Studies on physiological, biochemical and molecular factors associated with drought tolerance in *Hevea* germplasm accessions

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BIOCHEMISTRY
(Faculty of Science)

By

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under the supervision and guidance of

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I hereby declare that the thesis entitled **"Studies on physiological, biochemical and molecular factors associated with drought tolerance in *Hevea* germplasm accessions"** is an authentic record of original research carried out by me under the supervision and guidance of Dr. Molly Thomas, Principal Scientist (Retd.), Crop Physiology Division, Rubber Research Institute of India, Kottayam-9 and Dr. K. Annamalainathan, Joint Director, Crop Physiology Division, Rubber Research Institute of India, Kottayam-9 in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY of the Mahatma Gandhi University, Kottayam and no part of this work has been presented for any degree, diploma or any other similar titles of any university.

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Smitha M. Xavier

Dedicated to my Family.....

CONTENTS

ABBREVATIONS

A	:	CO_2 assimilation rate
ABA	:	Abscisic acid
AC	:	Acre
ANOVA	:	Analysis of variance
APX	:	Ascorbate peroxidise
AsA	:	Ascorbic acid
ATP	:	Adenosine triphosphate
CAT	:	Catalase
cDNA	:	Complementary DNA
Ct	:	Threshold cycle
DEPC	:	Diethyl pyrocarbonate
DNA	:	Deoxyribonucleic acid
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
GAPDH	:	Glyceraldehyde 3 phosphate dehydrogenase
GB	:	Glycine betaine
g_s	:	Stomatal conductance
GSH	:	Glutathione
H. brasiliensis	:	*Hevea brasiliensis*
kb	:	Kilobase
LEA	:	Late Embryogenesis abundant
MAPK	:	Mitogen Activated Protein Kinase
mRNA	:	Messenger RNA

MT	:	Mato Grosso
NaCl	:	Sodium Chloride
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NR	:	Natural rubber
PAGE	:	Polyacrylamide gel electrophoresis
P_N	:	Net photosynthetic rate
POD	:	Peroxidase
Pro	:	Free proline
PSII	:	Photosystem II
qPCR	:	Quantitative PCR
RNA	:	Ribonucleic acid
RO	:	Rondonia
ROS	:	Reactive oxygen species
RQ	:	Relative quantification
RRII	:	Rubber Research Institute of India
RRIM	:	Rubber Research Institute of Malaysia
RT-PCR	:	Reverse transcription PCR
RWC	:	Relative water content
SDS	:	Sodium dodecyl sulphate
sHSP	:	Small heat shock protein
SOD	:	Superoxide dismutase
TCA	:	Trichloroacetic acid
TEMED	:	Tetra methyl ethylene diamine
TF	:	Transcription factor
WUE	:	Water use efficiency

Units

°C	:	Degree Celsius
g	:	Gram(s)
hr	:	Hour(s)
kDa	:	Kilo dalton
l	:	Litre(s)
M	:	Molar
min	:	Minutes
mol	:	Mole(s)
nM	:	Nanomole
rpm	:	Revolutions per minute
v/v	:	Volume per volume
w/v	:	Weight per volume

Prefixes

k	:	Kilo
m	:	Milli
μ	:	Micro

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Plants being frequently exposed to different adverse environmental stresses due to their sessile nature have evolved various defence mechanisms at multiple levels to overcome stress conditions. It is imperative to dissect various regulatory mechanisms of stress responses and identify the key factors involved in this process to develop stress tolerant plants. Drought is amajor abiotic stress which negatively influences plant growth, development and productivity. In the changing climate and global warming scenario, development of new cultivars which are more resistant to the environmental stress conditions is essential.

Hevea brasiliensis, the major commercial source of natural rubber is widely cultivated in the traditional rubber growing regions of Kerala and Kanyakumari District of Tamil Nadu, where the climatic conditions are optimum for its growth and productivity. Being an important industrial raw material, global demand of natural rubber is found increasing. But the constraints in the availability of cultivable land in traditional rubber growing regions necessitate the extension of rubber cultivation to non-traditional areas including drought prone regions. For this, the identification of genotypes which can withstand extreme climatic conditions is a prime requirement. Germplasm collections are potential sources of genetic diversity and provide valuable resource of genes conferring tolerance to various biotic and abiotic stresses. Proper screening and characterization of germplasm collections provide resource materials for plant breeding program and crop improvement. Also screening of germplasm accessions for intrinsic drought tolerance traits will help in identifying suitable clones/accessions for the non traditional drought prone regions. The evaluation of *Hevea* germplasm accessions using physiological, biochemical and molecular

factors will also facilitate the development of early evaluation screening tools, so that it will be easier to identify genotypes with desired characters.

This study was initiated with an objective to evaluate and screen *Hevea* germplasm accessions using physiological, biochemical and molecular factors which are associated with drought tolerance. Also the study aimed at identifying a few phenotypic and genotypic markers for early screening of *Hevea* for drought tolerance. An initial screening has performed and germplasm accessions were short listed and ranked on the basis of physiological and biochemical parameters. The shortlisted germplasm accessions were further subjected to detailed investigation by biochemical as well as molecular trait analyses. The studies on antioxidant defense system comprising of antioxidant enzymes, secondary metabolites and osmolytes showed their strong association in enhancing drought tolerance especially in tolerant clones/germplasm accessions. The presence of 23 kDa chloroplast stress protein also showed differential expression under drought stress. An *in vitro* analysis of genomic DNA showed significant fragmentation under high light induced osmotic stress in susceptible clones/ accessions.

The molecular studies involved the qPCR analyses of fourteen drought related genes. The transcripts *NAC tf, LEA5, WRKY tf, DNA bp, ERF, MAPK, TIP* and *HbsHSP23.8* were found drought responsive and showed an upregulation under drought stress in *Hevea* clones/germplasm accessions. Among these, the transcripts *NAC tf, LEA 5, WRKY tf, DNA bp* and *ERF* showed significant upregulation in tolerant clones/accessions alone, thus showed their strong association in enhancing drought tolerance.

The accessions RO 3261 and AC 612 can be selected as potential drought tolerant accessions with relatively better drought tolerance capacity, on the basis of physiological, biochemical and molecular analyses, and they

can be further used for crop improvement programmes in *Hevea*. The transcripts *NAC tf, LEA 5, WRKY tf, DNA bp* and *ERF* showed strong association with drought tolerance and hence can be used as markers for early screening of *Hevea* genotypes for drought tolerance.

Keywords: Antioxidant enzymes, drought tolerance, gene expression, germplasm accessions, *Hevea brasiliensis*, qPCR.

Chapter 1
Introduction

Hevea brasiliensis Muell. Arg. (Para rubber tree), commonly known as "rubber tree" is a tall deciduous perennial tree belongs to the family Euphorbiaceae. Among 2500 rubber producing plant species, *Hevea brasiliensis* is the only successful commercial source of natural rubber (NR) (Mooibroek and Cornish, 2000; Hayashi, 2009). It accounts for more than 99% of the world's natural rubber production. The other two species which are known to produce rubber with high molecular weight are guayule (*Parthenium argentatum*) and the Russian dandelion (*Taraxacum koksaghyz*) (Beilen and Poirier, 2007). The genus *Hevea* comprises of 11 inter-crossable species. *Hevea brasiliensis* is a native of the Amazon rain forest of South America where a wet equatorial climate exists (Gonçalves *et al.*, 2009).

Natural rubber (chemically, *cis*-1,4-polyisoprene) is an important industrial raw material for more than 40,000 products. It is produced in the milky cytoplasm (latex) of specialized cells called laticifers. NR consists of 94% *cis*-1,4-polyisoprene and 6% proteins, minerals, carbohydrates and lipids (Sakdapipanich, 2007; Hayashi, 2009). NR has superior elasticity, abrasion resistance, resilience, efficient heat dispersion and impact resistance compared to synthetic rubber, because of the presence of high *cis*-bond content and high molecular weight (more than $1x10^6$ Da). A major quantity of natural rubber produced is consumed by the automobile tyre industry due to its higher strength, low heat build up and better resistance to wear and flex cracking (Hayashi, 2009). Natural rubber is ecofriendly when compared to synthetic rubber. It sequesters significant quantity of atmospheric CO_2 in tree biomass therefore, involved in emission reduction. In addition, rubber wood

is a major source of timber and it has high environmental acceptability both in domestic and international markets.

Hevea brasiliensis grows well in a warm humid climate (21–35°C) with a fairly distributed annual rainfall ofnot less than 200 cm. It is widely cultivated in south-east Asian countries like China, Thailand, Malayasia, Indonesia, India, Sri Lanka and Vietnam. In India, rubber is cultivated in about 8.11 lakh hectares with an annual production of about 5.62 lakh metric tonnes (Indian Rubber Statistics, 2016). The traditional rubber growing regions in India are Kerala state and Kanyakumari district of Tamil Nadu, where the climatic conditions are optimal and conducive for its cultivation. The increased global demand for natural rubber and the constraints in the availability of land in the traditional area forced its cultivation to be extended to non-traditional regions which include drought prone areas such as North Konkan, certain parts of Karnataka, Odisha, Madhya Pradesh and low temperature prevailing areas of north-eastern states. The drought prone non-traditional regions experience soil and atmospheric drought, high temperature combined with high intensity of solar light and low relative humidity (RH) during summer season, severely affects the growth and productivity of the crop (Jacob *et al.*, 1999). Drought stress concomitant with high intensity of sunlight inflict damage to the green leaves resulting in severe inhibition of photosynthesis (Devakumar *et al.*, 2002; Annamalainathan *et al.,* 2010). The ability of a plant to withstand water deficit is associated with numerous inherent traits that contribute to drought tolerance. An understanding of these traits which are more directly related to drought tolerance helps in easy identification of genetic materials suitable for such areas.

Germplasm collection provides original materials for crop improvement and plant breeding program. Because of genetic diversity as

well asthe possible occurrence of desirable genes for biotic and abiotic stress tolerance, germplasm collections are useful targets for plant breeders and other biologists. Recently, many of germplasm are being lost worldwide due to invasion of foreign species, habitat destruction, urbanization and reliance on a few high yielding varieties. The maintenance of germplasm of agricultural crops is very important. It helps in better understanding of the properties and performance of the crops, up to genomic level. Proper characterization and evaluation of germplasm is necessary to use them in breeding programs, without which, valuable genetic variations cannot be used in crop improvement programmes effectively. Understanding the characteristics and the magnitude of variability of important traits existing among plant genetic materials is vital for the effective utilization of such materials for breeding purposes. Conventional plant breeding depends on various phenotypic traits as markers for genetic analyses and cultivar identification (Lam *et al.*, 2012). The development of molecular and biochemical markers help researchers to identify genotypes and to assess and exploit the genetic variability (Whitkus *et al.*, 1998).

Hevea brasiliensis, was introduced to countries in South East Asia and Africa during late 19th century. The credit for domestication of *Hevea brasiliensis,* goes to Sir Henry Alexander Wickham, who is known as the father of natural rubber cultivation. He successfully transported 70,000 rubber seeds from the Amazonian forest to the Royal Botanical Gardens at Kew, England in 1876 (Lane, 1953; Wycherly, 1968), which is one of the most far reaching and successful introductions in the history of plant breeding. The beginning of rubber cultivation in India dates back to 1878 with the importing of rooted cuttings from Royal Botanic Gardens, Heneratgoda, Ceylon (Dean, 1987; Thomas and Panikkar, 2000).

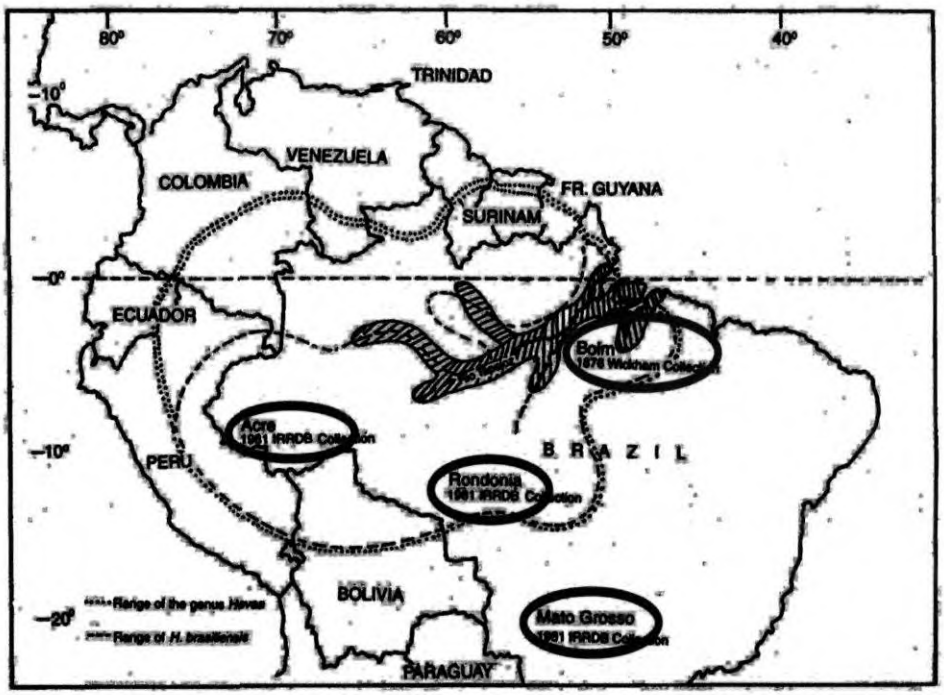

Fig.1.1. Distribution of genus *Hevea* in its primary centre of origin.

The genetic base of *Hevea* in South East Asia was very narrow, restricted to a less number of seedlings originally collected from a minuscule of the genetic range in Brazil referred to as the "Wickham base"(Simmonds, 1989). In 1981, the International Rubber Research and Development Board (IRRDB) conducted an expedition for collecting wild germplasm from the Amazonian forests of Brazil and this resulted in the broadening of genetic base of *Hevea* in major rubber growing countries. The wild *Hevea* germplasm accessions were collected from three western provinces of Brazil namely, Acre, Rondonia and Mato Grosso, in 16 different districts and in 60 different locations and it is referred as IRRDB'81 collection (Fig. 1.1). As a result, a total of 63,768 seeds, 1,413 meters of budwood from 194 high yielding trees and 1,160 seedlings were collected (Simmonds, 1989; Onokpise, 2004). India also received a share of this valuable genetic material

and at present 4548 wild *Hevea* germplasm accessions are being maintained at Rubber Research Institute of India (RRII) by *ex situ* conservation.

The germplasm collection provides a valuable resource of genes conferring tolerance to various biotic and abiotic stresses. In rubber tree, high latex yield is a major objective of breeding programs along with disease resistances and tolerance to abiotic stresses such as high and low temperatures, wind damage and moisture deficit. Screening and characterization of germplasm accessions for intrinsic drought tolerance traits will help in identifying suitable accessions for the non traditional drought prone regions. In the present changing climate and global warming scenario, identification of suitable clones/accessions with traits that confer environmental stress tolerance are essential for the traditional areas, as well. Several *Hevea* germplasm accessions have been identified with moderate to good drought tolerance potential (Nair *et al.*, 2005; Mercy *et al.*, 2010). The characterization of *Hevea* germplasm using molecular and biochemical markers will contribute to the knowledge of genetic relationships not only among wild accessions but also between accessions of wild and cultivated gene pool, and hence help to facilitate the breeding programs (Lam *et al.*, 2012). Also, the selected accessions will serves as desirable genetic resources for crop improvement programme.

In plants, the growth and development as well as yield attributes are greatly influenced by various environmental stress factors such as drought, extreme temperatures, high light, UV radiation, salinity, heavy metals, pathogen infection *etc.* Plant response to abiotic stress depends on various factors including the developmental stage, age, intensity of stress, plant species and the genotype (Le Gall *et al.*, 2015). To survive under stress conditions, plants have developed diverse complex mechanisms which include stress perception, signal transduction, transcriptional activation of

stress responsive target genes and synthesis of stress-associated proteins and other defence molecules. These adaptations help plants to cope with adverse environmental conditions through biochemical and physiological manifestations. An understanding of stress tolerance by studies on physiological, biochemical and molecular responses and identification of potential stress responsive genes involved in plant abiotic stress tolerance will give insight into the underlying tolerance mechanisms (Hasanuzzaman *et al.,* 2013; Wang *et al.,* 2016). This understanding is critical for the development of new varieties of crops, which are better adapted to adverse climatic conditions.

The water deficit caused by drought reduces growth and development in plants; thereby negatively affecting crop productivity worldwide. Drought is a meteorological term which is commonly defined as a period with reduced rainfall and limited water availability in the soil. Also, the rise in temperature leads to continuous loss of water from the atmosphere by transpiration or evaporation (Jaleel *et al.,* 2009; Singh and Laxmi, 2015). Plants have developed different mechanisms at morphological, physiological, biochemical, cellular and molecular levels which lead to adaptation and survival during periods of water deprivation (Fang *et al.,* 2015). Drought stress drastically affects various physiological activities in plants. The reduction of water content in plants results in diminished leaf water potential and turgor loss, stomatal closure and retarded growth. Other effects of drought include the reduction of photosynthesis, osmotic stress-imposed constraints on plant processes, and interference with nutrient availability as the soil dries (Huang *et al.,* 2012; Marco *et al.,* 2015). Drought stress progressively decreases CO_2 assimilation rates due to reduced stomatal conductance leading to mesophyll restrictions (Sheoran *et al.,* 2015). It decreases root proliferation, leaf size, extension of stems and disturbs plant

water relations and reduces water use efficiency (WUE). It also disrupts photosynthetic pigments and reduces the gas exchange leading to a reduction in plant growth and productivity (Anjum *et al.*, 2011).

Plants when exposed to excess light energy, which the leaves cannot dissipate or convert into biochemical energy, then there is a redirection of photon energy and this leads to the production of reactive oxygen species (ROS). The final result is substantial oxidative damage and the so-called oxidative stress.The important ROS are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$). ROS are generally produced in the cell organelles like chloroplast, mitochondria and peroxisomes (Apel and Hirt, 2004; Mittler *et al.,* 2002) as a result of metabolic activity, but is strictly controlled by scavenging systems. ROS also act as signalling molecules that can trigger cell responses. Excessive levels of ROS are responsible for various stress induced damages to macromolecules and cellular structure including RNA and DNA, enzymes, proteins, membrane lipids and ultimately leading to celldeath (Foyer and Noctor, 2005). Plants have evolved a complex antioxidant defence mechanism comprising of enzymatic and non enzymatic molecules to prevent oxidative damage due to ROS. The well known enzymatic antioxidants includes super oxide dismutase (SOD), catalase (CAT), and peroxidases like glutathione peroxidases (GPX) and ascorbate peroxidase (APX). In addition, numerous low molecular weight nonenzymatic antioxidant compounds such as glutathione, flavonoids, alkaloids, carotenoids and polyamines are also involved in defence against ROS (Liu *et al.,* 2011; Talbi *et al.,* 2015). Furthermore, osmotic adjustment *via* accumulation of low molecular weight water-soluble compounds known as osmolytes like proline, glycine betaine, sugars, inositols *etc.* play an

important role in stabilizing proteins and enzymes as well as in maintaining cell turgor (Krishnan *et al.,* 2008 ; Rodziewicz*et al.*, 2014).

In order to survive under water deficit stress conditions, plants respond by alteration in expression level of stress associated genes and regulate them through various complex transcriptional networks (Singh and Laxmi, 2015). A large number of drought responsive genes including dehydrins, heat shock proteins, transcription factors, late embryogenesis abundant proteins and aquaporins have been identified (Kaur and Asthir, 2017). Plant cells perceive stress stimuli *via* different sensors which in turn activate signalling pathways involving plant hormones, signal transducers, secondary messengers and transcriptional regulators (Danquah *et al.,* 2014; Gilroy *et al.,* 2014). Stress signals combine together to regulate stress inducible genes that encode proteins and enzymes directly involved in stress responsive metabolism (Casaretto *et al.,* 2016). Therefore, the elucidation of a molecular pathway for plant response to stress is essential to understand how plants respond and adapt themselves to various abiotic stress conditions. Also this understanding is important for crop improvement programme for drought tolerance and other abiotic stress using molecular techniques. Drought triggers the production of a phytohormone, abscisic acid (ABA), which in turn leads to stomatal closure and induces expression of stress associated genes. Drought inducible genes exist as ABA-independent and ABA-dependent regulatory systems (Yamaguchi-Shinozaki and Shinozaki, 2006). A large array of abiotic stress responsive genes has been identified in plants using molecular techniques such as microarray and transcriptome analyses, (Fowler and Thomashow, 2002; Nakashima *et al.*, 2007). These genes play a major role in the protection of the cells from stress by the production of different enzymes and proteins (functional proteins) and also in regulating signal transduction and gene expression during stress response

(regulatory proteins) (Lata and Prasad, 2011; Nakashima *et al.*, 2012). Identification and characterization of the key genes associated with plant stress responses is an essential prerequisite for engineering stress tolerant transgenic crops (Todaka *et al.*, 2015). Studies in *Hevea* clones revealed altered level of expression of drought responsive genes involved in imparting drought tolerance (Thomas *et al.*, 2011; 2012; Sathik *et al.*,2012; 2018, Luke *et al.*, 2015; 2017).

Development of genotypes with enhanced stress tolerance and wider adaptability with climate resilience traits is a cost-effective and eco-friendly approach to cope with drought stressed scenarios (Jha *et al.*, 2014). Due to constraints in the availability of land in traditional rubber growing areas, cultivation of *Hevea brasiliensis* is being extended to non traditional areas which experience extreme climatic conditions. Drought is the major climatic factor which affects crop productivity and also it is the factor that limits the performance of *Hevea brasiliensis* in non-traditional areas. Rubber plants, being a perennial crop have a long breeding cycle and hence early identification of suitable genotypes with desired traits is always a prerequisite. Studies have been conducted for screening of *Hevea* germplasm accessions for yield (Rao *et al.*, 2006; 2011) and abiotic stress tolerance such as cold (Rao *et al.*,2013; 2016) and drought (Nair *et al.*, 2005; 2011; Mercy *et al.*, 2010; 2013) tolerance. In this study, a detailed investigation has been conducted on physiological, biochemical and molecular factors associated with drought tolerance in *Hevea* germplasm accessions. An understanding of these characters which are more directly related to drought tolerance help in early identification of drought tolerant varieties. Furthermore, the identification of key candidate genes associated with drought tolerance can be used in marker assisted selection (MAS) programme which would help

the breeders to either develop crops with improved stress tolerance or to use them as markers for screening drought tolerant genotypes.

Objectives of the study

- To analyse the response of selected germplasm lines to drought stress.
- To assess the level of oxidative stress defence components including antioxidants, antioxidant enzymes and osmolytes under drought stress.
- To analyse the expression of drought stress responsive genes and proteins.
- Identification of a few drought tolerant genotypes which would be useful in developing improved tolerant *Hevea* clones.
- To pick out specific genotypic/phenotypic markers for screening drought tolerance in *Hevea*.

