a folding competent state. *EMBO Journal,* 16: 659-671.

Lee, G.J., Vierling, E. (2000). A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiology,* 122: 189-198.

Lee, K.W., Cha, J.Y., Kim, K.H., Kim, Y.G., Lee, B.H., Lee, S.H. (2012). Overexpression of alfalfa mitochondrial HSP23 in prokaryotic and eukaryotic model systems confers enhanced tolerance to salinity and arsenic stress. *Biotechnol Letters,* 34: 167-174.

Lenne, C., Block, M.A., Garin, J., Douce, R. (1995). Sequence and expression of the mRNA encoding HSP22, the mitochondrial small heat-shock protein in pea leaves. *Biochemical Journal,* 311(Pt 3): 805-813.

Levitt, M., Gerstein, M., Huang, E., Subbiah, S., Tsai, J. (1997). Protein folding: the endgame. *Annual Review of Biochemistry,* 66: 549-579.

Lewinsohn, T.M. (1991). The geographical distribution of plant latex. *Chemoecology,* 2: 64-68.

Lewis, M., Helmsing, P.J., Ashburner, M. (1975). Parallel changes in puffing activity and patterns of protein synthesis in salivary glands of *Drosophila. Proceedings of the National Academy of Sciences USA,* 72: 3604-3608.

Li, M., Ji, L., Yang, X., Meng, Q., Guo, S. (2012). The protective mechanisms of CaHSP26 in transgenic tobacco to alleviate photoinhibition of PSII during chilling stress. *Plant Cell Reports,* 31: 1969-1979.

Li, Y., Zhao, H.X., Duan, B.L., Korpelainen, H., Li, C.Y. (2011). Effect of drought and ABA on growth, photosynthesis and antioxidant system of *Cotinus coggygria* seedlings under two different light conditions. *Environmental and Experimental Botany,* 71: 107-113.

Lichtenthaler, H.K. (1987). Chlorophylls and Carotenoids: Pigments of Photosynthetic Biomembranes. *Methods in Enzymology,* 148: 350-382.

Li-feng Wang. (2014). Physiological and molecular responses to drought stress in rubber tree (*Hevea brasiliensis* Muell. Arg.). *Plant Physiology and Biochemistry,* 83: 243-249.

Lindquist, S. (1986). The heat shock response. *Annual Review of Biochemistry,* 55: 1151-1191.

Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, 25: 402-408.

Lobell, D., Field, C. (2007). Global scale climate - crop yield relationships and the impacts of recent warming. *Environmental Research Letters,* 2: 014002, 7 p.

Lopes-Caitar, V.S., de Carvalho, M.C., Darben, L.M., Kuwahara, M.K., Nepomuceno, A.L., Dias, W.P., Abdelnoor, R.V., Marcelino-Guimarães, F.C. (2013). Genome-wide analysis of the Hsp20 gene family in soybean: comprehensive sequence, genomic organization and expression profile analysis under abiotic and biotic stresses. *BMC Genomics,* 14: 577. doi: 10.1186/1471-2164-14-577.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin Phenol reagent. *Journal of Biological Chemistry,* 193(1): 265-275.

Lu, B., McClatchy, D.B., Kim, J.Y., Yates, J.R 3rd. (2008). Strategies for shotgun identification of integral membrane proteins by tandem mass spectrometry. Review. *Proteomics,* 8 (19): 3947-3955.

Luke, L.P., Sathik, M.B.M., Thomas, M., Kuruvilla, L., Sumesh, K.V. and Annamalainathan, K. (2015). Quantitative expression analysis of drought responsive genes in clones of *Hevea* with varying levels of drought tolerance. *Physiology and Molecular Biology of Plants,* 21(2): 179-186.

Ma, C., Haslbeck, M., Babujee, L., Jahn, O., Reumann, S. (2006). Identification and characterization of a stress-inducible and a constitutive small heat-shock protein targeted to the matrix of plant peroxisomes. *Plant Physiology,* 141: 47-60.

Madhava Rao, K.V. (2006). Introduction, In: *Physiology and molecular biology of stress tolerance in plants,* (Eds. Madhava Rao, K.V., Raghavendra, A.S., Janardhan Reddy, K.), Netherlands, Springer, pp. 1-14.

Malik, M.K., Slovin, J.P., Hwang, C.H., Zimmerman, J.L. (1999). Modified expression of a carrot small heat shock protein gene, hsp17.7, results in increased or decreased thermotolerancedouble dagger. *Plant Journal,* 20: 89-99.

Marchand, F.L., Mertens, S., Kockelbergh, F., Beyens, L., Nijs, I. (2005). Performance of high arctic tundra plants improved during but deteriorated after exposure to a simulated extreme temperature event. *Global Change Biology,* 11: 2078-2089.

Marco, F., Bitrian, M., Carrasco, P., Rajam, M.V., Alcazar, R., Tiburcio, A.F. (2015). Genetic engineering strategies for abiotic stress tolerance in plants. In: *Plant biology and biotechnology,* (Eds. Bahadur, B., Rajam, M.V., Sahijram, L., Krishnamurthy, K.V.). Springer, NY. pp. 579-609.

Masinovsky, Z., Lozovaya, G.I., Sivash, A.A. (1992). Some aspects of the early evolution of photosynthesis. *Advances in Space Research,* 12(4): 199-205.

Maxwell, K. and Johnson, N.G. (2000). Review Article. Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany,* 51(345): 659-668.

McHaourab, H.S., Godar, J.A., Stewart, P.L. (2009). Structure and mechanism of protein stability sensors: chaperone activity of small heat shock proteins. *Biochemistry,* 48: 3828-3837.

Mercykutty, V.C., Marattukalam, J.G., Saraswathyamma, C.K. and Meenakumari, T. (2002). *Identification of Hevea clones - A Manual,* Rubber Research Institute of India, Kottayam, Kerala, India.

Mertins, P., Qiao, J.W., Patel, J., Udeshi, N.D., Clauser, K.R., Mani, D.R., Burgess, M.W., Gillette, M.A., Jaffe, J.D., Carr, S.A. (2013). Integrated proteomic analysis of post-translational modifications by serial enrichment. *Nature Methods,* 10: 634-637.

Mirault, M.E., Goldschmidt-Clermont, M., Moran, L., Arrigo, A.P., Tissieres, A. (1978). The effect of heat shock on gene expression in *Drosophila melanogaster. Cold Spring Harbor Symposia on Quantitative Biology,* 42: 819-827.

Mogk, A., Schlieker, C., Friedrich, K.L., Schonfeld, H.J., Vierling, E., Bukau, B. (2003). Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *Journal of Biological Chemistry,* 278: 31033-31042.

Mohankrishna, T., Bhaskar, C.V.S., Rao, P.S., Chandrasekar, T.R., Sethuraj, M.R. and Vijayakumar, K.R. (1991). Effect of irrigation on physiological performance of immature plants of *Hevea brasiliensis* in North Konkan. *Indian Journal of Natural Rubber Research,* 4(1): 36-45.

Mooibroek, H., Cornish, K. (2000). Alternative sources of natural rubber. *Applied Microbiology and Biotechnology,* 53: 355-365.

Morimoto, R.I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & Development,* 12: 3788-3796.

Mu, C., Zhang, S., Yu, G., Chen, N., Li, X., Liu, H. (2013). Overexpression of small heat shock protein LimHSP16.45 in *Arabidopsis* enhances tolerance to abiotic stresses. *PLoS One,* 8: e82264.

Mydin, K.K. (2014). Genetic improvement of *Hevea brasiliensis*: sixty years of breeding efforts in India. *Rubber Science,* 27: 153-181.

Nagao, R.T., Czarnecka, E., Gurley, W.B., Schoffl, F., Key, J.L. (1985). Genes for low-molecular-weight heat shock proteins of soybeans: sequence analysis of a multigene family. *Molecular and Cellular Biology,* 5: 3417-3428.

Nair, D.B., Dey, S.K., Rajagopal, R., Vijayakumar, K.R., Sethuraj, M.R. (1996). Synergistic effect of heat and osmotic stress in causing membrane injury in *Hevea brasiliensis, Journal of Plant Biology,* 39: 177-181.

Nam, M.H., Huh, S.M., Kim, K.M., Park, W.J., Seo, J.B., Cho, K., Kim, D.Y., Kim B.G., Yoon, I.S. (2012). Comparative proteomic analysis of early salt stress-responsive proteins in roots of SnRK2 transgenic rice. *Proteome Science,* 10: 25.

Nelson, T., Harpster, M.H., Mayfield, S.P. and Taylor, W.C. (1984). Light-regulated gene expression during maize leaf development. *The Journal of Cell Biology,* 96: 558-564.

Nerland, A.H., Mustafa, A.S., Sweetser, D., Godal, T., Young, R.A. (1988). A protein antigen of *Mycobacterium leprae* is related to a family of small heat shock proteins. *Journal of Bacteriology,* 170: 5919-5921.

Neta-Sharir, I., Isaacson, T., Lurie, S., Weiss, D. (2005). Dual role for tomato heat shock protein 21: protecting photosystem II from oxidative stress and promoting color changes during fruit maturation. *Plant Cell,* 17: 1829-1838.

Nezhadahmadi, A., Hossain Prodhan, Z., Faruq, G. (2013). Drought tolerance in wheat. Review. *The Scientific World Journal,* 2013: 1-12.

O'Farrell, P.H. (1975). High Resolution Two-Dimensional Electrophoresis of Proteins. *Journal of Biological Chemistry,* 250(10): 4007-4021.

Oquist, G., Campbell, D., Clarke, A.K. and Gustafsson, P. (1995). The cyanobacterim *Synechococcus* modulates PSII function in response to excitation stress through D1 exchange. *Photosynthesis Research,* 46: 151-158.

Panaretou, B., Zhai, C. (2008). The heat shock proteins: their roles as multi-component machines for protein folding. *Fungal Biology Reviews,* 22, 110-119.

Papoian, G.A. (2008). Proteins with weakly funnelled energy landscapes challenge the classical structure-function paradigm. *Proceedings of the National Academy of Sciences USA,* 105: 14237-14238.

Parsell, D.A., Lindquist, S. (1993). The function of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual Review of Genetics,* 27: 437-496.

Patel, S., Vierling, E., Tama, F. (2014). Replica exchange molecular dynamics simulations provide insight into substrate recognition by small heat shock proteins. *Biophysical Journal,* 106: 2644-2655.

Perez-Alfocea, F., Ghanem, M.E., Gomez-Cadenas, A. and Dodd, I.C. (2011). Omics of root-to-shoot signaling under salt stress and water deficit. *OMICs: A Journal of Integrative Biology*, 15 (12): 893-901.

Perez-Clemente, M.R., Vives, V., Zandalinas, S.I., Lopez-Climent, M.F., Munoz, V. and Gomez-Cadenas, A. (2013). Biotechnological Approaches to Study Plant Responses to Stress. *BioMed Research International,* 2013: 654120.

Personat, J.M., Tejedor-Cano, J., Prieto-Dapena, P., Almoguera, C., Jordano, J. (2014). Co-overexpression of two heat shock factors results in enhanced seed longevity and in synergistic effects on seedling tolerance to severe dehydration and oxidative stress. *BMC Plant Biology,* 14: 56.