Chapter 2
Review of Literature

2.1. *Hevea brasiliensis*

Hevea brasiliensis Muell. Arg. (Para rubber tree), belonging to the family Euphorbiaceae, is the major commercial source of natural rubber (NR). Rubber molecules are produced in the latex vessels or laticifers of rubber tree. The latex, a cytoplasmic component of the laticifers, expels from the laticifers upon controlled wounding on the bark called tapping (Gomez and Moir, 1979; Li *et al.,* 2010). Natural rubber is chemically *cis*-1,4-polyisoprene which is used as an industrial raw material for the manufacture of more than 40,000 products. NR from *Hevea brasiliensis* is superior in many applications compared to synthetic rubber (Van Beilen and Poirier, 2007). No other synthetic substitute has comparable physical qualities like elasticity, resilience, resistance to high temperature, abrasion resistance, efficient heat dispersal and malleability at low temperature (Davis, 1997). These properties make NR difficult to be replaced by synthetic rubber in several applications such as surgical gloves, high performance tyres for trucks, aircrafts, racing cars and various engineering and consumer products (Mooibroek, 2000).

Natural rubber is biosynthesized in over 2000 plant species belonging to 300 genera of seven families *viz.*, Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae (Cornish *et al.,* 1993). The genus *Hevea* is basically consists of 11 inter-crossable species: *H. brasiliensis*, *H. camporum*, *H. guianensis*, *H.benthamiana*, *H. pauciflora*, *H. spruceana*, *H. microphylla*, *H. rigidifolia*,

H. nitida, *H. camargoana* and *H. paludosa* (Schultes, 1990; Priyadarshan and Gonçaalves, 2002; Clement-Demange *et al.*, 2007). *H. brasiliensis* remains as the only cultivated species of natural rubber because of its abundance in latex, high quality, high molecular weight polymer and convenience of harvesting.

Hevea brasiliensis is native to rain forests of the tropical region of the Great Amazonian basin of South America, which lies between equator and 15° S. This area is characterized by a wet equatorial climate ideal for rubber cultivation (Strahler, 1969) with 2000-4000 mm rainfall distributed across 100-150 rainy days per annum (Pushparajah, 1977), temperature of around 28 ± 2°C with a diurnal variation of about 7°C (Barry and Chorley, 1976) and sunshine hours of about 2000 hrs/per year at the rate of 6 hrs per day in all months (Pushparajah, 1977; Yew, 1982; Ong *et al.*, 1998). Rubber cultivation was introduced to South-east Asian countries by the end of 19th century (Limkaisang *et al.*, 2005). In India traditional rubber growing region attributes ideal and conducive agro climatic conditions for rubber cultivation where it resembles its natural habitat (Rao and Vijayakumar, 1992).

2.1.1. *Hevea* germplasm accessions

The introduction of the rubber trees into Asia began with the transfer of 70,000 seeds from the Amazonian forest to the Royal Botanical Gardens at Kew, England by Henry Wickham in 1876 (Wycherly, 1968). The development of the entire high-yielding cultivars of rubber trees through breeding programs in Southeast Asia was originated from this narrow genetic base known as "Wickham base". With the intention of broadening the original gene pool, the International Rubber Research and Development Board (IRRDB) made an expedition to Acre, Rondonia and Mato Grosso provinces of Brazil to collect wild germplasm in 1981, and distributed to various member countries including India for further conservation and

evaluation. India received a total of 4548 accessions which have been established in traditional and non-traditional areas in conservation-cum-source bush nurseries (Rao *et al.*, 2013). Since the mid of 1980's efforts have been put forward for the characterization, evaluation and utilization of these accessions (Mydin *et al.*, 2014). The germplasm accessions were evaluated for traits of interest for subsequent utilization in breeding programmes (Sankariammal *et al.*, 2010; Sankariammal and Mydin, 2011; Mydin *et al.*, 2012). Nineteen accessions were identified for better timber yield and four accessions for good quality timber (Reghu *et al.*, 2005; 2011). The wild accessions were also evaluated for growth and yield performances (Das*et al.*, 2014). Screening against biotic stress resulted in the identification of several accessions which are tolerant to *Phytophthora, Corynespora, Colletotrichum* and *Oidium* (Reghu *et al.*, 2011; Mydin *et al.*, 2011). Studies were also performed for the screening of these accessions for yield (Rao *et al.*, 2006; 2011) with better growth rate, increased number of lactifers and promising test tap yield at immature phase (Reghu *et al.*, 2012). Screening for abiotic stress tolerance were conducted for cold (Rao *et al.*, 2012, 2016) and drought (Nair *et al.*, 2005; 2011; Mercy *et al.*, 2010; 2013) and identified of a few accessions as potential parents for drought tolerance related breeding programmes (Mydin *et al.*, 2011).

2.2. Abiotic stress in plants

The growth and development of plants are greatly influenced by various environmental factors such as drought, salinity, flooding, cold, heat, UV radiation, heavy metals, *etc.* which are known as abiotic stresses. An increase or decrease in intensity of these environmental factors from their optimum levels adversely affect the overall plant growth as well as crop yield (Gao *et al.*, 2007; Shinozaki and Yamaguchi-Shinozaki, 2007; Bansal *et al.*, 2012). As an adaptive mechanism to combat with diverse

environmental cues, various signalling cascades get activated in the plant cell leading to altered cellular function and response (Singh *et al.,* 2015). Abiotic stresses which lead to shortage of cellular water such as drought, temperature extremes and high soil salinity are responsible for greater crop losses (Hare *et al.,* 1998). Under stressful conditions, plants respond by activating different tolerance mechanisms at multiple levels of organization such as morphological, anatomical, tissue and molecular levels by adjusting the membrane system as well as cell wall architecture and also by altering the cell cycle, cell division and by metabolic tuning (Atkinson *et al.,* 2012). Under mild stress, early plant response mechanisms prevent or mitigate cellular damage caused by the stress and re-establish homeostatic conditions and thus continue the growth of plants (Peleg *et al.,* 2011). Abiotic stress activates a multigene response that leads to changes in various proteins and primary and secondary metabolite accumulation (Rodziewicz *et al.,* 2014). Products of those genes may function in stress response and tolerance at the cellular level. Proteins may involve in biosynthesis of osmoprotectants, detoxification of enzyme systems and functions as chaperones towards direct protection from stress. In addition, activation of regulatory proteins such as transcription factors (TF), kinases and protein phosphatases and signalling molecules are essential in the concomitant regulation of signal transduction and stress-responsive gene expression (Wang *et al.,* 2009; Krasensky and Jonak, 2012).

2.2.1. Drought stress

Drought is a severe environmental stress that significantly restricts plant growth, development and productivity (Boyer *et al.,* 1982; Rivero *et al.,* 2007; Surendran *et al.,* 2017). Drought stress occurs when the available water in the soil is reduced to such critical levels and an atmospheric condition adds to continuous loss of water. The drought stress due to soil

moisture deficit is usually accompanied by high temperatures and solar radiation (Xu *et al.,* 2010). In plants, the water deficit caused by drought reduces growth and development, arising from the reduction of water content, diminished leaf water potential and turgor loss, closure of stomata, and decrease in cell enlargement and growth (Jaleel *et al.,* 2009). In addition to decreased plant growth and crop productivity, drought leads to changes in cell membrane, reduced photosynthesis, osmotic stress induced changes in various plant processes, decrease in biomass production, reduced yield and decreased nutrient availability from the soil (Huang *et al.,* 2012; Binott *et al.,* 2017). Under prolonged exposure to drought stress, plants exhibit leaf rolling followed by wilting and photo-bleaching of pigments that may eventually lead to death of the plant (Sahoo *et al.,* 2013). Reproductive stages, i.e., flowering and seed development are greatly influenced by dehydration stress (Samarah and Alqudah, 2011).

Drought stress tolerance is seen in all plants but its extent varies from species to species depending on the stress tolerance/adaptation mechanisms. Dehydration stress generates reactive oxygen species (ROS) in chloroplasts and mitochondria, which are toxic to the plants at high concentrations. The antioxidant enzymes as well as non enzymatic antioxidants play major roles in plant drought tolerance responses (Apel and Hirt, 2004). The synthesis of osmolytes increases in plants during stress conditions and their accumulation contribute to the protection of enzymes and membrane integrity as well asinthe regulation of osmotic pressure (Chen and Murata, 2011). The plant hormone abscisic acid (ABA) is produced under dehydration stress conditions and this induces stomatal closure and signalling of expression of various stress related genes (Yang *et al.,* 2011).At the molecular level, several drought stress responsive genes which code for proteins having either metabolic or regulatory functions have been identified and characterized in

various crops. The metabolic or functional proteins are mainly involved in protecting the cell from stresses such as detoxification, ion transporter, water channel, heat shock protein (HSP) and late embryogenesis abundant (LEA) protein (Joshi *et al.,* 2016). Various regulatory proteins including transcription factors (tfs), protein kinases and protein phosphatases regulate signal transduction as well as expression of genes in stress responses and thus protect the plants from drought stress (Hennig, 2012; Wani *et al.,* 2013).

2.2.2. Physiological and biochemical aspects of water deficit stress

Drought stress drastically affects various physiological traits in plants. Among these physiological responses, closing of stomata to avoid further water loss is a dominant and earliest response identified under drought stress conditions and it is vital for prevention of desiccation as well as CO_2 acquisition (Yin *et al.,* 2005; Medici *et al.,* 2007). Stomatal closure leads to decrease in internal CO_2 concentration (Ci) and inhibition of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme activity and ATP synthesis which inturn cause decrease of net photosynthetic rate under drought stress (Cornic, 2000; Dulai *et al.,* 2006). Stomatal closure in response to water deficit stress occurs due to decreased leaf turgor and atmospheric vapour pressure deficit (VPD) along with root-generated signals (Chaves *et al.,* 2009). A schematic representation of physiological responses induced in plants during drought stress depicted by Zingaretti *et al.,* (2013) is given in Fig 2.1.

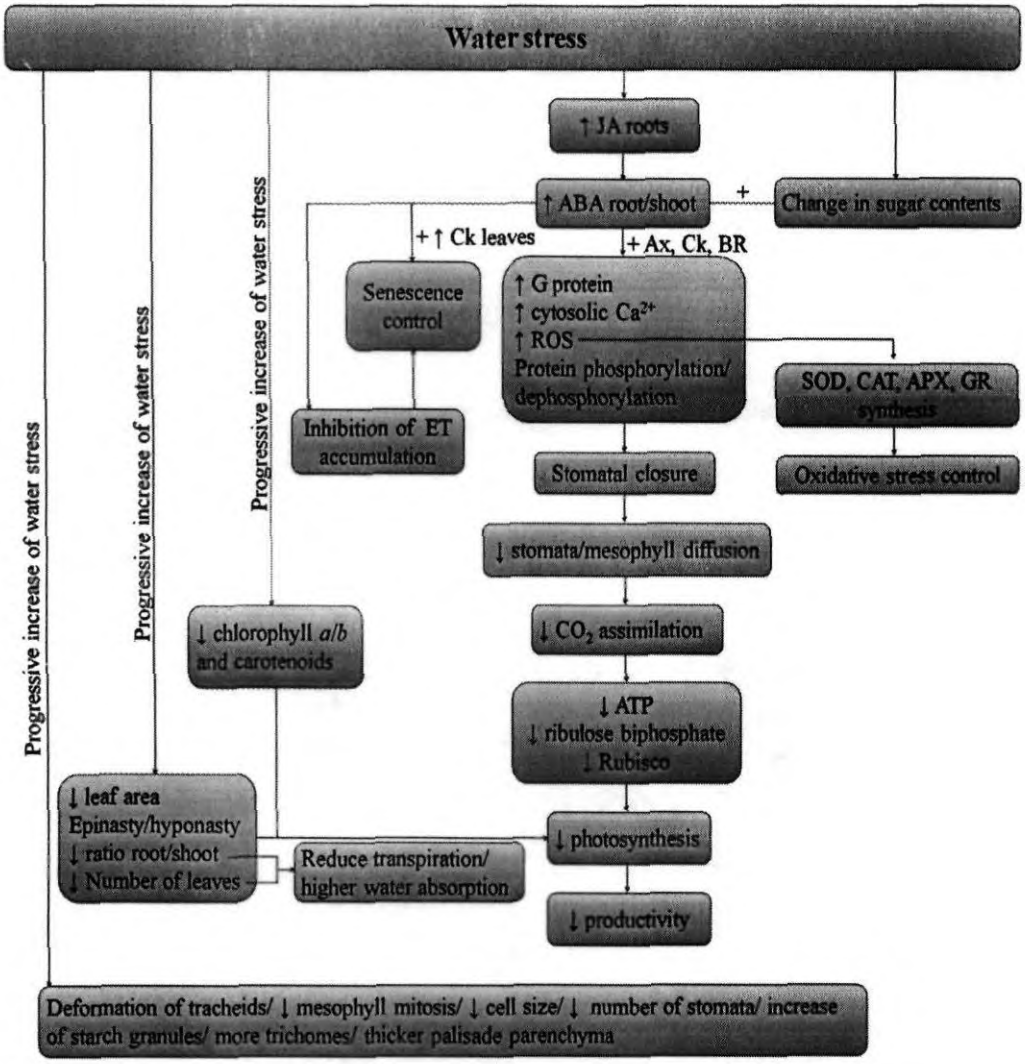

Fig.2.1. Physiological responses induced in plants under drought stress (Zingaretti *et al.*, 2013)

The rate of photosynthesis usually decreases during exposure to various stresses in higher plants (Chaves *et al.*, 2009). Reduced inhibition of photosynthesis under drought stress is of great importance for drought tolerance (Zlatev and Yordanov, 2004). The effect of drought stress on CO_2 assimilation rate (A), transpiration rate (E) and water use efficiency (WUE) has been investigated in many crops such as *Zea mays* (Ashraf *et al.*, 2007), *Brassica napus* L. (Kauser *et al.*, 2006) and mungbean genotypes (Ahmed *et*

al., 2002). Drought tolerant species maintain better water use efficiency by reducing the loss of water (Anjum *et al.,* 2011). It has been reported that photosystem II (PSII) plays an important role in the photosynthetic response to environmental stresses in higher plants (Baker *et al.*, 2004). The PSI and PSII reaction centres are the major generation sites for ROS in chloroplast (Asada, 2006). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is an enzyme involved in the CO_2 incorporation into organic compound and the competing photorespiratory carbon oxidation (Spreitzer and Salvucci, 2002). A decrease in Rubisco activity was identified as one of the non-stomatal reasons for lowering the photosynthesis rate (Flexas *et al.,* 2006).

Photosynthetic pigments play a major role in harvesting light and production of reducing powers. Drought stress causes substantial damage to photosynthetic pigments, and also leads to deterioration of thylakoid membranes (Anjum *et al.*, 2011). Alterations occur in the level of photosynthetic pigments in drought stressed plants, showing reduced or even no pigmentation (Mafakheri *et al.*, 2010; Din *et al.*, 2011). The content of both chlorophyll *a* and *b* changes under drought stress (Farooq *et al.,* 2009) and this directly affect plant biomass production. A decrease in photosynthetic pigments will drive a cut down in energy consumption and carbon demand for chlorophyll synthesis. Studies on chlorophyllase and peroxidase revealed that the decrease in the level of chlorophyll is attributed to its accelerated breakdown rather than its slow synthesis (Harpaz-Saad *et al.*, 2007; Kaewsuksaeng, 2011). Other pigments such as carotenoids play essential role in the antioxidant defense system under stress conditions. It also functions as an accessory pigment for photosynthesis and their concentration can be reduced as part of plant drought response (Chaves *et al.,* 2009; Silva *et al.,* 2010; Zingaretti *et al.,* 2013). The effect of drought stress on chlorophyll and carotenoid content has been investigated in cotton

(Mssacci, 2008) and *Catharanthus roseus* (Jaleel *et al.,* 2008). Under severe dessication stress the chlorophyll content decreased to a significant level in *Vaccinium myrtillus* (Tahkokorpi *et al.*, 2007) and in sunflower plants (Kiani *et al.,* 2008). An *in vitro* drought study in *Hevea* using polyethylene glycol (PEG) and light showed marked decrease in chlorophyll content of leaves and enhanced chlorophyll bleaching under stress (Nair *et al.*, 2011).

The Fv/Fm ratio determines the maximum quantum efficiency of PSII, the rate of linear electron transport and also it provides an indication of overall photosynthetic capacity (Tang *et al.*, 2007; Balouchi, 2010). The photosystem efficiency (Fv/Fm) ratio is a parameter which permits the detection of any damage to PSII and possible photoinhibition. Water deficit stress affects Fv/Fm and decreases the electron transport rate and the effective quantum yield of photosystem II (Ahmed *et al.,* 2002). Alterations in the proportion of photochemical and energy-dependent quenching lead to change of fluorescence kinetics under water deficit stress (Zlatev and Yordanov, 2004). Analysis of chlorophyll fluorescence and measurement of the Fv/Fm ratio under drought stress can be helpful in determining damage to light reaction systems in photosynthetic mechanisms.

Relative water content (RWC), leaf water potential (Ψw), rate of transpiration, stomatal resistance, leaf temperature and canopy temperature are the major attributes that influence plant water relations.Flower and Ludlow (1987) suggested RWC as an alternative measurement to study the plant water status which reflects the metabolic process in the tissues and a meaningful index for drought tolerance. In higher plants the photosynthetic rate was found decreased as the relative water content and leaf water potential decreases (Lawlor and Cornic, 2002). A decrease in RWC has been reported in a wide variety of plants in response to drought stress. When plants are subjected to drought, leaves exhibit large reductions in RWC and

water potential (Nayyar and Gupta, 2006). Also dehydration stress substantially decreases leaf water potential, transpiration rate and relative water content, with a concomitant increase in leaf temperature in plants (Siddique *et al.,* 2001). Studies reported that plant species maintaining better RWC under osmotic stress will be less susceptible to low water potential, and thus retain their growth and productivity (Joshi and Karan, 2013). It was observed that soil moisture surrounding the plant root system also will support the crop growth primarily (Tron *et al.,* 2015).

Epicuticular wax, a major component of cuticle, plays important role in plant abiotic and biotic stress tolerance as well as in defence mechanism against high temperature, ultraviolet radiation, high salinity, low temperature, bacterial and fungal pathogens and insects (Lee and Suh, 2015; Xue *et al.,* 2017). The surface of aerial plant parts, such as stem and leaves are covered by an outermost thin hydrophobic layer called cuticlar wax. It protects plants from non-stomatal water loss (Riederer and Schreiber, 2001), UV radiation (Reicosky and Hanover, 1978; Solovchenko and Merzlyak, 2003), insect attack (Eigenbrode and Espelie, 1995) and pathogen infection (Barthlott and Neinhuis, 1997). During drought conditions, epicuticular wax load (EWL) increases, thereby minimising cuticular transpiration and maximising leaf water retention (Zhang *et al.,* 2005; Goodwin and Jenks 2005; Seo and Park, 2011). A positive correlation between EWL and residual transpiration rate (RTR) had previously been demonstrated (Premachandra *et al.,* 1992; Chakhchar *et al.,* 2015). Epicuticular waxes were reported to enhance the plant resistance to environmental stresses by helping leaves in water retention (Goodwin and Jenks, 2005). The concentration of epicuticular wax deposition on leaves was often related to the degree of drought tolerance and transpiration rate (Zhang *et al.,* 2005; Kim *et al.,* 2007; Gonza´lez and Ayerbe, 2010).

2.2.3. Plant defence against reactive oxygen species (ROS) under drought

In plants various environmental constraints such as drought, salinity, flooding, heat and cold stresses results in enhanced production of reactive oxygen species (ROS) which leads to oxidative damage (Mittler, 2002). Plants being sessile organisms have to cope with drought stress at least at some point in their life cycle. During water deficit stress, enhanced ROS production occurs in three major cellular compartments including chloroplasts, peroxisomes and mitochondria. Other important sites of ROS production in plants are cytoplasm, endoplasmic reticulum and in apoplast at plasma membrane level (Gill and Tuteja, 2010). Prolonged exposure to drought stress will inevitably result in oxidative damage due to the excessive production of ROS (Smirnoff, 1993). They can oxidize different cellular components like DNA and RNA, proteins and lipids, and their unrestricted oxidation will ultimately results in cell death (Dismukes *et al.,* 2001; Vellosillo *et al.,* 2010; Karuppanapandian *et al.,* 2011). Under normal conditions reactive oxygen species are continuously produced in plants as byproducts of aerobic metabolism. In green plant parts major source of ROS is the chloroplast where the photosynthetic electron transport system may be overactive and is responsible for reduction of oxygen to different ROS (Foyerand Noctor, 2003). During photosynthesis, energy from the sunlight is captured and transferred to the light harvesting complexes photosystem II and photosystem I in the chloroplast thylakoidal membranes. The transfer of energy from triplet excited state chlorophyll to O_2 results in the formation of singlet oxygen ($^1O_2$). Whereas, the thylakoidal electron transport components get oxidized resulting in the reduction of O_2 (the Mehler reaction) thus forming superoxide (O_2^-) and H_2O_2 (Asada, 1987). Though such ROS generation occurs under normal condition, it is much more elevated under various stresses (Dat *et al.,* 2000; Boguszewska and Zagdańska, 2012).

Elevated level of ROS in plants leads to oxidative stress and cause damage to important cellular components. To avoid the accumulation of these compounds to toxic level, plants possess various detoxifying systems that comprises of a variety of antioxidant molecules and enzymes. There are two main classes of plant defences which can be classified as enzymatic systems and non-enzymatic (Boguszewska and Zagdańska, 2012). The enzymatic defence system comprise of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), mono dehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer, 1998). These enzymes functions in different subcellular compartments and respond in concert when cells are exposed to oxidative stress. Nonenzymic components of the antioxidative defence system include the major cellular redox buffers ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as tocopherol, carotenoids, and phenolic compounds (Apel and Hirt, 2004; Kar, 2011).

2.2.4. ROS scavenging by enzymatic systems

The enzymatic defensive system in plants, the most effective internal protective clean up system, helps to avoid injuries caused by ROS, thus guaranteeing normal cellular function (Horváth *et al.,* 2007). The assay of specific antioxidant enzyme activities or its expression analysis during drought stress has been generally utilized to assess the involvement of the scavenging system during drought stress (de Carvalho, 2008).

Superoxide dismutase (SOD) activity has been reported to be increased in plants under various environmental stress conditions, including drought and metal toxicity (Sharma and Dubey, 2005; Mishra *et al.,* 2011). Increased SOD activity was found in wheat (Badiani *et al.,* 1990), rice (Sharma and Dubey, 2005) pea (Mittler *et al.,* 1994), common and tepary

bean (Turkan *et al.,* 2005) and in olive trees (Sofo *et al.,* 2005) under water deficit stress. Overexpression of SODs in transgenic plants resulted in enhanced tolerance to drought and salinity (Badawi *et al.,* 2004) indicating that SODs have a critical role in the survival of plants under environmental stresses. Increased activity of SOD is often correlated with enhanced tolerance of the plant against environmental stresses. It was suggested that SOD can be used as an indirect selection criterion for screening drought-resistant plant materials and overproduction of SOD has been reported in enhanced oxidative stress tolerance in plants (Zaefyzadeh *et al.,* 2009).

Peroxidases are generally localized in vacuole, cell wall, cytosol, and apoplast, and are considered to be involved in a range of processes related to ROS-induced stress. Also they playmajor roles such as lignification of cell wall, degradation of indole-3-acetic acid (IAA), oxidation of hydroxy-cinnamyl alcohol into free radical intermediates, wound healing, regulation of plant cell elongation, biosynthesis of ethylene and polysaccharide cross linking (Sharma *et al.,* 2012; Prakasha and Umesha, 2016). Enhanced peroxidase activity has been reported under different stress conditions including drought (Mafakheri, 2011). Its activity varies depending upon plant species and stress condition. High peroxidase activity was reported in drought tolerant varities of *Camptotheca acuminate* (Ying *et al.,* 2015). Enhanced POD activity was observed in soybean plants under water deficit stress (Zhang *et al.,* 2006). Increased POD activity had also been reported under drought stress conditions in liquorice (Pan *et al.,* 2006), sun flower (Gunes *et al.,* 2008), and poplar (Xiao *et al.,* 2008). In *Hevea*, increased peroxidase activity was reported in high yielders as well as in trees with high girth which was also found associated with their intrinsic drought tolerance capacity (Sreelatha *et al.,* 2003).