Piques, M., Schulze, W.X., Hohne, M., Usadel, B., Gibon, Y., Rohwer, J., Stitt, M. (2009). Ribosome and transcript copy numbers, polysome occupancy and enzyme dynamics in *Arabidopsis*. *Molecular Systems Biology,* 5: 314.

Praveen, S., Nutan, K.K., Soda, N., Nongpiur, R.C., Roy, S., Singla-Pareek, S.L. and Pareek, A. (2015). Towards understanding abiotic stress signaling in plants: convergence of genomic, transcriptomic, proteomic, and metabolomic approaches. In: *Elucidation of abiotic stress signaling in plants: Functional genomics perspectives,* (Ed. Pandey, K.G.). Springer, NY. pp. 3-40.

Premakumari, D. and Saraswathyamma, C.K. (2000). The para rubber tree. In: *Natural Rubber: Agromanagement and crop processing*, (Eds. George, P.J. and Jacob, C.K.). Rubber Research Institute of India, Kottayam, India, pp. 29-35.

Priyadarshan, P.M. (2011). *Biology of Hevea Rubber,* CAB International, Oxfordshire. pp. 1-10.

Priyadarshan, P.M., Sudhasoumyalatha, M.K., Sasikumar, S., Dey, S.K., Varghese, Y.A. (2000). Evaluation of *Hevea brasiliensis* clones for yielding trends in Tripura. *Indian Journal of Natural Rubber Research,* 11: 67-72.

Pushparajah, E. (1983). Problems and potentials for establishing *Hevea* under difficult environmental conditions. *Planter,* Kuala Lumpur, 59: 242-251.

Pushparajah, E. (2001). Natural rubber. In: *Tree Crop Ecosystems, Vol. 19, Ecosytems of the World* series, (Ed. Last, F.T.). Elsevier Science, Amsterdam, pp. 379-407.

Rahaman, W.A., Shivakumaran, S. (1998). Studies of carbon sequestration in rubber. *UNCTAD/IRSG Workshop,* Rubber forum, Bali, Indonesia. pp. 56-65.

Rahdari, P., Hoseini, S.M. (2012). Drought stress: a review. *International Journey of Agronomy and Plant Production,* 3: 443-446.

Ranasinghe, M.S., Milburn, J.A. (1995). Xylem conduction and cavitation in *Hevea brasiliensis. Journal of Experimental Botany,* 46: 1693-1700.

Rao, S. P. and Vijayakumar, K.R. (1992). Climatic requirements. In: *Natural Rubber: Biology, Cultivation and Technology,* (Eds. Sethuraj, M.R. and Mathew, N.M.). Elsevier Science Publishers, Amsterdam, Netherlands. pp. 200-219.

Reddy, G. B., Das, K. P., Petrash, J. M. and Surewicz, W. K. (2000). Temperature-dependent chaperone activity and structural properties of human alphaA- and alphaB-crystallins. *Journal of Biological Chemistry,* 275: 4565- 4570.

Reddy, P.S., Kavi Kishor, P.B., Seiler, C., Kuhlmann, M., Eschen-Lippold, L., Lee, J., Reddy, M.K., Sreenivasulu, N. (2014). Unraveling regulation of the small heat shock proteins by the heat shock factor HvHsfB2c in barley: its implications in drought stress response and seed development. *PLoS One,* 9: e89125.

Reed. C.F. (1976). Information summaries on 1000 economic plants. Typescripts submitted to the USDA.

Reeves, S.G. and Hall, D.O. (1973). The stochiometry (ATP/2e ratio) of non-cyclic photophosphorylation in isolated spinach chloroplasts. *Biochimica et Biophysica Acta,* 314: 66-78.

Rhodes, D., Hanson, A. (1993). Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annual Review of Plant Biology,* 44: 357-384.

Rodriguez, M., Canales, E., Borras-Hidalgo, O. (2005). Molecular aspects of abiotic stress in plants. *Biotecnologia Aplicada,* 22: 1-10.

Rodziewicz, P., Swarcewicz, B., Chmielewska, K., Wojakowska, A., Stobiecki, M. (2014). Influence of abiotic stresses on plant proteome and metabolome changes. *Acta Physiologiae Plantarum,* 36: 1-19.

Rost, B. and Liu, J. (2003). The PredictProtein server. *Nucleic Acids Research,* 31: 3300-3304.

Russnak, R.H., Jones, D., Candido, E.P. (1983). Cloning and analysis of cDNA sequences coding for two 16 kilodalton heat shock proteins (hsps) in *Caenorhabditis elegans*: homology with the small hsps of *Drosophila. Nucleic Acids Research,* 11: 3187-3205.

Salas-Munoz, S., Gomez-Anduro, G., Delgado-Sanchez, P., Rodriguez-Kessler, M., Jimenez-Bremont, J.F. (2012). The *Opuntia streptacantha* OpsHSP18 gene confers salt and osmotic stress tolerance in *Arabidopsis thaliana. International Journal of Molecular Sciences,* 13: 10154-10175.

Salehi-Lisar, S.Y., Bakhshayeshan-Agdam, H. (2016). Drought Stress in Plants: Causes, Consequences, and Tolerance. In: *Drought Stress Tolerance in Plants*, Vol 1 (Eds. Hossain, M., Wani, S., Bhattacharjee, S., Burritt, D., Tran, LS.). Springer, Cham, pp. 1-16.

Salehi-lisar, S.Y., Motafakkerazad, R., Hossain, M.M., Rahman, I.M.M. (2012). Water stress in plants: causes, effects and responses. In: *water stress*, (Eds. Ismail, M., Mofi Zur Rahman), In Tech, DOI: 10.5772/39363.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B., Bennett, J. (2002). A proteomic approach to analyzing drought-and salt-responsiveness in rice. *Field Crops Research,* 76: 199-219.