2.2.5. ROS scavenging by non-enzymatic antioxidant systems

The nonenzymatic antioxidant defence system comprises of low molecular weight compounds which include numerous vitamins, secondary metabolites and other phytochemicals which protect the plants against ROS activity. The most important non-enzymatic antioxidants are ascorbic acid (AsA), glutathione (GSH), carotenoids, tocopherols and phenolic compounds including flavonoids and polyphenols (Blokhina and Fagerstedt, 2010).

Ascorbic acid (vitamin C) is one of the most studied antioxidant which is very powerful, and abundant. It is present in majority of plant cell types, organelles and apoplast (Smirnoff and Wheeler, 2000). Under physiological conditions, vitamin C predominantly exists in its reduced form, ascorbic acid (AsA); it also exists in its oxidized form, dehydroascorbic acid (DHA), in trace quantities in leaves and chloroplasts (Smirnoff and Wheeler, 2000; Cárcamo *et al.,* 2004). AsA donate electrons to a wide range of enzymatic as well as non-enzymatic reactions, and this makes it an important ROS detoxifying compound in the aqueous phase. Ascorbic acid has a key role in removal of H_2O_2*via* AsA-GSH cycle (Pinto *et al.,* 2003). Oxidation of ascorbic acid occurs in two sequential steps, first is the production of monodehydroascorbate (MDHA) followed by the formation of dehydroascorbate (DHA). AsA can directly scavenge $O_2^{\cdot -}$, $OH^{\cdot}$, and $^1O_2$, and can reduce H_2O_2 to H_2O *via* the APX reaction (Noctor and Foyer, 1998). In turf grass, AsA concentration significantly increased during water deficiency (Shao *et al.,* 2006). In chloroplasts, AsA acts as a cofactor of violoxanthin de-epoxidase, thereby dissipating excess excitation energy (Smirnoff, 2000). Dehydroascorbic acid also functions as a signaling molecule regulating stomatal closure (Blokhina and Fagerstedt, 2010). Ascorbic acid has a role in the defence against oxidative stress. Studies in transgenic plants and mutants also confirmed the role of ascorbic acid-

glutathione cycle as well as ascorbic acid in oxidative stress. The amount of ascorbic acid was reported high in plants under various abiotic stresses (Yazdanpanah *et al.,* 2011).

Glutathione (GSH) is a tripeptide (*γ*-glutamylcysteinyl-glycine) which is one of the most important low molecular weight non-protein thiol that plays crucial role in intracellular defence against ROS induced oxidative damage. Its presence has been detected in all cell compartments such as chloroplasts, cytosol, vacuoles, mitochondria and endoplasmatic reticulum (Foyer and Noctor, 2003). Glutathione exists either in its reduced form (GSH) with a free thiol group or in its oxidized form (GSSG) with a disulfide between two identical molecules. The balance between the GSH and glutathione disulfide (GSSG) is the central component in maintaining cellular redox state (Li and Jin 2007). The reducing power of GSH helps it to perform major biological processes like cell growth/division, signal transduction, enzymatic regulation, synthesis of proteins and nucleic acids, detoxification of xenobiotics, conjugation of metabolites, synthesis of phytochelatins for metal chelation and the expression of stress responsive genes. Also it functions as an antioxidant free radical scavenger by reacting chemically with $O_2^{\bullet-}$, $^{\bullet}OH$ and H_2O_2. GSH protects macromolecules like proteins, lipids, and DNA either by the formation of adducts with reactive electrophiles (glutathiolation) or by acting as proton donor in the presence of ROS or organic free radicals, yielding GSSG (Asada, 1994). GSH in plant chloroplasts helps to protect the photosynthetic apparatus from oxidative damage (Boguszewska and Zagdańska, 2012). During drought stress, total GSH was increased in sunflower seedlings (Sgherri and Navari-Izzo, 1995), and under salt stress in groundnut (Jain *et al.,* 2002). Arabidopsis plants treated with GSH showed enhanced tolerance to drought stress (Chen *et al.,* 2012).

Carotenoids represent agroup of lipid soluble antioxidants which are able to detoxify different forms of ROS (Bartwal *et al.,* 2013). More than 600 carotenoids exists in nature, such as β-carotene, lycopene, lutein *etc.* which are important in the food and oil industries because of their powerful antioxidant activities (Kim *et al.,* 2013). In plants, they are synthesized in plastids, where they play diverse functions (Ruiz-Sola *et al.,* 2014). They serve as accessory pigments in the photosystems which absorbs light in the region, between 400-550 nm. Carotenoids protect the photosynthetic apparatus against toxic free radicals produced by plant abiotic stresses, especially singlet oxygen ($^1O_2$), and is considered as the first line defence of plants against O_2 toxicity. Also they are important for the PSI assembly and the stability of light harvesting complex protein as well as for thylakoid membrane stabilization (Sieferman-Harms, 1987). Carotenoids also serve as precursors for signaling molecules that influence plant development as well as biotic/abiotic stress responses (Li *et al.,* 2008).

Anthocyanins are water soluble pigments which are included in the vast group of plant secondary metabolites. They were reported to increase in response to drought, salinity and cold stress (Christie *et al.,* 1994; Chalker-Scott, 1999; Parida and Das, 2005). Plant tissues containing anthocyanins are rather resistant to drought stress (Chalker-Scott, 1999). Previous reports suggest that anthocyanins are generally involved in photoprotection under direct drought stress (Hoch *et al.,* 2001; Merzlyak *et al.*, 2008). A decrease in anthocyanin level was observed in the salt-sensitive species of potato (Daneshmand *et al.,* 2010). Plant tissues with higher anthocyanin content usually had higher resistance to water deficit stress. The purple cultivar of pepper was reported to be more tolerant to drought stress than the green cultivar (Bahler *et al.* 1991). In strawberry cell culture an increased level of anthocyanin was reported under low temperature (Zhang *et al.,* 1997).

2.2.6. Osmotic adjustment *via* osmolyte accumulation

The accumulation of low molecular weight water soluble organic compounds known as "compatible solutes" or "osmolytes" is one of the common strategies adopted by plants to combat with various environmental stresses (Rhodes and Hanson, 1993). The most common compatible solutes are amino acid like proline, sugars like mannitol, sorbitol and trehalose, polyols, polyamines and quaternary ammonium compounds like glycine betaine (Giri, 2011). The accumulation of osmolytes occurs under different stress conditions that cause cellular dehydration, like water deficit stress and salinity. Under dehydration stress, cell turgor is maintained by osmotic adjustment *via* the accumulation of osmolytes and improves water uptake from drying soil (Rhodes and Samaras, 1994). Compatible solutes are usually non-toxic even at high cellular concentrations and they provide protection to plants from stress by contributing to cellular osmotic adjustment, ROS detoxification, protection of membrane integrity and enzymes/protein stabilization (Bohnert and Jensen, 1996; Ashraf and Foolad, 2007). During abiotic stress, the tolerant or susceptible species show differential stress tolerance depending on the levels of accumulation of compatible solutes. Eventhough, osmolytes fall in different biochemical groups, their function is similar, the protection of plant against various stresses (Giri, 2011).

Proline is the most extensively studied among the compatible solutes, because of its great significance in the stress tolerance. Accumulation of proline has been reported to occur under various stress conditions like drought, high temperature, low temperature, salinity, heavy metal, UV irradiation, pathogen infection, nutrient deficiency and atmospheric pollution (Hare and Cress, 1997). Proline accumulation normally occurs in cytoplasm and it is an important response of plants

exposed to drought stress in order to reduce injury to cells (Anjum *et al.*, 2011). Apart from acting as an osmolyte for osmotic adjustment, it functions as molecular chaperons stabilizing the structure of proteins and its accumulation buffers cytosolic pH and maintains cell redox status (Hayat *et al.*, 2011). It also influences stress signalling as a part of adaptive response (Maggio *et al.*, 2002). Besides these assigned physiological roles, the accumulated free proline also function as a sink for excess reductant and a store of nitrogen and carbon for use after relief of water deficit stress (Zhu, 2002). Proline biosynthesis occurs in the cytosol and in the plastids while its degradation takes place in mitochondria (Elthon and Stewart, 1981). Proline accumulation during osmotic stress is mainly due to its increased synthesis and reduced degradation (Verbruggen and Hermans, 2008). Increased proline content was observed under drought stress in pea cultivars (Alexieva *et al.*, 2001). Free proline accumulation in drought tolerant petunia (*Petunia hybrida*) varieties under drought served as an osmoprotectant and induced drought tolerance (Yamada *et al.*, 2005). Elevated proline level under drought stress was also reported in chickpea cultivars (Mafakheri *et al.*, 2010) and *N. tabacum* (Dobrá *et al.*, 2011). An enhanced accumulation of proline was observed under water deficit stress in rice (Xiong *et al.*, 2012).

Glycine betaine (N, N, N-trimethyl glycine) is another compatible solute, which is a quaternary ammonium compound with zwitter ionic nature and is present in plants, animals and bacteria (Wahid *et al.*, 2007). Studies reported that glycine betaine (GB) plays a major role in enhancing plant tolerance towards a range of abiotic stresses including drought (Quan *et al.*, 2004). It is involved in maintaining water balance, protecting photosynthesis, stabilizing macromolecules and detoxifying reactive oxygen species (Chen and Murata, 2011). GB is mainly located in chloroplasts and plays a vital role in the stroma adjustment as well as protection of thylakoid membranes (Jagendorf and

Takabe, 2001). GB protects the photosystem II (PS II) complex under high salinity stress (Murata *et al.,* 1992) and at extreme temperatures or pH (Mohanty *et al.,* 1993). However, during stress conditions, crops like wheat, maize and barley does not accumulate significant amounts of glycine betaine. Moreover, some crops like rice do not accumulate glycine betaine at all (Giri, 2011). GB is synthesized either by oxidation of choline or N-methylation of glycine. In plants, the enzyme choline monooxygenase (CMO) converts choline into betaine aldehyde and then a NAD+ dependent enzyme, betaine aldehyde dehydrogenase (BADH) produces glycine betaine. These enzymes are found in chloroplast stroma and their activity was found increased under salinity stress. (Fariduddin *et al.,* 2013; Giri, 2011). Under abiotic stress conditions, GB accumulates at higher concentration in naturally occurring plants like spinach and sugarbeet and they functions as osmoprotectants in them (Fariduddin *et al.,* 2013). Increased level of glycine betaine was observed under drought stress in barley (Nakamura, 2001) and in higher plants (Jun *et al.,* 2000). GB was also been found to accumulate in a large number of plants exposed to salt and water stress like tomato, peas and beans (Sudhakar *et al.,* 1993; Girija *et al.,* 2002).

2.2.7. Proteomic changes in plants during drought stress

In plants, various environmental stress conditions leads to the activation of various stress responsive pathways at molecular level, which leads to significant changes in plant proteome. Proteins are involved in diverse functions such as enzymes and transcriptional factors, have protective functions, interact with other molecules, involve in energy transfer or radicals scavenging and take part in signaling pathways (Rodziewicz *et al.*, 2014). Based on their functions proteins can be classified into two groups, (i) functional proteins and (ii) regulatory proteins. The first group, functional protein, comprises of proteins having protective functions in stress tolerance which include HSPs, LEAs, proteins involved in the synthesis of

osmoprotectants, defence related proteins, reproduction and development related proteins, proteins involved in repair and protection from damage *etc.* The second group, regulatory proteins consists of proteins involved in regulation of signal transduction and transcription as part of stress response and include transcription factors, protein kinases and protein phosphatases (Ashoub *et al.,* 2013; Augustine, 2016).

Heat shock proteins are a large family of proteins which were initially identified in response to high temperatures and they are playing roles in maintaining cellular homeostasis both under stress and under optimal growth conditions. They are responsible for correct folding, translocation and degradation of proteins and prevent proteins from aggregation. HSPs were induced by different abiotic stresses including drought, high and low temperatures, light, salinity, ozone and heavy metal stress (Lee *et al.,* 2000; Wang *et al.,* 2003). Molecular chaperones/HSPs are located in both cytoplasm and organelles, such as mitochondria, chloroplast, nucleus and ER (Augustine, 2016). In plants, five major families of HSPs are recognized and they are classified based on their approximate molecular weight and according to their amino acid sequence homologies and functions: (1) HSP100 (Clp) family; (2) HSP90 family; (3) HSP70 (DnaK) family; (4) HSP60 (GroEL) family; (5) small HSP family (sHSP; 15–40 kDa) (Kotak *et al.,* 2007; Gupta *et al.*, 2010).

Among the HSPs in plants, small HSPs represent the most predominant and diverse family with respect to their sequence homology, cellular location and functions (Waters *et al.,* 1996). Small HSPs cannot refold non-native proteins, however they bind to partially folded or denatured proteins and prevent non-functional folding and aggregation. Also they are capable of degrading mis-folded proteins (Sun *et al.,* 2002). sHSPs in plants respond to a wide range of abiotic stresses including heat, cold,

salinity, drought and oxidative stress. In *Arabidopsis thaliana,* it was identified that the expression of cytosolic sHSP 17.1 generated more biomass and recovered more quickly from drought after rewatering (Zhang *et al.,* 2013). sHSPs were discovered to be induced by heat stress, the patterns of expression of sHSP were much complex and varied between organisms and developmental stages. Though majority of sHSP genes in Arabidopsis showed tremendous increase at elevated temperatures, At14.7, At15.4 and At21.7 showed no response at all to heat (Siddique *et al.,* 2008; Waters *et al.,* 2008). Rice transgenic seedlings with higher expression levels of *sHSP17.7* showed higher growth recovery potential after getting exposed to drought (Sato and Yokoya, 2008). sHSPs are abundant in plants, which functions in binding and stabilizing denatured proteins, suggesting that these sHSPs play an important role in enhancing plant stress tolerance (Sun *et al.,* 2002; Wang *et al.,* 2003). Studies on the chloroplastic factors responsible for drought tolerance showed that drought concomitant with light stress induced a 23 kDa chloroplast protein in young plants of *Hevea* (Annamalinathan *et al.,* 2006; 2010).

2.2.8. Stress induced changes in genomic DNA

Despite the very stable nature of plant genome, nuclear DNA is an inherently unstable molecule and can be damaged spontaneously, metabolically, or by various environmental stress factors (Gill *et al.,* 2015). Environmental stressors *viz.* UV-B, ozone, desiccation, low and high temperatures, ionizing radiations, salinity, air and soil pollutants, chemical mutagens and crosslinking agents, alkylating agents, aromatic compounds, fungal and bacterial toxins *etc.*, are important DNA damaging agents (Tuteja *et al.,* 2009). Damages to DNA mainly include single-strand DNA breaks (SSBs), double-strand DNA breaks (DSBs) and other intrinsic damages such as sugar-phosphate backbone breakage, loss of a base to form an abasic site

and chemical modification of a base to form a miscoding or non-coding lesion (Vonarx, 2000; Singh *et al.,* 2010).

Sunlight is the energy source for photosynthesis in plants and hence, it is impossible to avoid UV radiations. UV radiation is one of the most damaging agents for DNA and other biomolecules such as proteins and lipids (Prinsze *et al.,* 1990). DNA strongly absorbs UV-B radiation and therefore it is the most important target for UV-B induced damage. UV-B radiation damages nuclear, chloroplast, and mitochondrial DNA by inducing various DNA lesions including the generation of cyclobutane pyrimidine dimers (CPDs) which accounts for almost 75% of UV-B mediated total DNA damage and other photoproducts, pyrimidine (6-4) pyrimidone dimers, oxidized or hydrated bases, single-strand breaks, and others (Ballare *et al.,* 2001; Takahashi *et al.,* 2011). In addition, IR causes radiolysis of water, which generates hydroxyl radicals (·OH) which are highly reactive and causes damage to various important cellular components (Gill *et al.,* 2015).

The accumulation of reactive oxygen species (ROS) as by-products of normal cellular metabolism or as a result of environmental stress factors leads to DNA damage in the cell (Gill and Tuteja, 2010; Biedermann *et al.,* 2011). Accumulation of ROS at lower concentrations are employed as signals that mediate plant responses towards abiotic stress while at higher concentrations they causes significant threat that may eventually lead to programmed cell death (PCD) (Gechev and Hille, 2005). PCD is a highly regulated and organized process which involves the disassembly of cells through a series of distinct morphological changes, including cell and nuclear shrinkage, membrane blebbing, and disintegration into apoptotic bodies (Wituszynska and Karpinski, 2013). PCD in plants occurs as part of development, pathogen interaction, or abiotic stress, and they all share common apoptotic mechanisms. The biochemical feature of apoptosis is the

cleavage of DNA at internucleosomal sites, which generates oligonucleosomal fragments which can be detected by the formation of DNA ladders, multimers of 170 to 200 bp, on agarose gels (Stein and Hansen, 1999). In some other instances, plant cell death result in an apoptosis-like morphology which includes cell and nucleus shrinkage and/or membrane blebbing and formation of apoptotic body, in the absence of internucleosomal fragmentation. In these cases DNA is either degraded into approximately 50 kb fragments (Levine *et al.,* 1996), which can be considered as a precursor to oligonucleosomal fragmentation, or is degraded into more or less random sizes (McCabe *et al.*, 1997). DNA laddering phenomenon have been observed under various developmental processes (Danon *et al.* 2000), cold (Koukalova *et al.,* 1997), UV radiation (Danon and Gallois, 1998), salinity (Ling *et al.* 2009), heat stress (Balk *et al.,* 1999), in wheat roots after D-mannose treatment (Hameed *et al.* 2008), due to fungal infection and in cells or tissues exposed to abiotic stress treatments (Ryerson and Heath, 1996; Katsuhara, 1997).

2.2.9. Molecular responses in plants under drought stress

Plants have evolved various defence mechanisms at multiple levels in response to different abiotic stress conditions. The stress signalling pathway in response to an abiotic stress involves the perception of stress signals, signal transduction and expression of stress responsive genes which in turn activates the physiological and metabolic responses that provides tolerance or resistance towards a particular stress (Pérez- Clemente *et al.,* 2013; Wang *et al.,* 2016). During the process of stress signalling, the sensors or receptors located in the cell wall or membrane of plant cells perceive the stress stimulus. These extracellular signals are converted into intracellular ones through second messengers which include calcium ions, inositol phosphate, cyclic nucleotides (cAMP and cGMP), reactive oxygen species (ROS) *etc.* The second

messengers activate the corresponding signal transduction pathways (Chaves *et al.,* 2009; Bhargava and Sawant, 2013) which are mediated by protein kinases and phosphatases. As a result, the transcription factors are activated or suppressed, and they further bind specifically to *cis*-elements in the promoters of stress responsive genes and regulate their transcription (Danquah *et al.,* 2014). The presence of phytohormone abscisic acid (ABA) was reported to be increased under water deficit conditions which in turn causes stomatal closure and induces expression of various stress related genes (Yang *et al.,* 2011). Drought induced gene expression can occur either by ABA-dependent or ABA independent regulatory system (Aguado *et al.*, 2014).

Utilizing the functional genomics approaches such as microarray analysis and high throughput sequencing, various stress responsive genes have been identified and characterized in a wide variety of crops. These genes mainly code for proteins that have either functional (metabolic) or regulatory roles (Shinozaki *et al.,* 2003; Joshi *et al.,* 2016). The functional proteins include molecules that function in abiotic stress tolerance such as detoxification enzymes, water channel proteins, ion transporters, enzymes in osmolyte biosynthesis, HSPs and LEA protein (Joshi *et al.,* 2016). On the other hand, the regulatory class comprises of transcription factors (NAC, WRKY, AREB, AP2/ERF, bZIP, MYC and MYB), signaling protein kinases [mitogen activated protein kinases (MAPK), calcium-dependent protein kinases (CDPK), receptor protein kinases and transcription regulation protein kinases] and protein phosphatases (phosphoesterases and phospholipase), which synchronize signal transduction and expression of genes during stress responses (Wani *et al.,* 2013).

2.2.10. Transcriptional regulation of gene expression

Transcription factors constitute a group of regulatory proteins which modulate gene expression in response to drought stress at the transcriptional

level. TFs function as terminal transducers and directly regulate the expression of an array of downstream genes by interacting with the specific *cis*-elements in their promoter region (Yamaguchi-Shinozaki and Shinozaki, 2006). Members from diverse transcription factor families such as NAM-ATAF1/2-CUC2 (NAC), Ethylene- responsive element binding protein (AP2/EREBP), basic leucine zipper (bZIP), MYB, and zinc finger have been reported to be involved in the drought responses. Various transcription factors are involved in plant abiotic stress responses either in abscisic acid (ABA)-dependent or ABA-independent pathway (Umezawa *et al.,* 2006; Golldack *et al.,* 2011).

The *NAC* gene family constitutes the largest family of plant specific transcription factors present in a wide range of plant species. *NAC tf* has a highly conserved NAC domain in the N-terminal region and a variable transcriptional regulatory region in the C-terminal region. The NAC domain in the N-terminal region is associated with DNA binding, nucleus-oriented localization, and the formation of homodimers or heterodimers with other NAC proteins, whereas the transcriptional regulatory region in the C-terminal region functions as a transcriptional activator or repressor (Olsen *et al.,* 2005; Puranik *et al.,* 2012). Genes in the NAC family have been shown to regulate a wide range of developmental processes, including embryo development (Duval *et al.*, 2002), seed development (Sperotto *et al.,* 2009), shoot apical meristem formation (Kim *et al.,* 2007), fiber development (Ko *et al.,* 2007), leaf senescence (Guo *et al.,* 2005; Breeze *et al.,* 2011), cell division (Kim *et al.,* 2006) and various biotic and abiotic stress responses (Nuruzzaman *et al.,* 2013). Comprehensive investigation aided by a number of complete plant genomic sequences has identified 117 *NAC* genes in Arabidopsis, 26 in citrus, 151 in rice, 163 in poplar, 79 in grape and 152 each

in soybean and tobacco (Rushton *et al.,* 2008; Hu *et al.*, 2010; Le *et al.,* 2011; Nuruzzaman *et al.,* 2013).

The WRKY family of transcription factors were also found extensively distributed in plants and characterized by the presence of one or two highly conserved WRKY domain of about 60 amino acid residues. The WRKY domain contains a conserved WRKYGQK motif at the N-terminus and a C2H2 or C2HC zinc-finger motif at the C-terminus and they can specifically bind to W-box *cis*-elements with a core sequence of C/TTGACC/T, located at the promoter region of many target genes. A large number of *WRKY tfs* have been identified in various plants, such as 74 in Arabidopsis (Ulker and Somssich, 2004), 102 in rice (Wu *et al.,* 2005), 104 in poplar (He *et al.,* 2012), 86 in *Brachypodium distachyon* (Wen *et al.,* 2014), 182 in soybean (Bencke-Malato *et al.,* 2014), and 116 and 102 genes in two different species of cotton (Dou *et al.,* 2014). *WRKY tfs* have been reported to be involved in plant developmental processes such as growth, seed germination, leaf senescence, and responses to various biotic and abiotic stresses (Rushton *et al.,* 2010). It has been demonstrated that *WRKY tfs* play major role in regulating plant responses to a variety of abiotic stresses such as drought, cold, salinity, heat *etc.* (Chen *et al.,* 2012; Rushton *et al.,* 2012; Tripathi *et al.,* 2014; Banerjee and Roy choudhury, 2015). Over-expression of some stress-responsive *WRKY* genes showed enhanced tolerance to abiotic stresses in transgenic plants. Transgenic Arabidopsis plants over-expressing *GmWRKY21* gene showed increased tolerance to cold stress, while over-expressing *GmWRKY54* gene enhanced tolerance to drought and salinity (Zhou *et al.,* 2008). Transgenic Arabidopsis plants over-expressing *VvWRKY11* exhibited improved tolerance to mannitol-induced osmotic stress (Liu *et al.,* 2011).