Samarappuli, L. and Yogaratnam, N. (1998). Tolerance of rubber plantations to drought and atmospheric warming: A review. *Journal of the Rubber Research Institute of Sri Lanka,* 81: 51-67.

Sanmiya, K., Suzuki, K., Egawa, Y., Shono, M. (2004). Mitochondrial small heat-shock protein enhances thermotolerance in tobacco plants. *FEBS Letters,* 557: 265-268.

Santhanagopalan, I., Basha, E., Ballard, K.N., Bopp, N.E., Vierling, E. (2015). In: *The Big Book on Small Heat Shock Proteins,* (Eds. Tanguay, R.M., Hightower, L.E.). Springer, Switzerland, pp. 119-153.

Satheesh, P.R., Jacob, J. (2011). Impact of climate warming on natural rubber productivity in different agro-climatic regions of India. *Natural Rubber Research,* 24(1): 1-9.

Sato, Y., Yokoya, S. (2008). Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHSP17.7. *Plant Cell Reports,* 27: 329-334.

Sauer, U., Durre, P. (1993). Sequence and molecular characterization of a DNA region encoding a small heat shock protein of *Clostridium acetobutylicum. Journal of Bacteriology,* 175: 3394-3400.

Savas, J.N., Stein, B.D., Wu, C.C., Yates, J.R 3rd. (2011). Mass spectrometry accelerates membrane protein analysis. Review. *Trends in Biochemical Sciences,* 36(7): 388-396.

Scharf, K.D., Siddique, M., Vierling, E. (2001). The expanding family of *Arabidopsis thaliana* small heat stress proteins and a new family of proteins containing alpha-crystallin domains (Acd proteins). *Cell Stress Chaperones*, 6: 225-237.

Schindler, C. and Lichtenthaler, H.K. (1994). Is there a correlation between light-induced zeaxanthin accumulation and quenching of variable chlorophyll a fluorescence?. *Plant Physiology,* 32: 813-823.

Schirmer, E.C., Glover, J.R., Singer, M.A., Lindquist, S.L. (1996). Hsp100/Clp proteins: A common mechanism explains diverse functions. *Trends in Biochemical Sciences,* 21: 289-296.

Schlesinger, M.J. (1990). Heat shock proteins. *Journal of Biological Chemistry,* 265: 12111-12114.

Schoffl, F., Key, J.L. (1982). An analysis of mRNAs for a group of heat shock proteins of soybean using cloned cDNAs. *Journal of Molecular and Applied Genetics,* 1: 301-314.

Schoffl, F., Prandl, R., Reindl, A. (1998). Regulation of the heat shock response. *Plant Physiology,* 117: 1135-1141.

Schreiber, U., Bilger, W., Hormann, H., Neubauer, C. (1998). Chlorophyll fluorescence as a diagnostic tool: Basics and some aspects of practical relevance. In: *Photosynthesis: A comprehensive treatise,* (Ed, Raghavendra., A.S.). Cambridge: Cambridge University Press, pp. 320-336.

Schulze, E.D., Beck, E., Muller-Hohenstein, K. (2002). *Pflanzenökologie,* Spektrum Akademischer, Heidelberg.

Seki, M.A., Kamei, K., Yamaguchi-Shinozaki., Shinozaki. (2003). Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Current Opinion in Biotechnology,* 14 (2): 194-199.

Sethuraj, M.R. (1985). Physiology of growth and yield in *Hevea brasiliensis. Proceedings of the International Rubber Conference,* Kuala Lumpur, Malaysia. pp. 3-19.

Sethuraj, M.R., Potty, S.N., Vijayakumar, K.R., Krishnakumar, A.K., Sanjeeva Rao, P., Thapaliyal, A.P., Mohankrishna T., Gururaja Rao., G, Chaudhury, D., George, M.J., Soman, T.A., Meenattoor, J.R. (1989). Growth performance of *Hevea* in the non-traditional regions of India. *Proceedings RRIM Planters Conference,* Malayasia, pp. 212-227.

Sethuraj, M.R., Rao, C.G. and Raghavendra, A.S. (1984). The pattern of latex flow from rubber tree (*Hevea brasiliensis*) in relation to water stress. *Journal of cellular biochemistry Supplement,* 8B: 236.

Shao, H.B., Chu, L.Y., Jaleel, C.A., Zhao, C.X. (2008). Water-deficit stress-induced anatomical changes in higher plants. *Comptes Rendus Biologies,* 331: 215-225.

Shinozaki, K., Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross talk between two stress signalling pathways. *Current Opinion in Plant Biology,* 3: 217-223.

Shinozaki, K., Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany,* 58: 221-227.

Shinozaki, K., Yamaguchi-Shinozaki, K., Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology,* 6: 410-417.

Siddique, M., Gernhard, S., von Koskull-Doring, P., Vierling, E., Scharf, K.D. (2008). The plant sHSP superfamily: five new members in *Arabidopsis thaliana* with unexpected properties. *Cell Stress Chaperones,* 13: 183-197.

Singh, M., Srivastava, J.P., Kumar, A. (1990). Effect of water stress on water potential components in wheat genotypes. *Indian Journal of Plant Physiology,* 33: 312-317.

Smirnoff, N. (1993). The role of active oxygen in the response of plants to water deficit and dessication. *New Phytologist,* 125: 27-58.

Smirnoff, N. (1998). Plant resistance to environmental stress. *Current Opinion in Biotechnology,* 9: 214-219.

Smykal, P., Masin, J., Hrdy, I., Konopasek, I., Zarsky, V. (2000). Chaperone activity of tobacco HSP18, a small heat-shock protein, is inhibited by ATP. *Plant Journal,* 23: 703-713.