The *MYB tfs* are characterized by a highly conserved MYB domain for DNA-binding, which contains 1 to 4 imperfect repeats (*MYB* repeat) each with about 52 amino acid residues at the N-terminus. The C-terminus contains an activation domain which varies significantly among MYBs, leading to versatile regulatory roles of *MYB* family. Large numbers of *MYB tfs* have been identified in various plant species, such as 198 in Arabidopsis (Yanhui *et al.,* 2006), 229 in apple (Cao *et al.,* 2013), 183 in rice (Yanhui *et al.,* 2006), 209 in foxtail millet (Muthamilarasan *et al.,* 2014) and 177 in sweet orange (Hou *et al.,* 2014). *MYB tfs* are reported to have major roles in diverse physiological and biochemical processes including cell development and cell cycle, primary and secondary metabolism, hormone synthesis, signal transduction, and regulate plant responses to various biotic and abiotic stresses (Dubos *et al.,* 2010; Ambawat *et al.,* 2013). In Arabidopsis, *AtMYB15* improved freezing tolerance (Agarwal *et al.,* 2006); whereas, *AtMYB44*, *AtMYB60*, and *AtMYB61* improved drought tolerance by regulating stomatal movement (Cominelli *et al.,* 2005; Liang *et al.,* 2005; Jung *et al.,* 2008). Also, *AtMYB96* improved drought tolerance either by activating ABA and auxin signals (Seo *et al.,* 2009) or by enhancing cuticular wax synthesis (Seo *et al.,* 2011). In addition, *AtMYB102* is also reported to be playing a key role in responses to osmotic stress, salinity, and ABA application (Denekamp, 2003). In rice, *OsMYB2* was induced by salt, cold, and dehydration stress. The over-expression of *OsMYB2,* in transgenic plants showed enhanced tolerance to various stresses by the alteration of expression levels of numerous genes having diverse functions in stress response (Yang *et al.,* 2012). In Arabidopsis, salt and freezing tolerance was significantly enhanced by over-expressing either *GmMYB76* or *GmMYB177* from soybean (Liao *et al.,* 2008).

The AP2/ERF (APETALA2/ethylene responsive element) family is a large group of plant specific transcription factors and is characterized by the presence of the highly conserved DNA binding domain *AP2*/ethylene-responsive element-binding factor (ERF) that directly interact with GCC box and/or dehydration-responsive element (DRE)/C-repeat element (CRT) *cis*-acting elements at the promoter of downstream target genes (Riechmann and Meyerowitz, 1998). *AP2/ERFBP tfs* are involved in a variety of functions in plant developmental processes and stress responses which include plant hormone responses, cell proliferation, vegetative and reproductive development, regulation of biotic and abiotic stress responses *etc.* (Nakano *et al.*, 2006; Licausi *et al.*, 2010; Sharoni *et al.*, 2011). A large number of *AP2/ERFBP tfs* have been identified in different species such as 145 in Arabidopsis (Riechmann and Meyerowitz, 1998), 291 in Chinese cabbage (Song *et al.*, 2013), 163 in rice (Sharoni *et al.*, 2011), 171 in foxtail millet (Lata *et al.*, 2014), 200 in poplar (Zhuang *et al.*, 2008) and 116 in moso bamboo (Wu *et al.*, 2015). On the basis of number and similarity of *AP2/ERF* domains, these *AP2/EREBP tfs* are categorized into four major subfamilies: *AP2* (Apetala2), *RAV* (related to *ABI3/VP1*), *DREB* (dehydration-responsive element-binding protein), and *ERF* (Sakuma *et al.*, 2002; Sharoni *et al.*, 2011).

The DREB subfamily regulate the expression of a number of dehydration/cold-regulated (*RD/COR*) genes by interacting with *DRE/CRT* *cis*-elements (A/GCCGAC) located in the promoter region of *RD/COR* genes. These genes are found responsive to drought and low- temperature conditions, for example, *COR15A*, *COR6.6* and *RD29A/COR78* (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Lucas *et al.*, 2011). Based on the variation in some conserved motifs and their biological functions in various species, *DREB* TFs are further classified into two major subgroups: *DREB1/C*-repeat-

binding factor (*DREB1/CBF*) and *DREB2* and each of them is involved in different signal transduction pathway under abiotic stress conditions (Dubouzet *et al.,* 2003). *DREB1/CBF* genes are generally involved in low temperature stress responses in Arabidopsis and rice, while *DREB2* genes respond to dehydration, heat shock and high salinity (Lucas *et al.,* 2011). In Arabidopsis, *DREB1A/CBF3*, *DREB1B/CBF1* and *DREB1C/CBF2* are induced by cold stress (Stockinger *et al.,* 1997), while *DREB2A* and *DREB2B* are major *DREB2s* induced in response to dehydration, heat and high salinity (Nakashima *et al.,* 2000; Lim *et al.,* 2007). In transgenic potato plants, the expression of *StDREB2* significantly increased the level of drought tolerance than the wild-type control plant (Bouaziz *et al.,* 2015).

The ERF subfamily functions in plant stress tolerance by regulating the stress-responsive genes through interaction with the *cis*-element GCC boxes with a core sequence of AGCCGCC (Ohme-Takagi and Shinshi, 1995; Hao *et al.,* 1998). A multitude of ERF genes are induced by various abiotic stresses, such as drought, cold, high salinity and osmotic stress (Xu *et al.,* 2008). In rice, *OsERF4a* has been reported as a positive regulator of plant growth and drought tolerance (Joo *et al.,* 2013). Some ERF genes function in both biotic and abiotic stress tolerance and this is because of their involvement in various hormonal signalling pathways including ethylene, JA, or SA (Liang *et al.,* 2008). For example, overexpression of *TaPIE1* in wheat significantly increased resistance to both pathogen and freezing stress (Zhu *et al.,* 2014). While overexpression of *GmERF3* in tobacco enhanced resistance against infection by pathogen and tobacco mosaic virus (TMV) as well as improved tolerance to drought and salinity (Zhang *et al.,* 2009).

RAV protein (related to ABI3/VP1) which is unique in higher plants, contains an AP2 domain in the N-terminal region and a B3 domain in the C-terminal region (Kagaya *et al.,* 1999). It has been reported that the AP2 and

B3 domains of RAV1 were bind to CAACA and CACCTG motifs. The RAV genes, namely *RAV1* and *RAV2*, were initially identified in Arabidopsis, based on the sequence information of maize homologous gene VIVIPAROUS1 (*Vp1*) (McCarty *et al.,* 1989). Earlier studies in Arabidopsis, reported that RAV1 protein may be a negative regulator of growth and development (McCarty *et al.,* 1991). However, later studies reported that *AtRAV1* positively regulates leaf senescence, leading to earlier leaf etiolate (Woo *et al.,* 2010), involved in cold responses (Yamasaki *et al.,* 2004), and also they control the flowering time under long-day growth conditions (Osnato *et al.,* 2012). In addition, *RAV1* and *RAV2* genes display biphasic expression patterns in Arabidopsis, in response to internal and external stimulations (Kagaya *et al.,* 2009). A study in tomato (*Solanum lycopersicum*) indicated that RAV enhances plant tolerance to the bacterial wilt (Li *et al.,* 2011). Previous reports suggested *GmRAV* as a negative regulator acts on both photosynthesis and growth in soybean (Glycine max) (Zhao *et al.,* 2008). Studies also indicated that *AtRAV1*, *AtRAV2*, *CaRAV1* and *SlRAV2* were strongly induced in plants treated with bacterial pathogen, SA, high salt and mannitol (Sohn *et al.,* 2006; Kagaya *et al.,* 2009; Li *et al.,* 2011).

Signalling molecules

Mitogen-activated protein kinase (MAPK) cascades are tightly controlled signaling cascades in eukaryotes (Pitzschke, 2015) and they play major roles in plant growth, development, and defence mechanisms (Zhang *et al.,* 2016). The MAPKs are located in the cytoplasm as well as in the nucleus of cells and are involved in different signal transduction pathways (Rodriguez *et al.*, 2010; Joshi *et al.*, 2011). In plants both developmental signals and environmental cues trigger the activation of MAPK cascades (Sinha *et al.*, 2011; Xu and Zhang, 2015). MAPKs are serine/threonine kinases able to phosphorylate a wide range of substrates, and MAPK along

with phosphatases, function as on/off signal switcher to regulate the activity of many downstream targets including other kinases and/or transcription factors to control cell signaling in plant stress responses to environmental cues (Rodriguez *et al.,* 2010; Sinha *et al.*, 2011; Xu and Zhang, 2015). In rice *(Oryza sativa)*, a Raf-like MAPKKK protein, named DSM1 functions as a potential scavenger of the reactive oxygen species (ROS), thereby increases plant tolerance to dehydration stress (Ning *et al.,* 2010). Drought stress also resulted in the activation of *OsMSRMK2* and *OsMAPK5* in rice plants (Agrawal *et al.,* 2002; Xiong and Yang, 2003). In Arabidopsis, the MAP kinase *MPK6* is identified to enhance plant tolerance to dehydration via the regulation of RNA decapping activity (Xu and Chua, 2012). Also in Arabidopsis, the expression of *AtMEKK1* and *AtMPK3* found enhanced under drought stress (Mizoguchi *et al.,* 1996). Moustafa *et al.* (2008) reported that Arabidopsis MPK2, MPK3, MPK4, MPK5, MPK12 and MAPKKK4 were induced by water stress. In maize, a MAP kinase, *ZmMPK3*, was identified as a drought responsive gene (Wang *et al.*, 2010). Zhang *et al.,* (2011) identified a cotton MAP kinase, *GhMPK2* that functions in reducing water loss and adjusting osmotic pressure under drought conditions. In apple, the MAP kinase, *MaMPK* was upregulated at transcriptional and protein levels with an increased activity in the drought tolerant genotypes (Peng *et al.,* 2006).

Phosphatases are major signalling components in plants and they transduce a wide range of environmental signals. Plant protein phosphatases are protein serine/threonine phosphatases and they belong to three classes: (i) phosphoprotein phosphatases, (ii) phosphoprotein metallophosphatases and (iii) aspartate-based protein phosphatases (Kerk *et al.,* 2008). PP2C (type 2C protein phosphatases) belongs to phosphoprotein metallo phosphatase class, which is an Mg^{2+}/Mn^{2+}-dependent enzyme (Fuchs *et al.,* 2013).

Among plant protein phosphatases, PP2C has a major role in mediating abiotic stress triggered signalling pathways (Moorhead *et al.,* 2007; Singh *et al.,* 2010). In Arabidopsis, PP2Cs comprises of 80 members and in rice 90 members were identified. PP2C in Arabidopsis, comprises of *ABI1, ABI2, HAB1, HAB2, AHG1* and *AtPP2CA*, and they have been reported as negative regulators of ABA signalling and responses. In rice, Group A PP2C member *(OsPP108/OsPP2C68*), was highly up-regulated under ABA, salt and drought stress conditions (Singh *et al.,* 2010). Salinity and water deficit stress resulted in high transcript level of *OsPP2A-1* and *OsPP2A-3* genes in leaves (Singh and Pandey, 2012). *OsPFA-DSP1* is a dual specificity protein tyrosine phosphatase, and has been characterised in rice as well as in tobacco (Liu *et al.,* 2012). Expression level of *OsPFA-DSP1* was up regulated under different stress treatments such as ABA, PEG and NaCl. In addition, *OsPFA-DSP1* in transgenic plants has been found to negatively regulate drought stress responses (Singh and Pandey, 2012). In transgenic plants of *B.napus*, the overexpression of *AtABI1* resulted in decreased tolerance to drought stress (Singh *et al.,* 2015).

ATP-binding cassette (ABC) proteins constitute a major class of powerful transporters that drive the exchange of compounds across different biological membranes, and in most cases against existing electrochemical gradients, utilizing energy released from ATP hydrolysis (Wilkens, 2015). ABC transporters are involved in diverse processes such as pathogen response, phytate accumulation in seeds, surface lipid deposition, and transport of the phytohormones auxin and abscisic acid and hence they play an essential role in plant development, plant nutrition, organ growth, response to diverse abiotic stress, and plant interaction with its environment (Kang *et al.,* 2011). ABC transporters shuttle a wide spectrum of substrates, such as lipids, amino acids, sugars, phytohormones, nucleosides, vitamins,

peptides, carboxylates, heavy metals, chlorophyll catabolites and xenobiotic conjugates across a variety of biological membranes (Kretzschmar *et al,*. 2011; Wilkens, 2015). ABC transporter genes, *AtABCG25* and *AtABCG40*, were reported to be responsible for ABA transport and responses in Arabidopsis (Kang *et al.,* 2010). *AtABCG40* of the ABCG subfamily and additional three genes of different subfamilies have been reported to function in stomatal regulation in Arabidopsis. Mutant plants of *AtMRP5/AtABCC5* were less sensitive to drought stress (Klein *et al.,* 2003), whereas mutant plants of *AtMRP4/AtABCC4* were more sensitive to water stress (Suh *et al.,* 2007).

Ferritins are iron storage proteins, found in all the living beings, except in yeast (Briat *et al.*, 2006; Arosio *et al.,* 2008) and they have a major role in sequestering or releasing iron upon demand (Sigel and Sigel,1998). Ferritins constitute a class of 450 kDa proteins that contains 24 subunits, which are present in all cell types (Theil, 1987). Plant ferritins are targeted to mitochondria and plastids (Zancani *et al.*, 2004; Briat *et al.*, 2010). In plants, ferritin gene expression was induced in response to various biotic and abiotic stress conditions such as pathogen infection, drought, cold, heat and salinity. In Arabidopsis four ferritin genes have been identified: *AtFER1, AtFER2, AtFER3* and *AtFER4*. Ferritin genes were induced by treatment with H_2O_2, iron and abscisic acid (ABA) in Arabidopsis; however, not all four *AtFER* genes were induced (Petit *et al.*, 2001). Ferritin expression was found enhanced in response to drought stress in soybean nodules (Clement *et al.*, 2008). Overexpression of ferritin significantly enhanced abiotic stress tolerance in grapevine plants (Zok *et al.*, 2009). In wheat, *TaFER-5B* was induced by PEG, H_2O_2 and excess iron treatment suggesting that *TaFER-5B* may be involved in abiotic stress responses (Zang *et al.,* 2017). In chickpea, ferritin designated *CaFer1* showed high expression under conditions of iron deficiency and water deficit (Parveen *et al.*, 2017).

Caspases are cysteine proteases which are the central components in the process of apoptosis in animal cells. True caspases have not yet explained in plants (Lord and Gunawardena, 2012), however similar enzymatic activities were detected in plant extracts also. Two broad groups of caspase-like proteases identified in plant systems are cysteine endopeptidases (Rojo *et al.,* 2004) and serine endopeptidases (Coffeen and Wolpert, 2004). Cysteine endopeptidases are further divided into two groups, vacuolar processing enzymes (VPE) (Sanmartin *et al.,* 2005) and metacaspases (Bozhkov *et al.,* 2010; Lord and Gunawardena, 2012). The large number of metacaspases identified in plant genomes indicates that the members of this protease family might have evolved in specialized functions in plant tissues (Lam and Zhang, 2012). Metacaspases play major roles in different plant species in response to various biotic and abiotic stresses (Watanabe and Lam, 2011; Zhang *et al.,* 2013; Fagundes *et al.,* 2015). The cell death mediated by metacaspases occurs in response to herbicide-induced stress, ultraviolet light, ROS and senescing flowers (Vercammen *et al.,* 2007; He *et al.,* 2008; Ahmad *et al.,* 2012). In Arabidopsis, several metacaspase genes (*AtMC*) are involved in stress responses. Type I metacaspase, *AtMC1* functions as a positive regulator of hypersensitive cell death (Coll *et al.,* 2010; De Pinto *et al.,* 2012). Watanabe and Lam (2011) reported that *AtMC4* positively regulated PCD under biotic and abiotic stresses. *AtMC4* and *AtMC5* were rapidly induced in infection caused by bacterial pathogens (Baskett, 2012). In rice (*Oryza sativa*) metacaspase (*OsMC*) genes are expressed under biotic and abiotic stress. *OsMC1* was expressed in plants under cold stress, whereas, *OsMC6* and *OsMC8* in plants under drought stress. *OsMC5* was found increased in *Magnaporthe oryzae* resistant plants, and *OsMC6* was expressed when rice leaves were damaged by beef army worms (Ning *et al.,* 2002; Gupta *et al.,* 2012; Dona *et al.,* 2013; Wang and Zhang, 2014).

Aquaporins (AQPs) are transmembrane proteins, which belong to the major intrinsic protein (MIP) superfamily. The main function of aquaporins in plants is the transport of water and other small neutral molecules across cellular biological membranes (Maurel, 2007; Kapilan *et al.,* 2018). In plants, majority of AQPs are located in either the tonoplast or the plasma membrane and few are localized in the endomembranes (Kapilan *et al.,* 2018). Based on the amino acid sequence and membrane localization, AQPs in higher plants are classified into five subfamilies: (i) plasma membrane intrinsic proteins (PIPs), (ii) tonoplast intrinsic proteins (TIPs), (iii) nodulin-26 like intrinsic proteins (NIPs), (iv) small basic intrinsic proteins (SIPs), and (v) X intrinsic proteins/uncharacterized-intrinsic proteins (XIPs) (Hussain *et al.,* 2011). TIPs were the first proteins identified with water-transporting function in *Arabidopsis thaliana* and are the most abundant AQPs in the vacuolar membrane. The molecular weight of TIPs ranges from25 and 28 kDa and an isoelectric point of about 6.0 (Johnson *et al.,* 1990; Maurel *et al.,* 2008). TIP genes showed differential expression in response to environmental constraints, such as salinity, drought and to ABA (Alexandersson *et al.,* 2005; Regon *et al.,* 2014). *AtTIP1;1* from Arabidopsis was the first plant aquaporin characterized. *TIP1;1* is the most expressed AQP in both maize and Arabidopsis, which is expressed in all plant organs, prominently in roots and leaves, and seems to be associated with cell elongation (Chaumont *et al.,* 1998). In rice, *OsTIP1;1, OsTIP2; 2, OsTIP2;1, OsTIP1;2, OsTIP4;1* and *OsTIP3;1* were found to be highly expressed under drought condition. Under salt stress condition, the same genes were found to be highly expressed in rice, however, the gene *OsTIP1;2*, which was particularly expressed in drought condition, was found to be expressed very low under salt stress condition. In Zea Mays, the genes *ZmTIP3;1* and *ZmTIP4;2* were found to be highly expressed only in drought condition,

whereas the gene *ZmTIP4;1* was found to be down regulated under this condition (Gattolin *et al.,* 2011; Regon *et al.,* 2014).

Protective proteins

Late embryogenesis abundant (LEA) proteins constitute a large group of hydrophilic proteins with an important role in abiotic stress tolerance, particularly drought tolerance in plants (Magwanga *et al.,* 2018). These proteins were first reported to accumulate during plant seed dehydration, at the later stages of embryogenesis (Pedrosa *et al.,* 2015). LEA proteins were found to accumulate in tissues of plants during some stages of plant development in response to water deficit stress. LEAs are widely distributed proteins in the plant kingdom, from algae to angiosperms, and also found in some bacterial species in response to dehydration. Based on their amino acid sequences, LEA proteins have been classified into seven different groups, each containing distinctive motifs (LEA1 - LEA7 (Battaglia and Covarrubias, 2013). LEAs appear predominantly in the cytosol and also in mitochondria, chloroplast, lipid bodies and nucleus (Rorat, 2006). LEA genes have been reported to be significantly induced by abiotic stresses, such as drought, cold and salinity, and their over expression in transgenic plants has resulted in increased tolerance towards abiotic stresses (Liu *et al.,* 2013). Desiccation tolerance is a common attribute of all proteins belonging to this family (He *et al.,* 2012). Over expression of the barley LEA gene *HVA1* in rice plants imparted dehydration stress tolerance. Transgenic rice plants over expressing the above gene showed growth rate stability, as well as better recovery of growth compared to control plants under conditions of water deficit stress (Xu *et al.,* 1996). Transgenic wheat expressing the *HVA1* showed increased drought tolerance, with biomass productivity (Sivamani *et al.,* 2000). Under field conditions the *OsLEA3-1* gene was found overexpressed in rice plants and showed higher grain yield than the wild-type during water deficit stress

(Xiao *et al.,* 2007). A higher survival rate was observed in transgenic Arabidopsis plants with *BnLEA4-1* gene (Dalal *et al.,* 2009) whereas, in transgenic poplar the expression of the *TaLEA* gene improved cell membrane protection (Gao *et al.,* 2013). Enhanced tolerance to salinity and drought was reported in tobacco plants expressing *Rab16A* LEA gene from salttolerant rice (Roy Choudhury *et al.,* 2007). Reduced loss of water under drought stress was observed in transgenic plants of *Salvia miltiorrhiza* overexpressing the *SmLEA* gene (Wu *et al.,* 2014). Over-expression of *SiLEA14* gene in foxtail millet enhanced tolerance to osmotic stress, and also it contributed to the increase of free proline and soluble sugar content against drought stress (Wang *et al.,* 2014). Under drought stress, transgenic plants of Arabidopsis overexpressing the *JcLEA* gene showed higher relative water content and less damage to the cell membrane, compared to control plants (Liang *et al.,* 2013).

2.2.11. Drought stress studies in *Hevea*

Drought stress has been reported to affect growth and yield performances of *Hevea* (Sethuraj *et al.,* 1984; Huang and Pan, 1992; Sreelatha *et al.,* 2007; 2011). Increased atmospheric temperatures concomitant with high light and low relative humidity during peak summer season affected the overall performance of the crop (Chandrasekhar *et al.,* 1990; Devakumar *et al.,* 1998; Jacob *et al.,* 1999). Gas exchange parameters have been reported to be severely inhibited under drought stress. Sumesh *et al.* (2011) observed that clone RRIM 600, a relatively drought tolerant clone had lesser inhibition of photosynthesis compared to clone RRII 414, a relatively drought susceptible clone. Increased activity of peroxidase and ascorbate peroxidase along with decreased activity of polyphenol oxidase were associated with drought tolerance in *Hevea* (Sreelatha *et al.,* 2003). Investigations related to biochemical parameters indicated that the metabolic

activity was inhibited in clone RRII 105 during drought stress (Sreelatha *et al.,* 2007). Under drought condition increased SOD expression and significant reduction in oxidative stress was observed in the *Hevea* transgenic plants over expressed with SOD gene compared to control plants (Jayashree *et al.*, 2011). The evaluation of modern *Hevea* clones, using physiological and biochemical parameters revealed better drought tolerance potential of RRII 430 (Thomas *et al.*, 2014) among the RRII 400 series clones.