Sobhana, P. (1998). Physiology of rooting and stock-scion interaction in *Hevea. Ph.D Thesis,* Kerala University, Kerala, India.

Sobhana, P., James Jacob and Sethuraj, M R. (2007). Rootstock-scion relationships in bud grafted plants with special reference to *Hevea brasiliensis. Natural rubber research,* 20(1&2): 94-107.

Sobhana, P., Nair, N.U., George, M.J. and Sethuraj, M.R. (1980). Effect of scion on root growth, cation exchange capacity of roots and mineral uptake in *Hevea brasiliensis. International Rubber Conference India,* Kottayam, India.

Song, N.H., Ahn, Y.J. (2010). DcHsp17.7, a small heat shock protein from carrot, is upregulated under cold stress and enhances cold tolerance by functioning as a molecular chaperone. *Hort Science,* 45: 469-474.

Song, N.H., Ahn, Y.J. (2011). DcHsp17.7, a small heat shock protein in carrot, is tissue-specifically expressed under salt stress and confers tolerance to salinity. *New Biotechnology,* 28: 698-704.

Soto, A., Allona, I., Collada, C., Guevara, M.A., Casado, R., Rodriguez-Cerezo, E., Aragoncillo, C., Gomez, L. (1999). Heterologous expression of a plant small heat-shock protein enhances *Escherichia coli* viability under heat and cold stress. *Plant Physiology,* 120: 521-528.

Speers, A.E., Wu, C.C. (2007). Proteomics of integral membrane proteins-theory and application. Review. *Chemical Reviews,* 107 (8): 3687-3714.

Sreelatha, S., James Jacob., Prakash, G., Simon, S.P., Annamalainathan, K. and Chandrasekhar, T.R. (2003). Antioxidant defense Systems and drought tolerance in *Hevea brasiliensis. Indian Journal of Natural Rubber Research,* 16(1&2): 93-101.

Sreelatha, S., Mydin, K.K., Simon, S.P., Krishnakumar, R., Jacob, J. and Annamalainathan, K. (2011). Seasonal variations in yield and associated biochemical changes in RRII 400 series clones of *Hevea brasiliensis. Natural Rubber Research*, 24: 117-123.

Sreelatha, S., Simon, S.P., MuraleedharaKurup, G., Vijayakumar, K.R. (2007). Biochemical mechanism associated with low yield during stress in *Hevea* clone RRII 105. *Journal of Rubber Research,* 10(2): 107-115.

Stengel, F., Baldwin, A.J., Painter, A.J., Jaya, N., Basha, E., Kay, L.E., Vierling, E., Robinson, C.V., Benesch, J.L. (2010). Quaternary dynamics and plasticity underlie small heat shock protein chaperone function. *Proceedings of the National Academy of Sciences USA,* 107: 2007-2012.

Subramaniam, A. (1995). The chemistry of natural rubber latex. In: *latex allergy. Immunology and Allergy Clinics of North America* (Ed. Fink, J., Saunders). Philadelphia, 15: 1-20.

Sumesh, K.V., Satheesh, P.R., Annamalainathan, K., Krishnakumar, R., Thomas, M. and Jacob, J. (2011). Physiological evaluation of a few modern *Hevea* clones for intrinsic drought tolerance. *Natural RubberResearch*, 24: 61-67.

Sun, A.Z., Guo, F.Q. (2016). Chloroplast retrograde regulation of heat stress responses in plants. Review. *Frontiers in Plant Science,* 7: 398.

Sun, L., Liu, Y., Kong, X., Zhang, D., Pan, J., Zhou, Y., Wang, L., Li, D., Yang, X. (2012). ZmHSP16.9, a cytosolic class I small heat shock protein in maize (*Zea mays*), confers heat tolerance in transgenic tobacco. *Plant Cell Reports,* 31: 1473-1484.

Sun, W., Bernard, C., van de Cotte, B., Van Montagu, M., Verbruggen, N. (2001). At-HSP17.6A, encoding a small heat-shock protein in *Arabidopsis*, can enhance osmotolerance upon overexpression. *Plant Journal,* 27: 407-415.

Sun, W., Van Montagu, M., Verbruggen, N. (2002). Small heat shock proteins and stress tolerance in plants. *Biochimica et Biophysica Acta,* 1577: 1-9.

Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R. (2014). Abiotic and biotic stress combinations. *New Phytologist,* 203: 32-43.

Taiz, L. and Zeiger, E. (1991). *Plant Physiology.* Third edition, Benjamin/Cummings, Redwood city, California, USA, 623p.

Tamburino, R., Vitale, M., Ruggiero, A., Sassi, M., Sannino, L., Arena, S., Costa, A., Batelli, G., Zambrano, N., Scaloni, A., Grillo, S. and Scotti, N. (2017). Chloroplast proteome response to drought stress and recovery in tomato (*Solanum lycopersicum* L.) . *BMC Plant Biology,* 17: 40.

Tejedor-Cano, J., Prieto-Dapena, P., Almoguera, C., Carranco, R., Hiratsu, K., Ohme-Takagi, M., Jordano, J. (2010). Loss of function of the HSFA9 seed longevity program. *Plant, Cell & Environment,* 33: 1408-1417.

Tester, M., Langridge, P. (2010). Breeding technologies to increase crop production in a changing world. *Science,* 327: 818-822.

Thomas, M., Sathik, M.B.M., Luke, L.P., Kuruvilla, L., Sumesh, K.V., Satheesh, P.R., Annamalainathan, K., Krishnakumar, R. and Jacob, J. (2011). Analysis of drought responsive gene expression in *Hevea brasiliensis. Natural Rubber Research,* 24(2): 247-252.

Thomas, M., Xavier, S.M., Sumesh, K.V., Annamalainathan, K., Nair, D.B. and Mercy, M.A. (2015). Identification of potential drought tolerant *Hevea* germplasm accessions using physiological and biochemical parameters. *Rubber Science*, 28 (1): 62-69.

Thulaseedharan, A., Kumari Jayasree, P., Venkatachalam, P. (2000). Biotechnological approaches for crop improvement in rubber. In: *Biotechnology in Horticultural and Plantation Crops,* (Eds. Chadha, K.L., Ravindran, P.N., Sahijram, L.). Malhotra Publishing House, Calcutta. pp. 323-351.

Tiroli-Cepeda, A.O., Ramos, C.H. (2010). Heat causes oligomeric disassembly and increases the chaperone activity of small heat shock proteins from sugarcane. *Plant Physiology and Biochemistry,* 48: 108-116.

Tissieres, A., Mitchell, H.K., Tracy, V.M. (1974). Protein synthesis in salivary glands of *Drosophila melanogaster:* relation to chromosome puffs. *Journal of Molecular Biology,* 84: 389-398.

Todaka, D., Shinozaki, K. and Yamaguchi-Shinozaki, K.(2015). Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. *Frontiers in Plant Science*, 6: 84.

Torok, Z., Goloubinoff, P., Horvath, I., Tsvetkova, N.M., Glatz, A., Balogh, G., Varvasovszki, V., Los, D.A., Verling, E., Crowe, J.H., Vigh, L. (2001). *Synechocystis* HSP 17 as an amphitropic protein that stabilizes heat-stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding. *Proceedings of the National Academy of Sciences USA,* 98 (6): 3098-3103.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proceedings of the National Academy of Sciences USA,* 76: 4350-4354.

Trent, J.D. (1996). A review of acquired thermotolerance, heat-shock proteins and molecular chaperones in Archaea. *FEMS Microbiology Reviews,* 18, 249-258.

Tsvetkova N.M., Horvath, I., Torok, Z., Wolkers, W.F., Balogi, Z., Shigapova, N., Crowe, L.M., Tablin, F., Vierling, E., Crowe, J.H., Vigh, L. (2002). Small heat-shock proteins regulate membrane lipid polymorphism. *Proceedings of the National Academy of Sciences USA,* 99: 13504-13509.

Tuberosa, R., Salvi, S. (2006). Genomics-based approaches to improve drought tolerance of crops. *Trends in Plant Science,* 11: 405-412.

Van Aken, O., Zhang, B., Carrie, C., Uggalla, V., Paynter, E., Giraud, E., Whelan, J. (2009). Defining the mitochondrial stress response in *Arabidopsis thaliana. Molecular Plant,* 2: 1310-1324.

van Montfort, R.L., Basha, E., Friedrich, K.L., Slingsby, C., Vierling, E. (2001). Crystal structure and assembly of a eukaryotic small heat shock protein. *Nature Structural Biology,* 8: 1025-1030.

Varghese, Y.A., John, A., Premakumari, D., Panikkar, A.O.N., Sethuraj, M.R. (1993). Early evaluation of *Hevea*: Growth and yield at the juvenile phase. *Indian Journal of Natural Rubber Research,* 6(1&2): 19-23.

Veinger, L., Diamant, S., Buchner, J., Goloubinoff, P. (1998). The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *Journal of Biological Chemistry,* 273: 11032-11037.

Verbon, A., Hartskeerl, R.A., Schuitema, A., Kolk, A.H., Young, D.B., Lathigra, R. (1992). The 14,000- molecular-weight antigen of *Mycobacterium tuberculosis* is related to the alphacrystallin family of low-molecular-weight heat shock proteins. *Journal of Bacteriology*, 174: 1352-1359.

Vierling, E. (1991). The role of heat shock proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology,* 42: 579-620.

Vierling, E., Mishkind, M.L., Schmidt, G.W., Key, J.L. (1986). Specific heat shock proteins are transported into chloroplasts. *Proceedings of the National Academy of Sciences USA,* 83: 361-365.

Vierling, E., Nagao, R.T., DeRocher, A.E., Harris, L.M. (1988). A heat shock protein localized to chloroplasts is a member of a eukaryotic superfamily of heat shock proteins. *EMBO Journal,* 7: 575-581.

Vijayakumar, K.R., Dey, S.K., Chandrasekhar, T.R., Devakumar, A.S., Mohankrishna,T., Rao, P.S., and Sethuraj, M.R. (1998). Irrigation requirement of rubber trees (*Hevea brasiliensis*) in the subhumid tropics. Agricultural Water Management, 35 (3): 245-259.

Vinocur, B., Altman, A. (2005). Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Current Opinion in Biotechnology,* 16: 123-132.

Wadhwa, R., Ryu, J., Gao, R., Choi, I.K., Morrow, G., Kaur, K., Kim, I., Kaul, S.C., Yun, C.O., Tanguay, R.M. (2010). Proproliferative functions of Drosophila small mitochondrial heat shock protein 22 in human cells. *Journal of Biological Chemistry,* 285: 3833-3839.

Walker, D.A. (1988). In: *The use of oxygen electrode and fluorescence probe in simple measurements of photosynthesis.* II ed., Oxygraphics Ltd., Sheffield, pp. 57-69.

Wang, D. and Luthe, D.S. (2003). Heat sensitivity in a bentgrass variant. Failure to accumulate a chloroplast heat shock protein isoform implicated in heat tolerance. *Plant Physiology,* 133: 319-327.

Wang, J.Z., Cui, L.J., Wang, Y., Li, J.L. (2009). Growth, lipid peroxidation and photosynthesis in two tall fescue cultivars differing in heat tolerance. *Biologia Plant*arum, 53: 237-242.