Reports on *Hevea* clones revealed the occurrence of altered level of expression of drought responsive genes in imparting drought tolerance. Genes such as *peroxidase, WRKY tf* and *LEA 5* proteins were reported to have stronger association with drought tolerance in *Hevea* (Thomas *et al.,* 2011; 2012; Sathik *et al.,* 2012; Luke *et al.,* 2015). Significant up-regulation of genes related to ROS scavenging systems and energy biosynthesis including *HbMnSOD, HbCuZnSOD, HbAPX, HbCAT, HbCOA, HbATP*, and *HbACAT* under drought stress was reported (Wang, 2014). Recent expression studies on MicroRNAs of *Hevea* 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). Annamalinathan *et al.* (2006; 2010) studied the chloroplastic factors responsible for drought tolerance in *Hevea* and reported that under drought stress, a novel 23 kDa chloroplast thylakoid protein was induced in young plants of rubber. Jun *et al.,* (2015) studied the expression of sHSP gene *HbsHSP23.8* from *Hevea* for chloroplast localized sHSP23.8 and observed that they enhanced tolerance to environmental stresses including drought in rubber plants. Pramod *et al.* (2017) reported that the expression of *HbsHSP23.8* in *Hevea* was significantly up-regulated by drought conditions. Gene expression analysis and transcriptome sequencing

of several genes facilitated the unravelling of the molecular factors involved in enhancing drought tolerance (Sathik *et al.,* 2018; Luke *et al.,* 2015; 2017). Several transcripts were found associated with drought tolerance which can be further used as markers for early screening for drought tolerance.

2.2.12. Evaluation of germplasm accessions for drought tolerance.

The wild *Hevea* germplasm accessions remains as an invaluable reservoir of genetic diversity. A large number of germplasm accessions have been identified with abiotic stress tolerance. Studies have been conducted for screening *Hevea* germplasm accessions for drought tolerance (Nair *et al.*, 2005; 2011; Mercy *et al.*, 2010; 2013; Thomas *et al.*, 2015). An early evaluation for drought tolerance using dry matter production and growth reported wide variability among *Hevea* wild accessions (Mercy *et al.*, 2006). An *in vitro* screening of *Hevea* germplasm accessions for intrinsic drought tolerance traits was conducted under 60% PEG incubation in presence of high light, using chlorophyll fluorescence, quantum yield of PS II and Fv'/Fm'. The results showed significant variation for these parameters among the wild accessions with less reduction in tolerant accessions (Nair *et al.*, 2005). A preliminary field evaluation of *Hevea* germplasm accessions was performed in the drought prone regions of Dapchari in Maharashtra. The response of these accessions towards drought stress was studied using the parameters like plant height, girth, number of whorls and leaves, leaf senescence and relative water content. Significant variations among *Hevea* germplasm accessions were observed and a few accessions were identified as tolerant accessions (Mercy *et al.*, 2010). Field screening of a set of 130 wild accessions was conducted along with four check clones for a period of six years at the drought prone area of Dapchari in Maharshtra. Growth performance of these accessions in the drought prone situation was assessed in terms field observations of plants and fourteen potential accessions were

identified as drought tolerant. The studies were continued to assess the yield potential of these accessions under drought stress (Mercy *et al.*, 2013). Nair *et al.* (2011) have conducted the evaluation of large number of germplasm accessions during summer under high light and drought stress conditions. Based on the extent of leaf yellowing and senescence, the germplasm accessions were ranked as top, middle and bottom level. The top and bottom ranked accessions were further subjected to *in vitro* stress using PEG and high light and PS II activity was studied. A drastic reduction in PS II quantum yield was observed in bottom ranked accessions. A direct relationship between percentage leaf yellowing and inhibition of PS II activity was also established (Nair *et al.*, 2011).

Screening of germplasm accessions for drought tolerance potential were reported generally on the basis of different physiological studies. Drought tolerance traits in plants associated with free radical scavenging system comprises of antioxidant enzymes, metabolites, osmolytes, protective proteins *etc.* and molecular responses. The expression of drought responsive genes was also widely reported, which were not yet studied in *Hevea* germplasm accessions. Hence, it is imperative to evaluate *Hevea* germplasm accessions for physiological, biochemical and molecular factors which were already reported to be drought tolerance traits in elite clones. This study attempted to validate various physiological and biochemical traits as well as expression analysis of several drought responsive transcripts in *Hevea* germplasm accessions which would further facilitate utilizing them as markers for early screening of drought tolerance. The present study would further enable the identification of a few drought tolerant germplasm accessions of *Hevea.*

Chapter 3

Screening and ranking a set of wild *Hevea* germplasm accessions for drought tolerance

3.1. Introduction

Hevea brasiliensis, which is the most important commercial source of natural rubber (NR), is widely cultivated in South-East Asian countries like Malaysia, Thailand, Indonesia, India, China, Sri Lanka, Vietnam, *etc.* Being an important raw material of automobile industry, the global demand of NR is found increasing day by day. The traditional rubber growing regions in India include Kerala state and Kanyakumari district of Tamil Nadu where the climatic conditions are more favourable for NR cultivation. In India, NR cultivation in the traditional areas is facing constraints in cultivable land availability and competition from other crops, and hence its cultivation needs to be extended to the non-traditional regions in India, which include drought prone areas such as North Konkan regions, parts of Karnataka, Odisha, Madhya Pradesh and low temperature prevailing areas in the North-Eastern states. Extending NR cultivation to these non-traditional regions requires identification of suitable climate resilient clones that can perform well in such regions.

Drought stress which occurs due to soil and atmospheric moisture deficiency is one of the most significant environmental factor that affects plant growth and productivity (Oberoi *et al.*, 2014). Under field conditions, water deficit stress is usually accompanied by high temperature and high light stresses. Plants have evolved various physiological and biochemical adaptations to adjust and adapt to different environmental stress conditions

(Osakabe *et al.,* 2014). Plant responses to drought stress is a complex phenomena which include various aspects like stress sensing and signaling, changes in growth and biomass allocation patterns, decreased stomatal conductance and CO_2 assimilation, osmoregulation and detoxification processes *etc.* (Chaves *et al.*, 2003). Plants respond to drought primarily by closing the stomata resulting in reduction of water loss and restriction of CO_2 diffusion leading to substantial reduction in assimilation (Lawlor and Tezara, 2009). Silva *et al.* (2007) has shown that parameters like chlorophyll fluorescence, chlorophyll index and leaf temperature can be used as potential tools for rapid and non-destructive screening for drought tolerance in crop species. Water deficit stress have a direct impact on the photosynthetic apparatus, as it disrupts all major components of photosynthesis including thylakoid electron transport, carbon reduction cycle and stomatal control of the CO_2 supply, together with an increased accumulation of carbohydrates and disturbance of water balance (Allen and Ort, 2001).

Chlorophyll is one of the major chloroplast components for photosynthesis, and relative chlorophyll content always has a positive correlation with the rate of photosynthesis. The reduction in chlorophyll content under dehydration stress has been considered as an indication of oxidative stress as well as photo-oxidation of pigments and chlorophyll degradation. Photosynthetic pigments in plants are essential for harvesting light and production of reducing powers. Both the chlorophyll *a* and *b* are reported to be photo oxidized under soil moisture deficit condition (Farooq *et al.*, 2009). Under water stress condition, photosynthetic pigments and electron transport components were altered (Anjum *et al.*, 2003), photosynthetic apparatus damaged (Fu and Huang, 2001) and activities of enzymes in Calvin cycle also diminished which ultimately lead to reduction in crop yield.

Cuticular wax, is considered as a protective layer in many plant species, and it plays significant role in plant abiotic and biotic stress tolerance and involved in defence mechanisms against high temperature, excessive ultraviolet radiation, high salinity, low temperature, pests, bacterial and fungal pathogens (Xue *et al.,* 2017). Relative quantity of epicuticular wax on the leaf surface is reported to be an important parameter associated with drought and heat tolerance in coconut genotypes (Rajagopal *et al.*, 1990).

Wild relatives of cultivated species are potential source of drought tolerance in different crop species (Shimshi *et al.*, 1982). The wild accessions of *Hevea* (about 4500) collected from its primary centre of origin, the Amazon forests, is an excellent repository of various useful traits including drought tolerance. Many *Hevea* germplasm lines have been already identified with moderate to good drought tolerance potential (Nair *et al.*, 2005; Mercy *et al.*, 2009; 2010). Acclimation of plants to water deficit is the result of diverse events, which lead to adaptive changes in plant growth and physio-biochemical processes, such as changes in plant growth rate, tissue osmotic potential and antioxidant defence mechanisms (Duan *et al.*, 2007). The susceptibility or tolerance of plants to drought stress varies in accordance with degree of stress, different accompanying stress factors, genotypical variation in species, and their developmental stages (Demirevska *et al.,* 2009). Great differences are observed within species in their tolerance towards drought stress and hence the screening of *Hevea* germplasm accessions for drought tolerance is worth for identifying its drought tolerance potential. An understanding of the physiological, biochemical and genetic mechanisms allowing plants to cope with environmental challenges is of vital importance towards developing crops with improved stress tolerance traits (Langridge and Reynolds, 2015). In the present study, attempts were

made to evaluate the drought tolerance potential of 18 *Hevea* germplasm accessions using specific physiological and biochemical parameters.

3.2. Materials and Methods

3.2.1. Plant material and stress induction

Six month old polybag plants of 18 (14 relatively drought tolerant and 4 susceptible) germplasm accessions, short listed based on extent of leaf yellowing and leaf lamina scorching, were selected for this study along with relatively drought tolerant (RRIM 600 and RRII 430) and susceptible (RRII 105 and RRII 414) check clones. The plants (n=4) were subjected to water deficit stress by withholding irrigation for 7 days during summer season. The net CO_2 assimilation rate (A) and chlorophyll fluorescence were measured using portable photosynthesis system (LI-6400, LI-COR, USA) at a fixed CO_2 concentration of 400 ppm. Light intensity of 500 μmol m^{-2} s^{-1} was provided using the leaf chamber fluorometer (LCF – 40, Li-COR, USA) attached to the photosynthesis system (Li-6400, Li-COR).

Leaf samples were collected on the seventh day of drought imposition for biochemical analyses. Physiologically mature leaves were collected from the selected plants for estimation of pigments and epicuticular wax contents. The leaf epicuticular wax content was determined according to the method described by Ebercon *et al.*, 1977. Total cholophyll was estimated by the method of Arnon (1949) and the carotenoids were estimated according to Lichtenthaler (1987).

3.2.2. Estimation of leaf epicuticular wax

The leaf epicuticular wax content was determined following the method of Ebercon *et al.*, 1977. Leaf discs with 2.5 cm diameter were taken and leaf epicuticular wax was extracted by immersing in chloroform for 20 seconds. The extract was then evaporated on a boiling water bath. After

evaporation, 5 ml of potassium dichromate ($K_2Cr_2O_7$) reagent was added and then kept in a boiling water bath for 30 min. The reaction mixture was allowed to cool and then 12 ml of de ionized water was added to each tube. Mixed thoroughly and allowed few minutes for colour development. The optical density was measured spectrophotometrically at 590 nm. The wax content was calculated using carnauba plant wax as standard and the leaf epicuticular wax was expressed in wax load per unit area of leaf ($\mu g\ cm^{-2}$).

3.2.3. Estimation of chlorophyll

Total chlorophyll content was estimated by the method of Arnon (1949). The chlorophylls were extracted in Acetone: Dimethyl sulphoxide (1:1) solution. Leaf discs were 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. The chlorophyll contents were calculated using the following equation and were expressed in mg $cm^{-2.}$

Calculations:

Chlorophyll *a*: (12.7_{A663})-$(2.69_{A\ 645})$

Chlorophyll *b*: (22.9_{A645})-$(4.68_{\ A\ 663})$

Total Chlorophyll: $(20.2_{\ A645} + 8.02_{A\ 663})$

3.2.4. Estimation of carotenoids

Total carotenoids contents were estimated by the method of Lichenthaler (1987). The total carotenoids were extracted in Acetone: Dimethyl sulphoxide (1:1) solution. The following calculations were used for estimation of carotenoids.

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

Significance of genotypic difference for different parameters was worked out statistically by ANOVA using Cropstat V.7.2. Software. The

accessions were evaluated for their drought tolerance potential by ranking them using ranksum method.

3.3. Results

Different parameters associated with drought tolerance traits were studied in *Hevea*germplasm accessions after subjecting them to water deficit stress for a period of one week. These accessions were selected from a previous study on screening of germplasm lines based on extent of leaf yellowing (Nair *et al.*, 2011). The wild accessions used in this study were found to have varied response to the different drought related parameters *viz.* photosynthetic rate, maximum quantum yield, leaf wax content and pigments. The results are given in Table 3.1.

Table 3.1. CO_2assimilation rate (A), maximum quantum yield of PSII (Fv/Fm), total chlorophyll, carotenoids and epicuticular wax content in *hevea* germplasm accessions after drought stress imposition for seven days

Sl. No.	Clones/ Accessions	A (µmol m^{-2} s^{-1})	Fv/Fm	Chlorophyll (mg cm^{-2})	Carotenoids (mg cm^{-2})	Wax (µg cm^{-2})
1	**RRII 105**	1.28	0.785	0.0485	0.01773	38.27
2	**RRIM 600**	2.41	0.803	0.0379	0.05813	57.50
3	**RRII 414**	0.26	0.484	0.0332	0.01620	37.16
4	**RRII 430**	2.06	0.750	0.0468	0.01843	53.91
5	**RO 2432**	4.06	0.786	0.0404	0.01643	79.59
6	**RO 3157**	3.46	0.814	0.0533	0.02327	91.13
7	**RO 3976**	1.28	0.773	0.0405	0.01543	49.36
8	**RO 3261**	7.51	0.803	0.0647	0.02707	68.28
9	**RO 1425**	3.11	0.754	0.0539	0.02097	53.78

10	**MT 1619**	1.43	0.743	0.0291	0.01240	32.53
11	**AC 2009**	2.18	0.773	0.0414	0.01807	56.77
12	**MT 196**	1.21	0.805	0.0368	0.01643	42.48
13	**AC 612**	5.38	0.812	0.0702	0.02577	64.58
14	**RO 2360**	0.90	0.773	0.0329	0.01453	35.29
15	**MT 2210**	1.36	0.790	0.0363	0.01447	52.17
16	**AC 173**	2.84	0.769	0.0366	0.01690	48.18
17	**RO 1406**	5.14	0.816	0.0430	0.01717	71.51
18	**RO 3184**	4.00	0.811	0.0558	0.02027	61.70
19	**AC 4149**	1.73	0.780	0.0367	0.01727	39.24
20	**RO 2286**	2.15	0.718	0.0365	0.01413	44.70
21	**AC 4084**	0.63	0.715	0.0442	0.01670	27.26
22	**RO 3242**	0.00	0.790	0.0302	0.01157	30.47
CD (P≤ 0.05)		1.92	0.0134	0.051	0.02566	12.62

The data on CO_2 assimilation rate (A) is given in Table 3.1 and Fig.3.1. The rate of photosynthesis in the genotypes under drought conditions varied significantly which ranged from zero to 7.51 μmol m^{-2} s^{-1}. The accession RO 3261 recorded the highest and RO 3242 the lowest A. Eight accessions showed higher photosynthetic rate than the drought tolerant check clone RRIM 600. The data on maximum quantum yield of PSII (Fv/Fm) is given in Table 3.1 and Fig.3.2. The accessions showed significant genotypic difference for Fv/Fm. The accession RO 1406 recorded the highest and AC 4084 the lowest values. Two accessions showed higher Fv/Fm than the relatively tolerant clone RRIM 600.

The data on photosynthetic pigments (total chlorophyll and carotenoids) are given in Table 3.1 and Figs. 3.3 and 3.4. The accessions showed significant genotypic difference for total chlorophyll content in leaves. The accession AC 612 recorded the highest where as MT 1619 recorded the lowest chlorophyll content. The check clone RRIM 600 recorded the highest carotenoid content. Among the accessions, RO 3261 and RO 3242 recorded the highest and the lowest carotenoid contents respectively. The variability present among the accessions for the epicuticular wax content (ECW) is shown in Table 3.1 and Fig. 3.5. The total wax content of the genotypes varied significantly which ranged from 91.13 μg cm^{-2} to 27.26 μg cm^{-2}. The highest ECW content was present in the accession RO 3157 (91.13 μg cm^{-2}) followed by RO 2432 (79.59 μg cm^{-2}) where as the lowest was in the accession AC 4084 (27.26 μg cm^{-2}). Six accessions showed higher wax content than RRIM 600.

3.3.1. Ranking of accessions

The accessions were ranked based on five different parameters *viz.* photosynthetic rate, maximum quantum yield of PSII, total chlorophyll, carotenoid and epicuticular wax content. The ranksum for the accessions and their ranks are shown in Table 3.2. Five accessions (RO 3261, AC 612, RO 3157, RO 3184 and RO 1406) scored higher ranks than the drought tolerant check clone RRIM 600. The accessions RO 3242 and MT 1619 were the accessions which came in the bottom ranks (more susceptible). The top three accessions RO 3261, AC 612 and RO 3157 were selected as potential accessions with relatively better drought tolerance capacity.

Table.3.2. Ranking of selected genotypes of *Hevea brasiliensis* based on parameters related to drought tolerance

Clones / Accessions	A	Fv/Fm	Chlorophyll	Carotenoids	Wax	Total	Rank
RO 3261	21	20	20	20	18	99	1
AC 612	20	20	21	19	17	97	2
RO 3157	16	20	17	18	21	92	3
RO 3184	17	20	19	16	16	88	4
RO 1406	19	21	13	11	19	83	5
RRIM 600	13	19	9	21	15	77	6
RO 1425	15	15	18	17	12	77	6
RO 2432	18	18	10	8	20	74	7
RRII 430	10	15	15	15	13	68	8
AC 2009	12	15	12	14	14	67	9
AC 173	14	18	6	10	9	57	10
RRII 105	6	16	16	13	5	56	11
AC 4149	9	18	7	12	6	54	12
RO 3976	6	17	11	5	10	49	13
MT 196	4	19	8	7	7	45	14
MT 2210	7	19	4	3	11	44	15
RO 2286	11	13	5	2	8	39	16
AC 4084	2	13	14	9	0	38	17
RRII 414	1	12	13	6	4	36	18
RO 2360	3	17	1	4	3	28	19
MT 1619	8	14	0	1	2	25	20
RO 3242	0	18	1	0	1	20	21

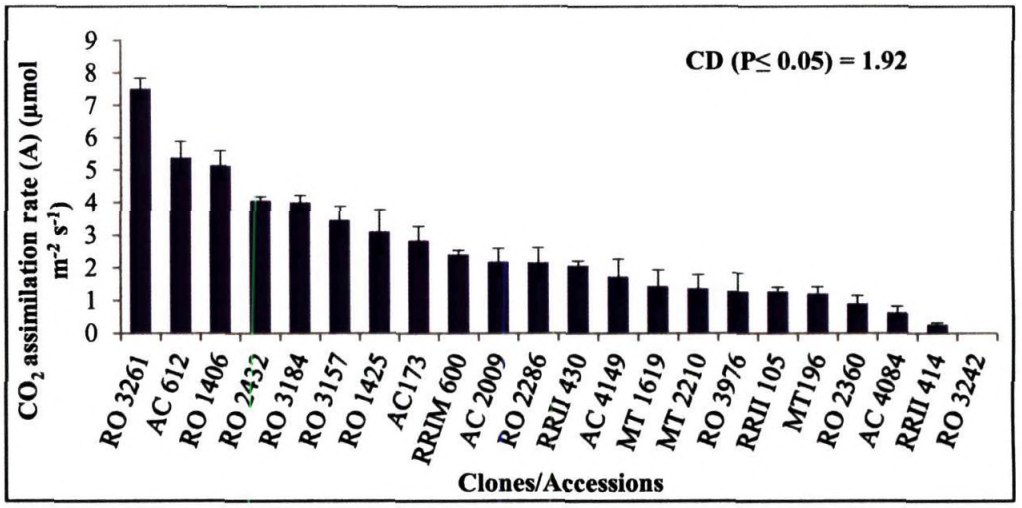

Fig. 3.1. CO_2 assimilation rate (A) in different *Hevea* genotypes after drought imposition for seven days

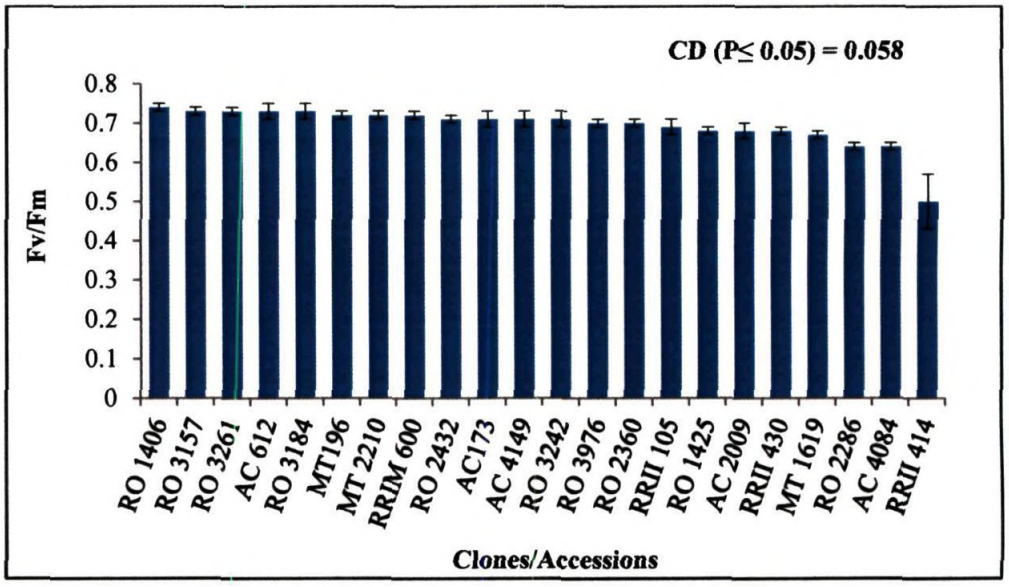

Fig. 3.2. Maximum potential quantum yield of PS II (Fv/Fm) in different *Hevea* genotypes after drought imposition for seven days

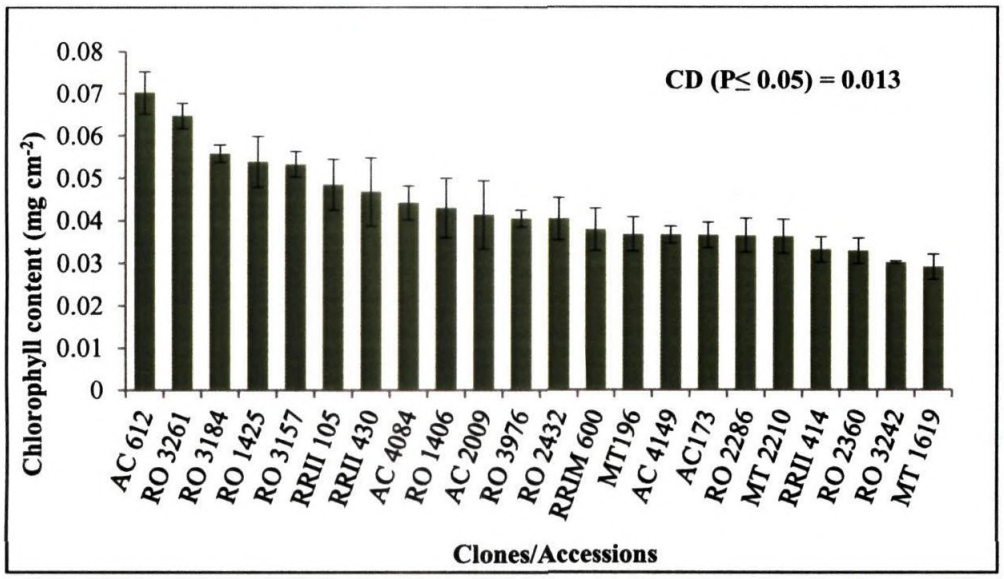

Fig. 3.3. Total chlorophyll in different *Hevea* genotypes after drought imposition for seven days