Wang, W., Vinocur, B., Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta,* 218: 1-14.

Wang, W., Vinocur, B., Shoseyov, O., Altman, A. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science,* 9: 244-252.

Wang, W.X., Pelah, D., Alergand, T., Shoseyov, O., Altman, A. (2002). Characterization of SP1, a stress-responsive, boiling-soluble, homo-oligomeric protein from Aspen (*Populus tremula* L.). *Plant Physiology,* 130: 865-875.

Wang, X., Cai, X., Xu, C., Wang, Q. and Dai, S. (2016). Drought-responsive mechanisms in plant leaves revealed by proteomics. *International Journal of Molecular Sciences,* 17: 1706.

Wani, S.H., Sah, S.K., Hossain, M.A., Kumar, V. and Balachandran, S.M. (2016). Transgenic approaches for abiotic stress tolerance in crop plants. In: *Advances in Plant Breeding Strategies: Agronomic, Abiotic and Biotic Stress Traits,* (Eds. Al-Khayri, J.M., Jain, S.M., Johnson, D.V.). Springer, Switzerland, pp. 345-397.

Waters, E.R., Aevermann, B.D., Sanders-Reed, Z. (2008). Comparative analysis of the small heat shock proteins in three angiosperm genomes identifies new subfamilies and reveals diverse evolutionary patterns. *Cell Stress Chaperones,* 13: 127-142.

Waters, E.R., Lee, G.J., Vierling, E. (1996). Evolution, structure and function of the small heat shock proteins in plants. *Journal of Experimental Botany,* 47: 325-338.

Waters, E.R., Vierling, E. (1999a). Chloroplast small heat shock proteins: evidence for a typical evolution of an organelle-localized protein. *Proceedings of the National Academy of Sciences USA,* 96: 14394-14399.

Waters, E.R., Vierling, E. (1999b). The diversification of plant cytosolic small heat shock proteins preceded the divergence of mosses. *Molecular Biology and Evolution,* 16: 127-139.

Webster, C. C. and Paardekooper, E. C. (1989). The Botany of the Rubber Tree. In: *Rubber* (Eds. Webster, C.C. and Baulkwill, W. J.). Longman Scientific and Technical, Essex, pp. 57-84.

Wehmeyer, N., Hernandez, L.D., Finkelstein, R.R., Vierling, E. (1996). Synthesis of small heat-shock proteins is part of the developmental program of late seed maturation. *Plant Physiology,* 112: 747-757.

Wehmeyer, N., Vierling, E. (2000). The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. *Plant Physiology,* 122: 1099-1108.

Wilkins, M.R., Sanchez, J.C., Gooley, A.A., Appel, R.D., Humphery-Smith, I., Hochstrasser, D.F., Williams, K.L. (1996). Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnology and Genetic Engineering Reviews.* 13: 19-50.

Witzel, K., Weidner, A., Surabhi, G.K., Borner, A., Mock, H.P. (2009). Salt stress- induced alterations in the root proteome of barley genotypes with contrasting response towards salinity. *Journal of Experimental Botany,* 60: 3545-3557.

Wu, C.C., Yates, J.R 3rd. (2003). The application of mass spectrometry to membrane proteomics. Review. *Nature Biotechnology,* 21: 262-267.

Wycherley, P. R. (1968). Introduction of *Hevea* to the Orient. *The Planter* (Kuala Lumpur), 44: 127- 37.

Wycherley, P.R. (1992). The Genus *Hevea*- Botanical Aspects. In: *Natural Rubber: Biology, Cultivation and Technology,* (Eds. M.R. Sethuraj and N.M. Mathew). Elsevier Science Publishers, Amsterdam, Netherlands. pp. 50-66.

Xiong, L., Ishitani, M. (2006). Stress signal transduction: components, pathways, and network integration. In: *Abiotic stress tolerance in plants: toward the improvement of global environment and food*, (Eds. Rai A.K., Takabe, T.). Springer, Dordercht, The Netherlands. pp 3-29.

Xoconostle-Cazares, B., Ramirez-Ortega, F.A., Flores-Elenes, L., Ruiz-Medrano, R. (2010). Drought tolerance in crop plants. *American Journal of Plant Physiology,* 5: 241-256.

Xue, Y., Peng, R., Xiong, A., Li, X., Zha, D., Yao, Q. (2009). Yeast heat-shock protein gene HSP26 enhances freezing tolerance in *Arabidopsis*. *Journal of Plant Physiology,* 166: 844-850.

Yachdav, G., Kloppmann, E., Kajan, L., Hecht, M., Goldberg, T., Hamp, T., Honigschmid, P., Schafferhans, A., Roos, M., Bernhoefr, M., Richter, L., Ashkenazy, H., Punta, M., Schlessinger, A., Bromberg, Y., Schneider, R., Vriend, G., Sander, C., Ben-Tal, N., Rost, B. (2014). PredictProtein-an open resource for online prediction of protein structural and functional features. *Nucleic Acids Research,* 42: W337-W343.

Yagishita, N., Hirata, Y., Mizukami, H., Ohashi, H. and Okochi, K. (1986). Characterization of graft induced changes of capsicin content in *Capsicum annuum* L., *Euphytica,* 34: 297-301.

Yagishita, N., Hirata., Mizukami, H., Oshashi, H. and Yamashita, K. (1990). Genetic nature of low capsicin content in the variant strains induced by grafting in *Capsicum annuum* L., *Euphytica*, 46: 249-252.

Yamaguchi-Shinozaki, K., Shinozaki, K. (2005). Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends in Plant Science,* 10: 88-94.

Yan, K., Shao, H., Shao, C., Chen, P., Zhao, S., Brestic, M., Chen, X. (2013). Physiological adaptive mechanisms of plants grown in saline soil and implications for sustainable saline agriculture in coastal zone. *Acta Physiologiae Plantarum,* 35: 2867-2878.

Yeh, C.H., Chang, P.F., Yeh, K.W., Lin, W.C., Chen, Y.M., Lin, C.Y. (1997). Expression of a gene encoding a 16.9-kDa heat-shock protein, Oshsp16.9, in *Escherichia coli* enhances thermotolerance. *Proceedings of the National Academy of Sciences USA,* 94: 10967-10972.

Yeh, C.H., Chen, Y.M., Lin, C.Y. (2002). Functional regions of rice heat shock protein, Oshsp16.9, required for conferring thermotolerance in *Escherichia coli. Plant Physiology,* 128: 661-668.

Yin, C.C., Ma, B., Collinge, D.P., Pogson, B.J., He, S.J., Xiong, Q., Duan, K.X., Chen, H., yang, C., Lu, X., Wang, Y.Q., Zhang, W.K., Chu, C.C., Sun, X.H., Fang, S., Chu, J.F., Lu, T.G., Chen, S.Y., Zhang, J.S. (2015). Ethylene responses in rice roots and coleoptiles are differentially regulated by a carotenoid isomerase-mediated abscisic acid pathway. *Plant Cell,* 27: 1061-1081.

Yoon, H.J., Kim, K.P., Park, S.M., Hong, C.B. (2005). Functional mode of NtHSP17.6. a cytosolic small heat-shock protein from *Nicotiana tabacum. Journal of Plant Biology,* 48: 120-127.

Young, A.J. (1991). The photoprotective role of carotenoids in higher plants. *Physiologia Plantarum,* 83: 702-708.

Young, A.J. (1993). Occurence and distribution of carotenoids in photosynthetic systems. In: *Carotenoids in photosynthesis,* (Eds. Young, A.J. and Britton, G.). Chapman & Hall, London, pp. 16-71.

Young, J.C., Moarefi, I., Hartl, F. U. (2001). Hsp90: a specialized but essential protein-folding tool. *Journal of Cell Biology,* 154: 267-273.

Young, R.A., Elliott, T.J. (2002). Stress proteins, infection and immune surveillance. *Cell,* 59: 5-8.

Zare, M., Azizi, M.H., Bazrafshan, F. (2011). Effect of drought stress on some agronomic traits in ten barley (*Hordeum vulgare*) cultivars. *Technical Journal of Engineering and Applied Sciences,* 1: 57-62.

Zeppel, M.J., Anderegg, W.R., Adams, H.D. (2013). Forest mortality due to drought: latest insights, evidence and unresolved questions on physiological pathways and consequences of tree death. *New phytologist,* 197: 372-374.

Zhang, Q. (2007). Strategies for developing Green Super Rice. *Proceedings of the National Academy of Sciences,* USA, 104: 16402-16409.

Zhao, C., Shono, M., Sun, A., Yi, S., Li, M., Liu, J. (2007). Constitutive expression of an endoplasmic reticulum small heat shock protein alleviates endoplasmic reticulum stress in transgenic tomato. *Journal of Plant Physiology,* 164: 835-841.

Zheng, W., Schafer, N.P., Davtyan, A., Papoian, G.A., Wolynes, P.G. (2012). Predictive energy landscapes for protein-protein association. *Proceedings of the National Academy of Sciences USA,* 109: 19244-19249.

Zhong, L., Zhou, W., Wang, H., Ding, S., Lu, Q., Wen, X., Peng, L., Zhang, L., Lu, C. (2013). Chloroplast small heat shock protein HSP21 interacts with plastid nucleoid protein pTAC5 and is essential for chloroplast development in *Arabidopsis* under heat stress. *Plant Cell,* 25: 2925-2943.

Zhou, S., Li, M., Guan, Q., Liu, F., Zhang, S., Chen, W., Yin, L., Qin, Y., Ma, F. (2015). Physiological and proteome analysis suggest critical roles for the photosynthetic system for high water-use efficiency under drought stress in *Malus* . *Plant Science,* 236: 44-60.

Zhou, Y., Chen, H., Chu, P., Li, Y., Tan, B., Ding, Y., Tsang, E.W., Jiang, L., Wu, K., Huang, S. (2012). NnHSP17.5, a cytosolic class II small heat shock protein gene from *Nelumbo nucifera*, contributes to seed germination vigor and seedling thermotolerance in transgenic *Arabidopsis*. *Plant Cell Reports,* 31: 379-389.

Zhou, Y., Lam, H.M., Zhang, J. (2007). Inhibition of photosynthesis and energy dissipation induced by water and high light stresses in rice. *Journal of Experimental Botany,* 58: 1207-1217.

Zhu, J.K. (2002). Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology,* 53: 247-273.

Zivcak, M., Kalaji, H.M., Shao, H.B., Olsovska, K., Brestic, M. (2014). Photosynthetic proton and electron transport in wheat leaves under prolonged moderate drought stress. *Journal of Photochemistry and Photobiology. B, Biology,* 137: 107-115.

Zlatev, Z., Lidon, F.C. (2012). An overview on drought induced changes in plant growth, water relations and photosynthesis. *Emirates Journal of Food and Agriculture,* 24: 57-72.

Zongdao, H. and Xueqin, Z. (1983). Rubber cultivation in China. *Proceedings RRIM Planters' Conference,* pp. 31-43.

Zou, J., Liu, C., Liu, A., Zou, D., Chen, X. (2012). Overexpression of OsHsp17.0 and OsHsp23.7 enhances drought and salt tolerance in rice. *Journal of Plant Physiology,* 169: 628-635.