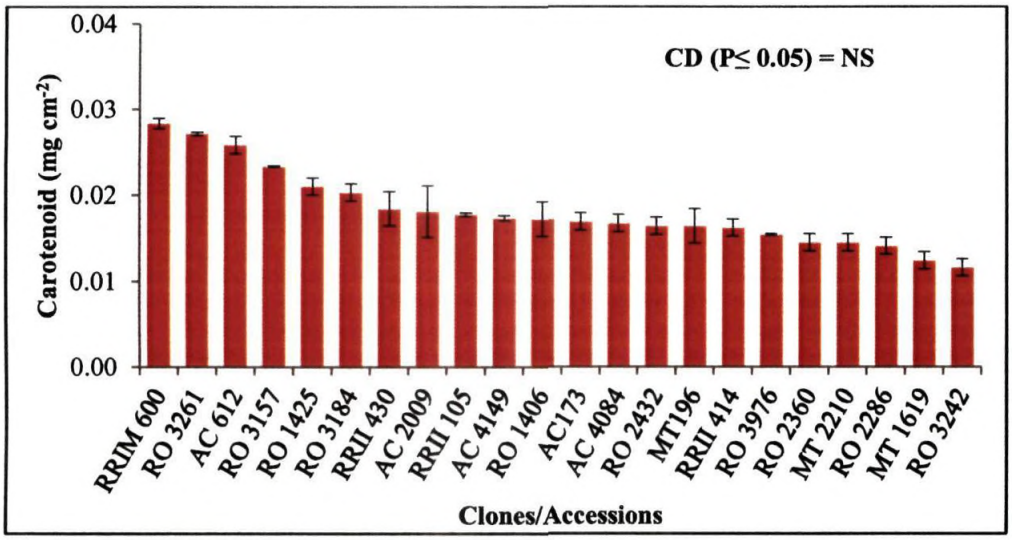

Fig. 3.4. Carotenoid content in different *Hevea* genotypes after drought imposition for seven days

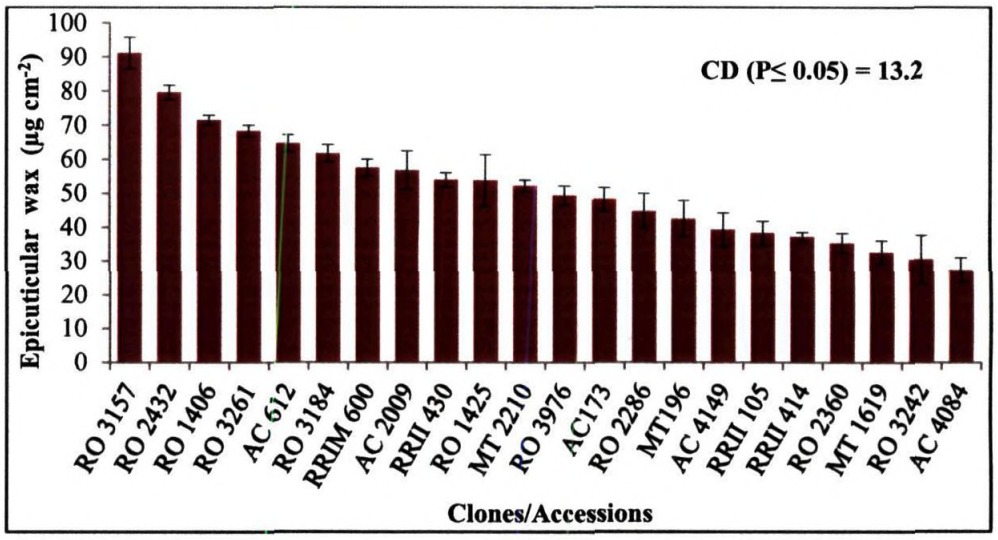

Fig. 3.5. Epicuticular wax content in different *Hevea* genotypes after drought imposition for seven days

3.4. Discussion

Drought tolerance is found largely varying among genotypes within crop species (Siddiqui *et al.,* 2015; Yang *et al.,* 2015). Significant genotypic differences for various drought related characters were noticed in *Hevea* (Nazeer *et al.*, 1992). Decreased growth rate under drought stress conditions is more pronounced in stress sensitive plants than in tolerant ones (Demiral and Turkan, 2005). Measurement of gas exchange and chlorophyll fluorescence parameters in plants during water and high temperature stresses showed that water deficit caused a significant decrease (17-20%) in the rate of CO_2 uptake and O_2 evolution (Yordanov *et al.*, 1998). During stress period, absorption of light is likely to exceed that required for photosynthetic assimilation and can lead to photo inhibition of the photosynthetic apparatus (Methy *et al.*, 1996; Jacob *et al.*, 1999). Drought tolerance capacity has been found to vary among different *Hevea* clones (Annamalainathan *et al.*, 2010).

Photosynthetic rate is greatly influenced by adverse environmental conditions such as water deficit and high solar light. Lower photosynthetic ability is one of the reasons responsible for reduction in crop productivity.

Under drought stress, plants close their stomata to avoid further water loss which may ultimately results in reduced CO_2 assimilation. A decrease in internal CO_2 concentration (Ci) and ATP synthesis along with inhibition of ribulose-1, 5-bisphosphate carboxylase/oxygenase enzyme activity lead to a decrease of net photosynthetic rate under drought stress (Tezara *et al.,* 1999; Silva *et al.,* 2010; Dulai *et al.,* 2006). Reduction in the level of photosynthetic pigments chlorophyll *a* and *b* and accessory pigment carotenoids will also lead to reduced photosynthetic rate as they will drive a cutdown in energy consumption and carbon demand for chlorophyll synthesis in stressed plants and this directly affect plant biomass production (Silva *et al.,* 2010; Chaves *et al.,* 2009, Zhang *et al.,* 2012). Photosynthetic gas exchange parameters in *Hevea* showed a declining trend under water deficit stress conditions (Sangsing *et al.*, 2004; Alam *et al.*, 2006; Sumesh *et al.*, 2011). Drought induced reduction in photosynthetic rate in field grown *Hevea* plants has earlier been reported by Devakumar *et al.* (2002) and Alam *et al.* (2006). The present study is in agreement with the earlier reports showing reduction in CO_2 assimilation rate (A) among the genotypes. The reduction was found less in the tolerant accessions RO 3261, AC 612 and RO 1406.

Several studies in *Hevea* reported the negative impact of high light intensity and high temperature on overall performance of the clones in drought prone areas (Nair *et al.*, 1998; Jacob *et al.*, 1999). The maximum potential quantum yield is an important and reliable measure of the structural and functional integrity of PSII under a given environmental condition. Nair *et al.* (2005) used this as a reliable parameter for screening drought tolerance potential of wild *Hevea* germplasm accessions. In this study also the accessions showed significant genotypic difference for Fv/Fm and the higher value was observed in RO 1406.

Environmental stresses such as drought, lead to the degradation of photosynthetic pigments (Parida and Das, 2005). Monitoring the reduction in the contents of photosynthetic pigments in drought stressed plants can be used as a biomarker of stress (Schiop *et al.,* 2015). Numerous reports are available on decreased levels of chlorophylls under water deficit stress in different species (Al Hassan *et al.,* 2015; Yang *et al.,* 2015). The chlorophyll level was found higher in the accessions AC 612 and RO 3261. The results obtained are in agreement with previous reports, and the genotypes showed significant difference under drought stress. Cartenoids are essential components in the photosynthetic apparatus in plants where they protect against photo oxidative damage and contribute to the light harvesting in photosynthesis (Goodwin, 1980). However in this study carotenoids did not show any significant difference among the genotypes studied. The clone RRIM 600 showed higher level of carotenoids among the clones and accessions studied.

Researchers suggest that cuticular wax is important in maintaining plant water status in various species (Jetter *et al.*, 2006; Yeats and Rose, 2013; Lee and Suh, 2015). Jefferson *et al.* (1988) observed increased wax production in drought stressed alfafa plants and identified it as a potential drought resistance selection criterion. The role of ECW in the maintenance of water balance has been reported in different crops (Rajagopal *et al.*, 1990; Zhang *et al.*, 2005) and in wild accessions of *Hevea* (Nair *et al.*, 2005). Higher wax content helps in the adaptation of the plant to drought conditions by reducing the stomatal conductance and transpiration rate (Rao *et al.*, 1988). In this study also high epicuticular wax deposition was observed in tolerant genotypes under water deficit stress. The accessions RO 3157, RO 3242 and RO 1406 showed higher levels of epicuticular wax content.

3.5. Conclusions

Identifying suitable germplasm accessions with stress tolerance and yield sustainability is important to enhance crop productivity. In this study, a set of 18 *Hevea* germplasm accessions short listed based on previous observations and were further evaluated for their drought tolerance potential using different physiological and biochemical parameters. The *Hevea* germplasm accessions showed significant variation for CO_2 assimilation rate (A), Fv/Fm, total chlorophylls and epicuticular wax contents. The data revealed a wide genetic variability among the accessions as indicated by a wide range obtained for these parameters in the ranking. Based on the study, six accessions RO 3261, AC 612, RO 3157, RO 3184, RO 1406 and RO 1425 were selected as top ranked drought tolerant accessions. The bottom ranked accessions RO 3242, MT 1619, RO 2360 and AC 4084 were selected as drought susceptible accessions. The results indicated that accessions RO 3261, AC 612 and RO 3157 are the top three drought tolerant accessions among the lines studied.

Chapter 4

Analyses of traits associated with drought tolerance in shortlisted *Hevea* germplasm accessions

4.1. Introduction

In plants, water deficit stress alone or in combination with high light intensity or other environmental stresses, disrupt photosynthesis and alters the normal homeostasis of cells which in turn leads to an increased production of reactive oxygen species (ROS). ROS play a dual role in plants in response to abiotic stresses; functioning as toxic by-products of stress metabolism, as well as important signal transduction molecules (Miller *et al.*, 2010). Reactive oxygen species are continuously produced in the cells as normal by-product of plant cellular metabolism. ROS production is localized in different cellular compartments including the chloroplasts, peroxisomes and mitochondria, the vital organelles with high oxidizing metabolic activities and intense rate of electron flow (Boguszewska and Zagdańska, 2012). During water deficit stress, prolonged exposure will inevitably result in oxidative damage due to over production and accumulation of reactive oxygen species (de Carvalho, 2008). The most common forms of ROS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) and each of them possess specific half-life as well as oxidizing potential. ROS molecules can be extremely reactive and toxic when accumulated in excessive levels and are responsible for various stress induced damages to macromolecules and cellular structure including enzyme activities, membrane lipid peroxidation, protein oxidation, RNA and DNA damage *etc.* Unrestricted oxidation of these cellular

components will ultimately results in cell death (Mittler *et al.,* 2002; Apel and Hirt, 2004). Hence, scavenging of ROS is necessary to protect the subcellular components as well as maintenance of normal growth and development.

In order to avoid the accumulation of reactive oxygen species to toxic level, animals and plants possess several detoxifying mechanisms that comprise a variety of antioxidant molecules and enzymes. The plant defence mechanisms against the deleterious effects of ROS can be classified as enzymatic and non-enzymatic systems. The enzymatic system includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione S-transferase (GST), dehydroascorbate reductase (DHAR), mono dehydroascorbate reductase (MDAR) *etc.* (Apel and Hirt 2004; Mittler *et al.* 2011; Ren *et al.* 2016). Whereas the non enzymatic system comprises of ascorbic acid (AsA), glutathione (GSH), carotenoids (CAR), α-tocopherol (vitamin E), anthocyanins *etc.* (Gill and Tuteja, 2010; Kaur *et al.,* 2013; Mattos and Moretti, 2015). Both of these plant defence mechanisms have been detected in most of the cellular compartments, showing the importance of ROS detoxification for cellular survival.

Relative water content (RWC) is considered as a measure of plant water status, which reflects the metabolic activity in tissues and the exposure of plants to dehydration stress decreases relative water content (Anjum *et al.,* 2011). Water deficit stress also affects photosynthesis as well as it leads to changes in the level of plant pigments like chlorophyll and carotenoid contents. Chlorophyll is one of the major chloroplast component of photosynthesis, and the decrease in chlorophyll content under drought stress has been considered a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation. Both

chlorophyll *a* and *b* are prone to soil moisture deficit and dehydration stress (Farooq *et al.,* 2009). Anthocyanins are water-soluble non photosynthetic pigments derived from flavonoids. They may be induced by various environmental factors including visible and UV-B radiation, cold temperatures, dehydration stress *etc.* Anthocyanins are found localized in leaf, stem and roottissues, which in turn help the plant to develop resistance to diverse abiotic stresses (Chalker-Scott, 1999).

Osmotic adjustment is another major plant response to water stress which involves the accumulation of low molecular weight water-soluble compounds known as osmolytes. The most common osmolytes or compatible solutes are polyols, sugars, aminoacids like proline, and quaternary ammonium compounds like glycine betaine. These compounds play an important role in stabilizing proteins and cell membranes as well as in maintaining cell turgor (Rodziewicz *et al.*, 2014). Proline accumulation has been reported in various biotic and abiotic stress conditions which include salinity, drought, high and low temperatures, UV radiation, heavy metal, pathogen infection, nutrient deficiency and atmospheric pollution (Hare and Cress, 1997). Glycine betaine has been reported to be involved in the protection of photosynthetic machinery, inhibition of ROS accumulation, maintaining membrane integrity and activation of some stress related genes (Chen and Murata, 2008). It maintains enzyme activity by the protection of quaternary structure of proteins from damaging effects of environmental stresses (Sakamoto *et al.*, 2002). The tolerant or susceptible species shows varying levels of stress responses depending on the level of accumulation of these compounds under different abiotic stress conditions (Giri, 2011; Chen and Murata, 2008).

A group of proteins called heat shock proteins (HSP) constitute a large family of proteins which are found highly expressed under stress

conditions. They have major roles in maintaining cellular homeostasis both under stress and under optimal growthconditions. Among the HSP, a sHSP or LMW HSP class (12-42 kDa) are ubiquitous molecular chaperones which are predominantly expressed under dehyration conditions (Hsieh *et. al.,* 1992). Annamalainathan *et al.* (2006) reported the presence of a consistently over expressing chloroplast stress protein (~23 kDa) and was found associated with drought responses in young plants of *Hevea brasiliensis.*

Plant nuclear DNA is highly unstable and can be damaged by various factors like UV-B radiation, low and high temperature, salinity, desiccation, ozone and air and soil pollutants. The over production and accumulation of reactive oxygen species(ROS) as by-products of normal cellular metabolism or asa result of various abiotic stress conditions can cause damage to DNA in the cell (Biedermann *et al.,* 2011; Petrov *et al.,* 2015). Increased accumulation of ROS may eventually lead to fragmentation of DNA followed by programmed cell death (PCD). The cleavage of DNA at internucleosomal sites, which generates oligonucleosomal fragments leads to the formation of DNA ladders, multimers of 170 to 200 bp, which can be detected on agarose gels (Stein and Hansen, 1999). DNA fragmentation has been observed in plants under high light and drought stress (Wituszynska and Karpinski, 2013).

The present study aims at evaluating the activity of antioxidant enzymes, non enzymatic antioxidants, osmolytes, photosynthetic and non photosynthetic plant pigments as well as expression of 23 kDa chloroplast stress protein in irrigated and drought stressed wild *Hevea* germplasm accessions. The accessions chosen were six top ranked (relatively drought tolerant) and four bottom ranked (relatively drought susceptible) based on the screening of 18 germplasm accessions studied in chapter 3. An *in-vitro* study was also performed to analyze high light and osmotic stress induced

fragmentation of genomic DNA in elite clones as well as in wild *Hevea* germplasm accessions.

4.2. Materials and Methods

4.2.1. Plant material and stress induction

Six month old polybag plants of 10 *Hevea* germplasm accessions (six relatively drought tolerant - RO 3261, AC 612, RO 3157, RO 3184, RO1406, RO 1425 and four susceptible - RO 3242, MT 1619, RO 2360, AC 4084) were selected for this study along with relatively drought tolerant (RRIM 600 and RRII 430) and susceptible (RRII 105 and RRII 414) check clones (Sumesh *et al.*, 2011). The plants grown in glass house were subjected to water deficit stress by with holding irrigation for seven days during summer season. A set of control plants were also maintained by irrigating on alternate days.The magnitude of water stress was assessed by measuring the CO_2 assimilation rate (A) and stomatal conductance (g_s) using a portable photosynthesis system (LI-6400, LI-COR, USA).

The leaf water status was assessed by analyzing the relative water content (RWC). Leaf samples were collected on the seventh day of drought imposition for biochemical analyses. Physiologically mature leaves were collected from selected plants (n=4) for the estimation of chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoids, anthocyanins,epicuticular wax, proline, glycine betaine, superoxide dismutase, total peroxidase, ascorbic acid and thiols.The differential expression of chloroplast stress protein in germplasm accessions was analyzed by SDS-PAGE. The presence of this protein was further confirmed by Western Blotting. Integrity of genomic DNA under high light and osmotic stress was analyzed by an *in-vitro* study using leaf discs from *Hevea* clones and wild germplasm accessions.

Cholorophyll was estimated by the method of Arnon (1949) and carotenoids were estimated according to Lichtenthaler (1987). Anthocyanins

were estimated according to Wagner (1979). Free proline content in the leaves was determined following the method of Bates *et al.* (1973). Glycine betaine estimation was done in dried leaf powder as per the method of Grieve and Grattan (1983). All data were statistically analyzed using ANOVA with IBM-SPSS analytical software package version 16.0 (IBM Corporation, USA).

4.2.2. Relative water content (RWC)

Relative water content (RWC) was studied following the method of Barrs and Weatherley (1962). Physiologically mature leaves from the top whorl of both stressed and irrigated plants were used for the analysis. After harvesting leaf samples, leaf discs were taken and weighed. This weight was noted as fresh weight (FW). The leaf discs were kept in a petridish and rehydrated in deionized water for 24 hrs. By the end of incubation period, the moisture on the surface of leaf discs were removed and weighed. This was marked as turgid weight (TW). Leaf discs were then dried overnight at 70°C in oven. After the completion of 24 hrs or attaining consistent stable weight, the leaf discs were allowed to cool down to room temperature and were weighed and marked as the dry weight (DW).

Calculation:

$$RWC = (FW-DW)/(TW- DW) \times 100$$

4.2.3. Estimation of chlorophyll

Chlorophyll content of control and drought stressed plants were estimated by the method of Arnon (1949) as mentioned in 3.2.3.

4.2.4. Estimation of carotenoids

The carotenoid contents of control and drought stressed plants were estimated by the method of Lichenthaler (1987) as explained in 3.2.4.

4.2.5. Estimation of Anthocyanins

Anthocyanin was determined by following the method of Wagner (1979). Leaf discs were kept overnight at 4° C in acidified methanol (0.3% HCl in methanol). It was then filtered and measured the O.D at 550 nm. Anthocyanins were calculated using the extinction coefficient $\varepsilon_{550} = 33,000$ [cm^2/mol] and expressed in μmol/cm^2.

4.2.6. Estimation of leaf epicuticular wax

The leaf epicuticular wax content was determined according to the method described by Ebercon *et al.* (1977) as mentioned in 3.2.2.

4.2.7. Estimation of free proline content

Free proline content in the leaves was determined following the method of Bates *et al.* (1973). 500 mg leaf samples (4 replicas) were ground to fine powder using liquid nitrogen and homogenized with 3 ml 5% sulphosalicyclic acid. The extracts were collected in tubes and centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was used for estimation. 2 ml of supernatant was mixed with 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent. The reaction mixture was then heated in a boiling water bath at 100 °C for 1 hr. After cooling the reaction mixtures, 4 ml of toluene was added and then transferred to a separating funnel. After thorough mixing, the chromospheres containing toluene was separated and its absorbance was read at 520 nm against toluene blank. Proline content in the samples was calculated using proline as standard and was expressed in μg/g FW.

4.2.8. Estimation of glycine betaine

Glycine betaine estimation was done in dried leaf powder as per the method of Grieve and Grattan (1983). Extract was prepared by mechanically shaking finely ground dry plant material (0.5 g) with 20 ml of deionized water for 48 hrs at 25°C. It was then filtered and diluted 1:1 with 2 N

sulphuric acid. 0.5 ml of the extract was cooled in ice water for 1 hr. Cold potassium iodide-iodine reagent (0.2 ml) was added and the mixture was gently mixed with vortex mixture. The samples were stored at 0-4°C for 16 hrs. After that samples were centrifuged at 10,000 g for 15 minutes at 0°C. The supernatant was carefully aspirated with micropipette and discarded. The periodite crystals were dissolved in 9 ml of 1,2-dichloro ethane (reagent grade). Vigorous vortex mixing was done to effect complete solubility in developing solvent. After 2.0-2.5 hrs the absorbancewas measured at 365 nm with UV-visible spectrophotometer. Standards of glycine betaine were prepared in 2 N sulphuric acid and the procedure for sample estimation was followed and GB content was expressed inmg/g DW.

4.2.9. Assay of superoxide dismutase (SOD)

The assay of superoxide dismutase was performed by following the method of Giannopolitis and Ries (1977). 200 mg of fresh leaf tissue was homogenized in 3.5 ml of 50 mM phosphate buffer in a pre-chilled mortar and pestle. ~50 mg polyvinyl pyrrolidone was added during homogenizing. Centrifuged the extract at 15,000 rpm for 20 minutes at 4°C and this extract was used for enzyme assay.

Reagents

1. 50 mM phosphate buffer pH 7.4
2. Na_2CO_3
3. Methionine
4. EDTA
5. Riboflavin
6. Nitroblue Tetrazolium (NBT)

Test: 1.6 ml buffer was pipetted in test tubes and added 0.1 ml sodium carbonate, 0.3 ml methionine, 0.3 ml EDTA, 0.3ml riboflavin and 0.1 ml enzyme.

Control: 1.6 ml buffer was pipetted in test tubes and added 0.1 ml sodium carbonate, 0.3 ml methionine, 0.3 ml EDTA, 0.3ml riboflavin and 0.1 ml enzyme (without light).

Light control: 1.7 ml buffer was pipetted in test tubes and added 0.1 ml sodium carbonate, 0.3 ml methionine, 0.3 ml EDTA, 0.3ml riboflavin (without enzyme).

Test and LC were placed under light for 30 minutes. The control tubes were placed in dark. The OD of test, control and light control were read at 560 nm. First read test, light control and then control.

Calculation:

$$\text{SOD (units)} = \frac{100 - [(\text{OD Sample}/\text{OD LC})*100] / 50}{\text{mg protein}}$$

OD Sample = OD test – OD control.

4.2.10. Assay of total peroxidase

The assay of total peroxidase was done by following the method of Guilbault (1976).

Reagents

1. 50 mM phosphate buffer pH 7.4
2. 0.1 mM H_2O_2
3. O-dianisidine (10 mg/ 10 ml methanol)

The reaction mixture contains 2.5 ml phosphate buffer, 100 µl o-dianisidine and 50 µl enzyme. The reaction was started by adding 50 µl H_2O_2. The

oxidation of o-dianisidine follows and the change in OD at 420 nm for 3 min at 30 sec interval was monitored. A blank without enzyme served as the control. The total peroxidase activity was expressed as change in OD/ min/ mg protein.

4.2.11. Estimation of ascorbic acid.

Ascorbate content was determined according to the modified method of Kampfenkel *et al.,* (1994). 250 mg leaf samples (4 replicas) were ground to fine powder in liquid nitrogen and homogenized in 2 ml 6% trichloro acetic acid and centrifuged at 10,000 rpm for 10 min at 4°C.300 µl of supernatants were taken for estimation. To this added 150µl of 10 mM DTT and 750µl of 0.2 M phosphate buffer (pH-7.4) and incubated at room temperature for 10 min. Then added 100µl of 0.5% N-ethylmaleimide (NEM) to all tubes and mixed well by vortexing. Again incubated at room temperature for 10 min. To each sample then added 600µl of 10% (w/v) TCA, 600µl of 42% (v/v) H_3PO_4, 600µl of 4% bipyridyl dissolved in 70% (v/v) ethanol and 400µl of 3% (w/v) $FeCl_3$. After vortex mixing, samples were incubated at 42°C for 40 minutes and the absorbance at 525 nm was recorded. A set of ascorbic acid standard was performed following the same methodand ascorbic acid content was expressed in mg/g.

4.2.12. Estimation of glutathione

Estimation of glutathione was done by following the method of Boyne and Ellman (1972). For the extraction of glutathione ~ 250 mg of leaf samples (3 replicas) were ground to fine powder using liquid nitrogen and homogenized it with 1.5ml 5% sulphosalicyclic acid. The extracts were collected in tubes and centrifuged at 12,000 rpm for 20 minutes at 4°C. For estimation, 0.1ml of supernatant was pipetted in test tubes and made up to 2ml with 2.5% trichloroacetic acid. 0.1ml DTNB and 2 ml Tris were added

to all tubes. Standards were also made up to 2 ml and treated in the same way. Mixed well and optical density was measured at 412 nm.

4.2.13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Isolation of chloroplasts

Type II (broken) chloroplast were isolated by following the method of Reeves and Hall (1973). The leaf samples were homogenised with liquid nitrogen in a mortar and pestle into powder. During grinding a pinch of crystalline PVP was added. The powdered samples (3 g) were extracted with 5 ml of ice cold grinding buffer consisting of 20 mMTrisHCl (pH 7.8), 10 mMNaCl, 5 mM $MgCl_2$, 350 mM mannitol, 2 mM ascorbate and filtered through four layered cheese cloth and centrifuged at 800 g for 2 min at 4°C. The pellet was removed and the supernatant was spun at 3500g for 5 min at 4°C and the resulting pellet was suspended in 1ml of grinding buffer (pH 7.8) as chloroplast suspension.

Chloroplast protein extraction

The chloroplast suspension (1 ml) was made upto 10 ml using 100% acetone and refrigerated for 1hr inside freezer followed by centrifugation in order to remove pigments and lipids. The pellet was later suspended in 10% TCA (1ml) 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 diethyl-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 used for further analysis.

SDS-PAGE

Isolated chloroplast 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). For resolving gels, 10% polyacrylamide gel was prepared by mixing 12.05 ml of water, 10 ml of 30% acrylamide mixture, 7.5ml 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. After polymerization of stacking gel, the comb was carefully removed from the top of gel. The gel was shifted to a buffer tank and the reservoirs were filled with 1X Tris-Glycine running buffer prepared by mixing 25mM 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 distilled 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 10,000rpm for 2 min to remove un-dissolved materials, if any. The supernatant was loaded in the gel along with standard molecular weight protein marker (Genei, Bangalore) in buffer tank

in the presence of running buffer. 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 distilled water and transferred to staining solution.

Staining and destaining

After electrophoresis, chloroplast protein profiles were visualized by soaking the gel in staining solution containing 0.5g of Coomassie Brilliant Blue R-250 (CBB) in 80 ml of methanol, 100 ml of distilled water and 20 ml of glacial acetic acid for 4 hrs. The gel was then washed with distilled water and destained using destaining solution containing ethanol, distilled water and glacial acetic acid in 40:50:10 ratio till the background is clear and appearance of sharp bands.The electrophoregrams were photographed using gel documentation system.

4.2.14. Western blotting analysis of chloroplast stress protein

Chloroplast proteins (30 μg) were electrophoretically separated using 10% SDS-polyacrylamide gels as described above. Chloroplast protein 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 glassrod

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 distilled 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 horse radish 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 tetramethyl benzidine (TMB)/ hydrogen peroxide (H_2O_2) for localization (Genei, Bangalore).

4.2.15. DNA fragmentation analysis

Genomic DNA isolation

Leaf samples were harvested and leaf discs were incubated in petri dishes containing deionized water. One set was kept in dark was kept at dark at room temperature (25°C), another set was exposed to high light in a growth chamber for three hours. The following conditions were maintained in the growth chamber; light intensity: 1000 μmol m^{-2} s^{-1}, RH: 60% and temperature: 30°C and a third set was kept in the open field for three hours with sun light intensity ranging from 1600 to 1800 μmol m^{-2} s^{-1} and temperature 34°C to 37°C.

The method described by Porebski *et al.,* (1997) with modification (Thomas *et al.,* 2001) was used for the extraction of DNA. The leaf samples were ground to a fine powder in pre-chilled mortar using liquid nitrogen. The powder was scooped into an Oakridge centrifuge tube and mixed with 5mL

pre-warmed (60°C) extraction buffer (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% CTAB pH- 8.0) containing 0.3% beta-mercaptoethanol, which was added to the buffer immediately before use. Following this, 50mg of polyvinyl pyrolidone (sigma) was added. After mixing the contents, the tubes were incubated in a water bath at 60°C for 30 min. Chloroform:isoamyl alcohol (24:1v/v) was added to the tubes when the contents of the tubes attained room temperature. After mixing thoroughly, the tubes were spun at 3000 rpm for 20 min at room temperature. Transferred the top aqueous solution to a new 15 ml centrifuge tubes using wide bore pipette tip. Repeat the extraction using choloroform:isoamylalcohol. Sodium chloride (5M, 0.5 volume of the final aqueous solution recovered) and ice cold ethanol (95%) were added to the extract and mixed well. The tubes were kept at -20°C for 10 min after mixing by inversion and were stored at 4°C overnight.The tubes were spun at 3000 rpm for 6 min and the pellet was washed with ice cold 70% ethanol. The DNA pellet was dried at room temperature and dissolved in 300 µl of TE buffer (100 mM Tris HCl, 1mM EDTA, pH-8.0) which was then transferred to 1.5ml eppendorf tube and 3 µl RNase A (10 mg/ml) was added. The tubes were incubated in a water bath at 37°C for 1hr. Protease K (3µl of 1mg/ml, sigma) was added to this and the incubation was continued at 37°C for 15 to 30 min. Neutral phenol (300 µl) was added to each eppendorf tube, mixed briefly and centrifuged at 12,000 rpm for 10 min. The upper layer was collected in a fresh 1.5 ml tubes and the DNA was precipitated by adding one-tenth volume of 2M sodium acetate and 2 volume of absolute ethanol and kept at -20°C overnight.The tubes were centrifuged at 12,000 rpm for 10 minutes and the DNA pellet was washed with ice cold 70% ethanol. The pellet was dried at room temperature and dissolved in 200 µl of TE buffer. Samples were stored in 1.5 ml microfuge at 4°C for long-term storage. The concentration of DNA in each sample was determined by using a Nanodrop spectrophotometer (USA).

DNA fragmentation visualization

The sample mixture was prepared by mixing 5 µl of loading buffer (0.25% bromophenol blue, 30% glycerol in TE buffer, pH-8.0) and 15 µl of the DNA (2 µg) and the samples were loaded on to a 1% agarose gel prepared in 1X TAE buffer (Tris- Acetate- EDTA) (Sam Brook *et al.*, 1989). Electrophoresis was carried out at 50 volts for nearly 4 hrs until the bromophenol blue dye front was migrated to the bottom of the gel. Wide range DNA marker (Sigma) was used as the molecular weight standard. Staining was carried out with 0.5 µg/ml ethidium bromide and the gels were photographed under UV light.

4.3. Results

The germplasm accessions for the study had been selected on the basis of initial screening of a set of wild germplasm accessions (Thomas *et al.*, 2015). The soil moisture percentage showed a reduction after seven days of drought imposition in glass house (Fig 4.1). The extent of drought stress was assessed by measurement of CO_2 assimilation rate (A) and stomatal conductance (g_s) and the results are given in figs.4.2 & 4.3.The irrigated control plants maintained a higher CO_2 assimilation rate (A) in tolerant germplasm accessions RO3261 and AC 612 as well as in tolerant check clones RRIM 600 and RRII 430 compared to susceptible ones (Fig. 4.2). After seven days of drought imposition by withholding irrigation, CO_2 assimilation (A) reduced in all plants and significant decline was observed in the susceptible clones/ accessions RO 3242 followed by RRII 105, RRII 414 and MT 1619. Stomatal conductance (g_s) was also significantly reduced after seven days of drought stress and reached almost near to zero in susceptible germplasm accessions, RO 3242, MT 1619, RO 2360 and susceptible check clones RRII 105 and RRII 414 (Fig. 4.3). The RWC showed a significant reduction under drought stress in all the clones/accessions compared to

irrigated plants (Fig. 4.4). The maximum reduction in RWC was observed in RO 3242 followed by RRII 414. The level of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids were found reduced under drought stress in all plants (Figs.4.5, 4.6, 4.7&4.8). The photosynthetic pigments showedno significant reduction in any of the clones/accessions studied after seven days of drought stress. The level of accessory pigment carotenoids also maintained a value near to irrigated control. The clone RRIM 600 maintained higher level of carotenoids among the clones/accessions studied. The pigment anthocyanin showed an increase under drought stress and it varied significantly (Fig.4.9). The tolerant germplasm accession RO3261 showed the highest level (19.91 μmol/cm^2) of anthocyanin content. The tolerant accession AC 612 showed a significant accumulation of anthocyanin under drought stress. Epicuticular wax, which is considered as a common trait associated with drought tolerance (Guo *et al.*, 2016) also showed significant level of accumulation under drought stress (Table 4.1 & Fig. 4.10). The tolerant accession RO 3261 showed maximum epicuticular wax content (101.36 μg/cm^2) under drought stress followed by tolerant clone RRII 430 and accessions AC 612 and RO 3157.

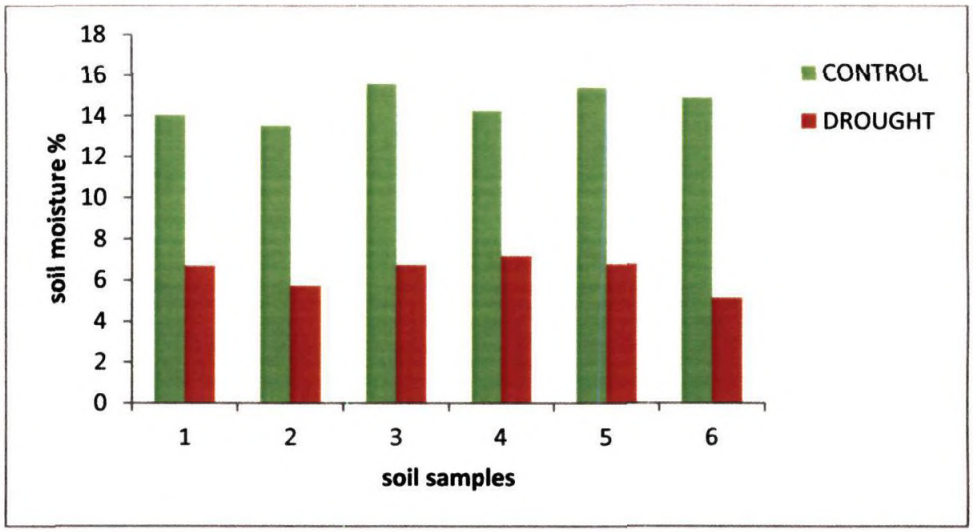

Fig. 4.1. Soil moisture % in six randomly collected soil samples (control and after seven days drought stress imposition) from poly bags kept in glass house.

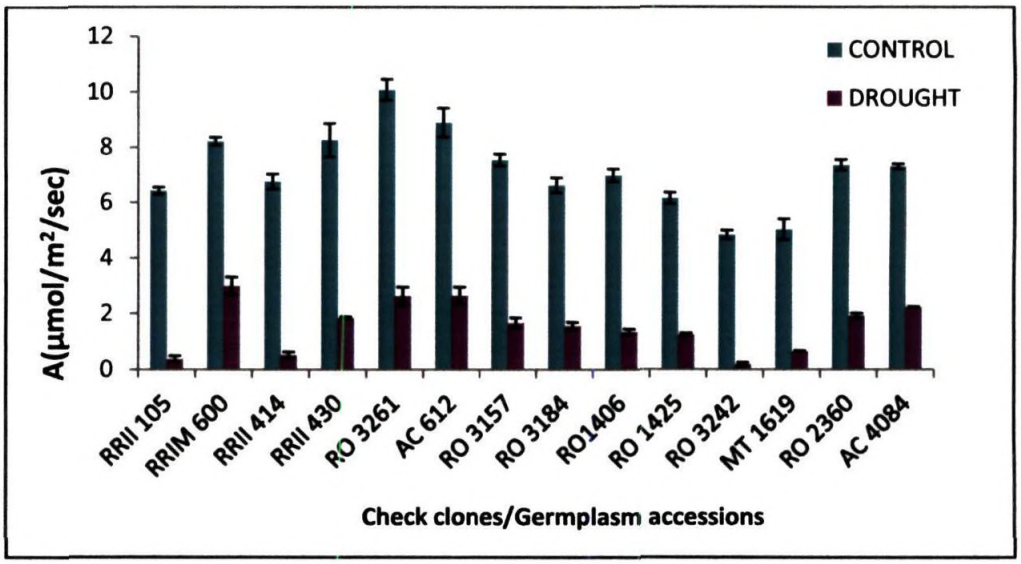

Fig. 4. 2. CO_2 assimilation rate (A) in different *Hevea* genotypes after seven days drought imposition in glass house

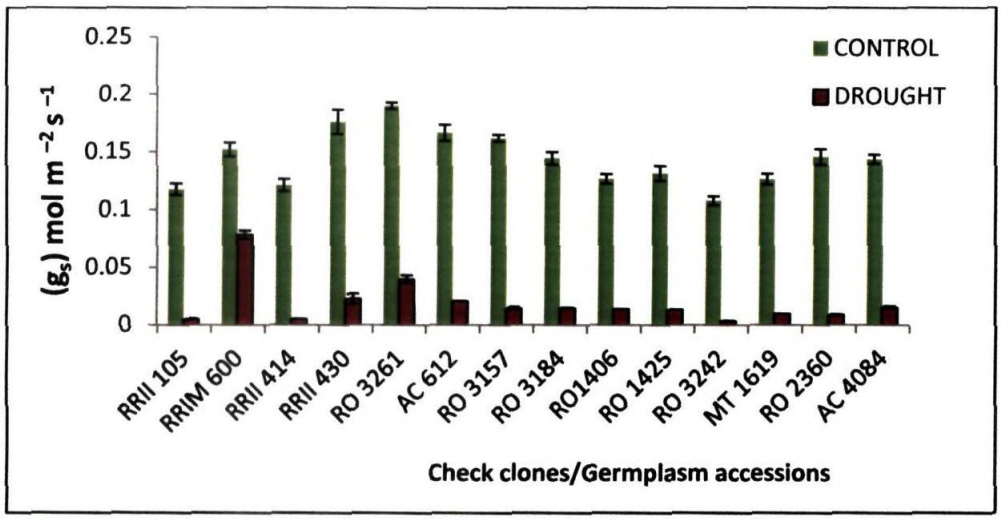

Fig. 4.3. Stomatal conductance (g_s) in different *Hevea* genotypes after seven days drought imposition in glass house

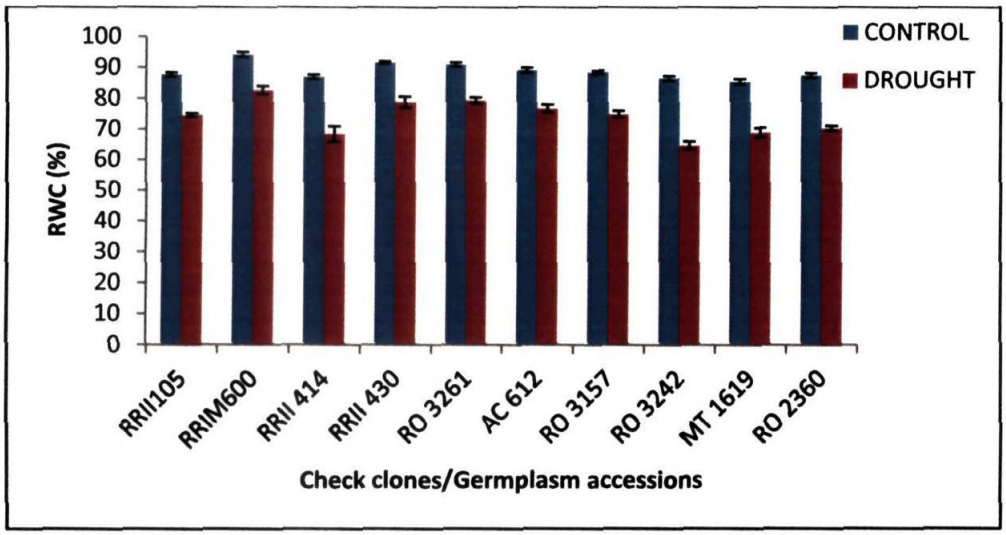

Fig. 4.4. Relative water content (%) in different *Hevea* genotypes after seven days drought imposition in glass house

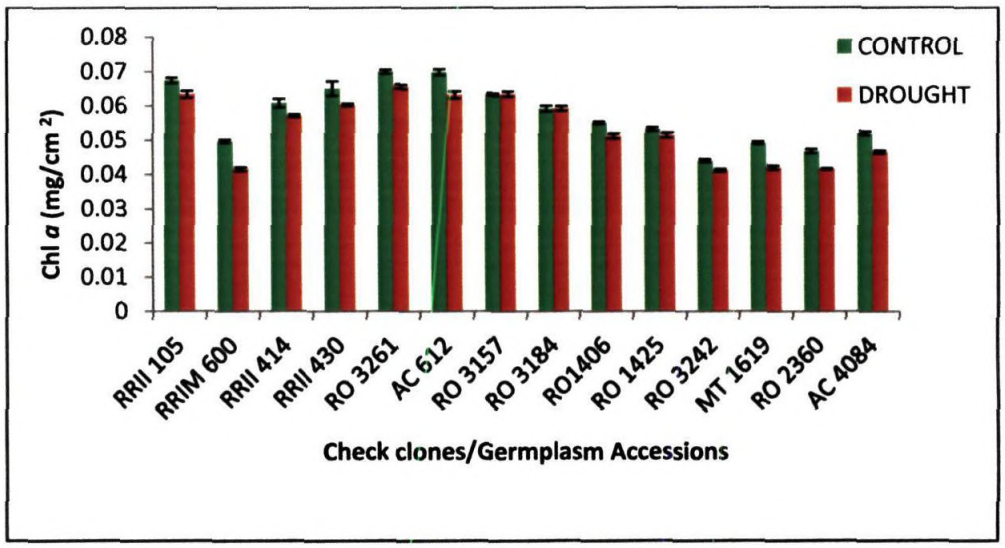

Fig.4.5. Chlorophyll *a* content in different *Hevea* genotypes after seven days drought imposition in glass house

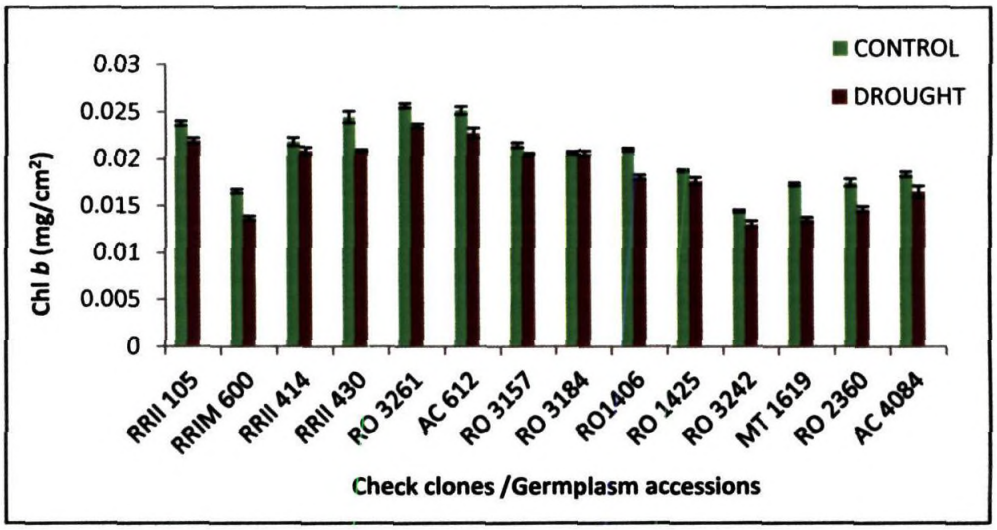

Fig. 4.6. Chlorophyll *b* content in different *Hevea* genotypes after seven days drought imposition in glass house

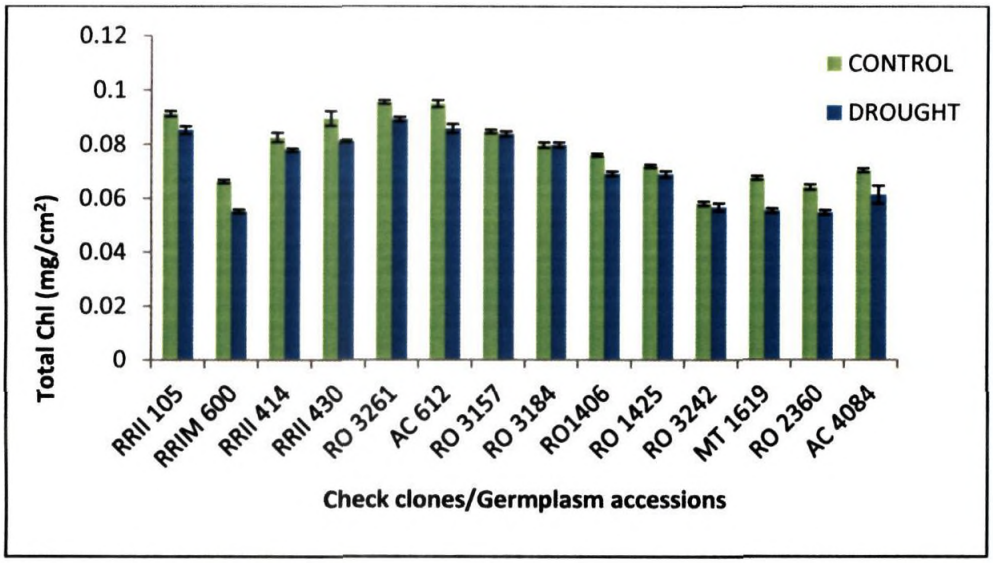

Fig. 4.7. Total chlorophyll content in different *Hevea* genotypes after seven days drought imposition in glass house

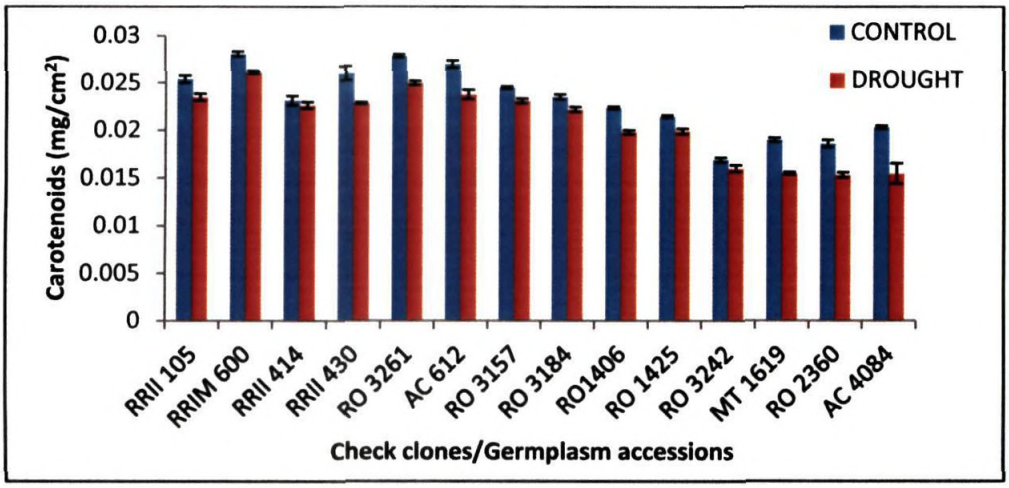

Fig. 4.8. Carotenoids in different *Hevea* genotypes after seven days drought imposition in glass house

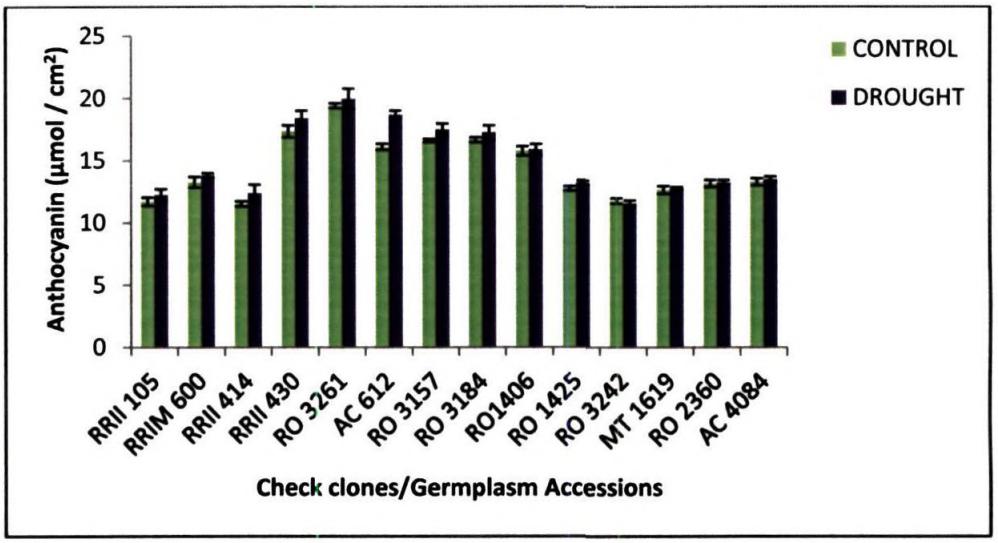

Fig.4.9. Anthocyanin content in different *Hevea* genotypes after seven days drought imposition in glass house

Table 4.1: Epicuticular wax, proline and glycine betaine content in control and after seven days drought stress imposition in glass house

Sl.No.	Clones	Epicuticular wax (µg/cm^2)		PROLINE(µg/g FW)		Glycine Betaine (mg/g DW)	
		CONTROL	DROUGHT	CONTROL	DROUGHT	CONTROL	DROUGHT
1	RRII 105	24.99±1.26	54.18±2.04	68.23 ± 3.3	77.96 ± 3.6	2.33 ± 0.06	2.71 ±0.05
2	RRIM 600	26.95±0.71	79.27±1.13	63.54 ± 5.3	87.26 ± 7.7	2.52 ± 0.04	3.30 ±0.27
3	RRII 414	25.84±0.63	50.22±1.21	65.14 ± 3.5	73.03 ± 2.9	2.21 ± 0.01	2.44 ±0.01
4	RRII 430	30.84±2.06	91.29±2.95	62.74 ± 5.8	83.31 ± 6.3	2.37 ± 0.04	3.26 ±0.13
5	RO 3261	31.52±1.92	101.36±2.10	87.77 ± 7.4	603.77 ± 10.5	2.56 ± 0.00	3.00 ±0.03
6	AC 612	27.70±1.26	90.08±2.43	86.74 ± 7.3	592.97 ± 9.9	2.31 ± 0.11	2.69 ±0.11
7	RO 3157	26.02±1.40	90.48±2.23	63.17 ± 4.3	459.26 ± 14.3	2.49 ± 0.16	2.85 ±0.06
8	RO 3184	31.77±1.08	86.79±1.92	113.14 ± 7.0	781.37 ± 4.0	2.17 ±0.13	2.76 ±0.29
9	RO1406	29.06±1.77	88.59±0.79	94.71 ± 5.3	672.51 ± 15.6	2.44 ± 0.04	2.97 ±0.11
10	RO 1425	27.17±1.42	78.84±1.36	143.53 ± 9.0	757.24 ± 17.1	2.60 ± 0.02	3.01 ±0.06
11	RO 3242	28.06±1.59	63.93±2.83	72.26 ± 6.7	165.04 ± 6.7	2.13 ±0.10	2.33 ±0.08
12	MT 1619	28.03±1.96	74.30±1.89	95.4 ± 5.1	246.23 ± 16.8	2.60 ± 0.02	2.63± 0.04
13	RO 2360	24.65±2.12	69.83±1.08	89.91 ± 7.9	341.57 ± 12.0	2.64 ± 0.10	2.93 ±0.08
14	AC 4084	30.01±1.68	79.32±1.47	59.7 ± 5.3	242.53 ± 8.3	2.55±0.06	2.88 ± 0.06
5%LSD		4.4056	5.4525	17.43	30.50	0.226	0.359
5%LSD clone×treatment		4.957		24.84		0.3003	

Table 4.2: SOD and peroxidase activity and content of ascorbic acid and thiols in control and after seven days drought stress imposition in glass house

Sl.No.	Clones	Total peroxidase (OD/min/mg protein)		SOD(units/mg protein)		Ascorbic acid (mg/g)		Thiols (mg/g)	
		CONTROL	DROUGHT	CONTROL	DROUGHT	CONTROL	DROUGHT	CONTROL	DROUGHT
1	RRII 105	0.389 ±0.017	0.766 ±0.021	3.52 ±0.19	5.19 ±0.16	1.296 ±0.07	2.248 ±0.18	1.935 ±0.12	2.969 ±0.19
2	RRIM 600	0.478 ±0.006	1.104 ±0.027	3.22 ±0.11	6.06 ±0.17	1.351 ±0.13	3.247 ±0.20	2.328 ±0.10	3.813 ±0.26
3	RRII 414	0.358 ±0.014	0.675 ±0.011	3.37 ±0.15	4.40 ±0.07	1.207 ±0.03	2.857 ±0.17	1.923 ±0.10	3.035 ±0.166
4	RRII 430	0.470 ±0.012	1.125 ±0.028	3.45 ±0.11	5.92 ±0.15	1.591 ±0.17	3.996 ±0.26	2.001 ±0.16	4.146 ±0.22
5	RO 3261	0.433 ±0.007	1.203 ±0.015	4.15 ±0.19	6.37 ±0.14	1.503 ±0.09	4.308 ±0.34	2.307 ±0.22	5.159 ±0.13
6	AC 612	0.422 ±0.007	1.082 ±0.004	3.95 ±0.14	6.41 ±0.13	1.920 ±0.12	4.022 ±0.11	2.190 ±0.20	5.184 ±0.05
7	RO 3157	0.400 ±0.011	1.002 ±0.013	3.59 ±0.19	5.43 ±0.19	1.525 ±0.09	3.605 ±0.07	2.214 ±0.31	5.018 ±0.24
8	RO 3184	0.384 ±0.007	1.015 ±0.017	3.64 ±0.21	5.11 ±0.19	1.730 ±0.22	3.596 ±0.17	1.913 ±0.34	4.139 ±0.28
9	RO1406	0.405 ±0.007	1.125 ±0.019	4.03 ±0.09	5.03 ±0.20	1.907 ±0.16	3.152 ±0.23	2.641 ±0.23	5.174 ±0.16
10	RO 1425	0.383 ±0.006	0.911 ±0.009	3.49 ±0.18	4.57 ±0.18	1.425 ±0.10	2.896 ±0.14	1.873 ±0.20	4.441 ±0.49
11	RO 3242	0.363 ±0.008	0.727 ±0.011	2.86 ±0.06	3.51 ±0.09	1.359 ±0.09	1.850 ±0.14	2.057 ±0.29	4.249 ±0.34
12	MT 1619	0.369 ±0.002	0.722 ±0.006	2.81 ±0.05	3.64 ±0.11	1.650 ±0.16	2.086 ±0.20	2.472 ±0.20	4.495 ±0.30
13	RO 2360	0.378 ±0.009	0.803 ±0.009	3.33 ±0.31	4.81 ±0.17	1.978 ±0.08	2.614 ±0.14	2.549 ±0.19	4.339 ±0.37
14	AC 4084	0.421 ±0.010	0.911 ±0.023	3.55 ±0.26	5.35 ±0.25	1.777 ±0.24	2.888 ±0.15	2.801 ±0.08	4.682 ±0.17
5%LSD		0.0266	0.0474	0.495	0.463	0.3844	0.5292	0.5916	0.7367
5%LSD clone × treatment		0.0384		0.479		0.4625		0.6681	

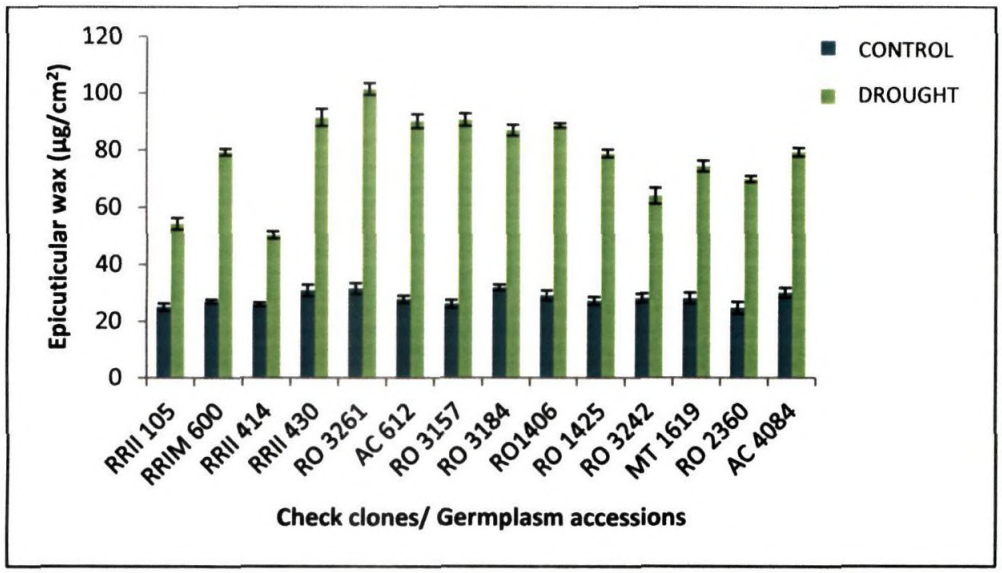

Fig. 4.10. Epicuticular wax content in different *Hevea* genotypes after seven days drought imposition in glass house

The compatible solutes proline and glycine betaine showed significant increase under drought stress (Table 4.1, Figs. 4.11 & 4.12). Free proline content in leaves of different *Hevea* genotypes is given in Fig. 4.11. The free proline content in leaves under irrigated condition ranged from 59 to 144µg/g FW. All the germplasm accessions showed significantly higher level of proline content compared to elite check clones. Under drought stress conditions, a tremendous increase has been observed in the level of proline which comes in the range of 450 to 760 µg/g FW in germplasm accesssions. The percentage of increase has been found high in all the tolerant germplasm accessions on comparison with susceptible accessions, however, the increase was not remarkably high in tolerant (RRIM 600 and RRII 430) as well as susceptible check clones (RRII 105 and RRII 414). A positive correlation can be observed between degree of drought stress tolerance and proline accumulation in the germplasm accessions.

The level of accumulation of glycine betaine was also found to be significantly increased upon drought stress imposition in different *Hevea* clones and accessions (Table 4.1 and Fig. 4.12). In tolerant check clones and germplasm accessions, percentage of increase was high, when compared to susceptible check clones and germplasm accessions. The tolerant clones RRIM 600 and RRII 430 recorded around 31 and 35% increase over their respective control plants. Among the germplasm accessions, 27% increase was observed in the tolerant accession RO 3184. The percentage increase was very less in a susceptible accession MT 1619 (1.2%). All the tolerant accessions showed percentage increase of glycine betaine in the range of 14% to 27%.

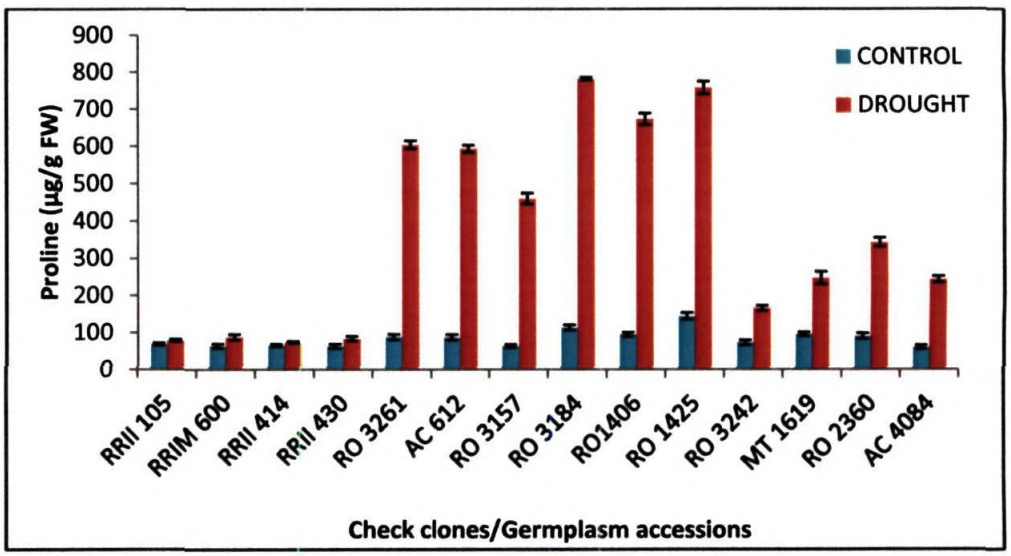

Fig. 4.11. Proline content in different *Hevea* genotypes after seven days drought imposition in glass house

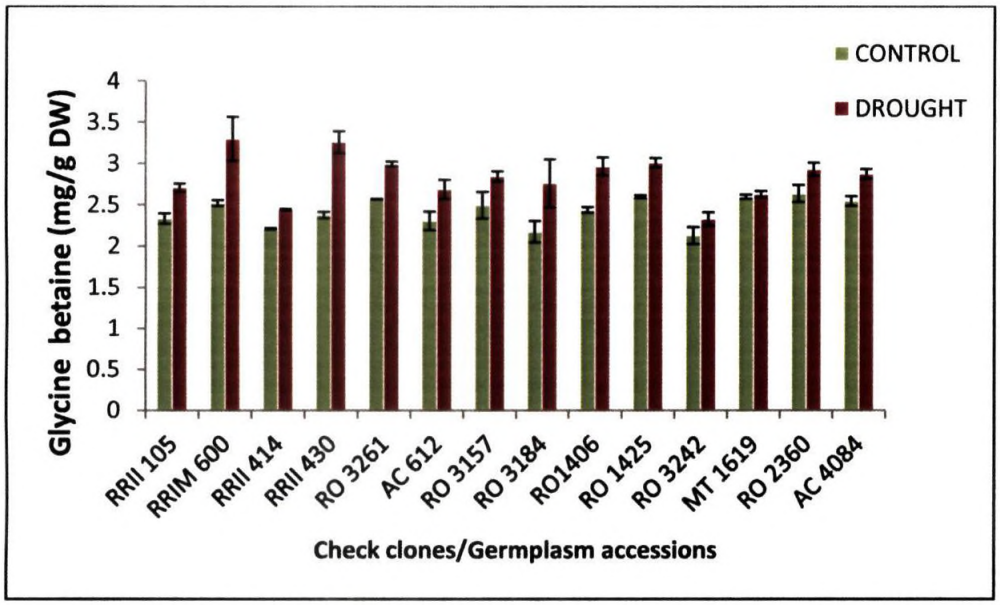

Fig. 4.12. Glycine betaine content in different *Hevea* genotypes after seven days drought imposition in glass house

Antioxidant enzymes superoxide dismutase (SOD) and total peroxidase activities were significantly increased under water deficit stress (Table 4.2, Figs. 4.13 &4.14). AC 612 showed maximum SOD activity (6.41 units/mg protein) under drought followed by RO 3261 (6.37 units/mg protein) and RRIM 600 (6.06 units/mg protein). The lowest rate of SOD activity was observed in RO 3242 (3.51 units/mg protein) followed by MT 1619 and RRII 414. Maximum peroxidase activity under drought stress condition was observed in RO 3261 (1.203 OD/min/mg protein) followed by RO 1406 and RRII 430 (1.125 OD/min/mg protein). The peroxidase activity was found lower in the case of RRII 414 (0.675 OD/min/mg protein) followed by MT 1619 (0.722 OD/min/mg protein) and RO 3242 (0.727 OD/min/mg protein). The non enzymatic antioxidants, ascorbic acid and glutathione were also found significantly increased under dehydration stress (Table 4.2, Figs. 4.15 & 4.16). An increased level of accumulation of ascorbic acid was found in tolerant accessions RO 3261 (4.31 mg/g)

followed by AC 612 (4.02 mg/g) and clone RRII 430 (4.00 mg/g). However, the increase was comparatively less in susceptibleaccession RO 3242 (1.85 mg/g), MT 1619 (2.09 mg/g) and clone RRII 105 (2.25 mg/g). A significant increase in accumulation of thiol was observed in allthe clones and accessions. Both tolerant and susceptible clones and accessions accumulated significant level of thiols under drought stress.

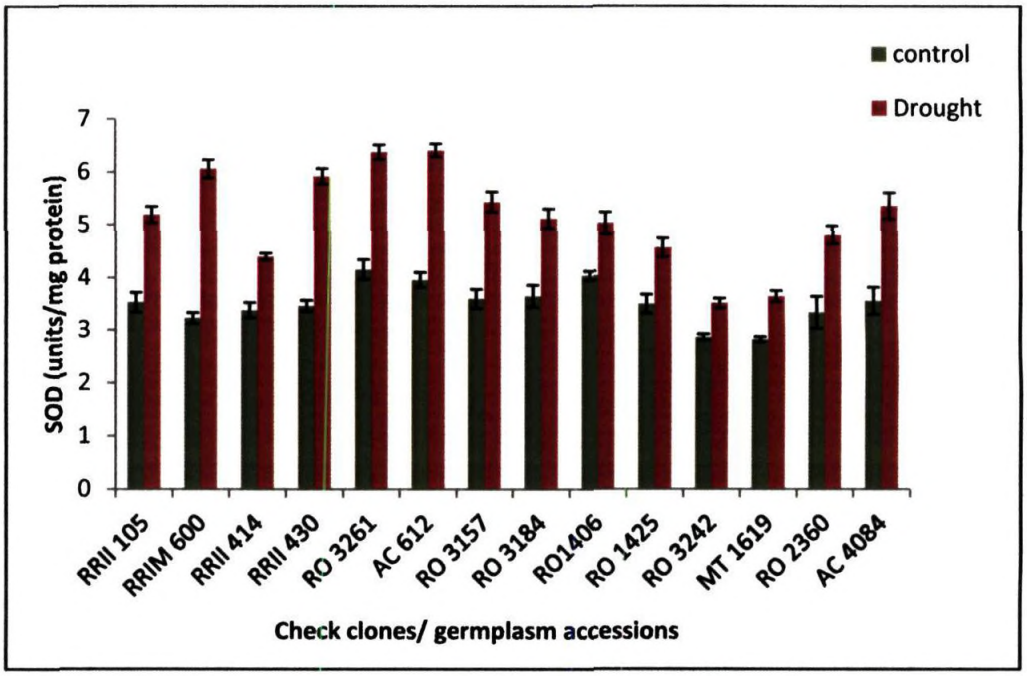

Fig. 4.13. Superoxide dismutase (SOD) activity in different *Hevea* genotypes after seven days drought imposition in glass house

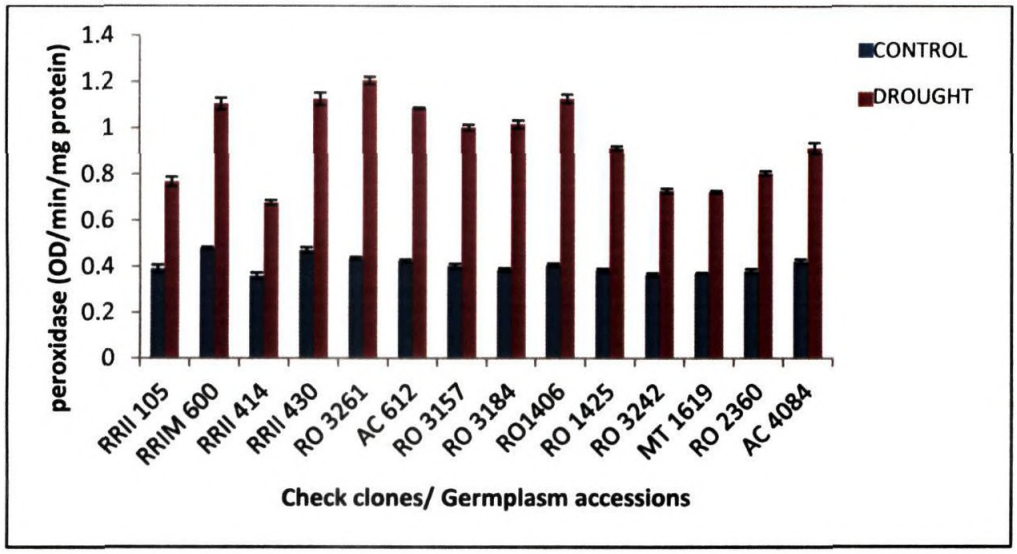

Fig. 4.14. Total peroxidase activity in different *Hevea* genotypes after seven days drought imposition in glass house

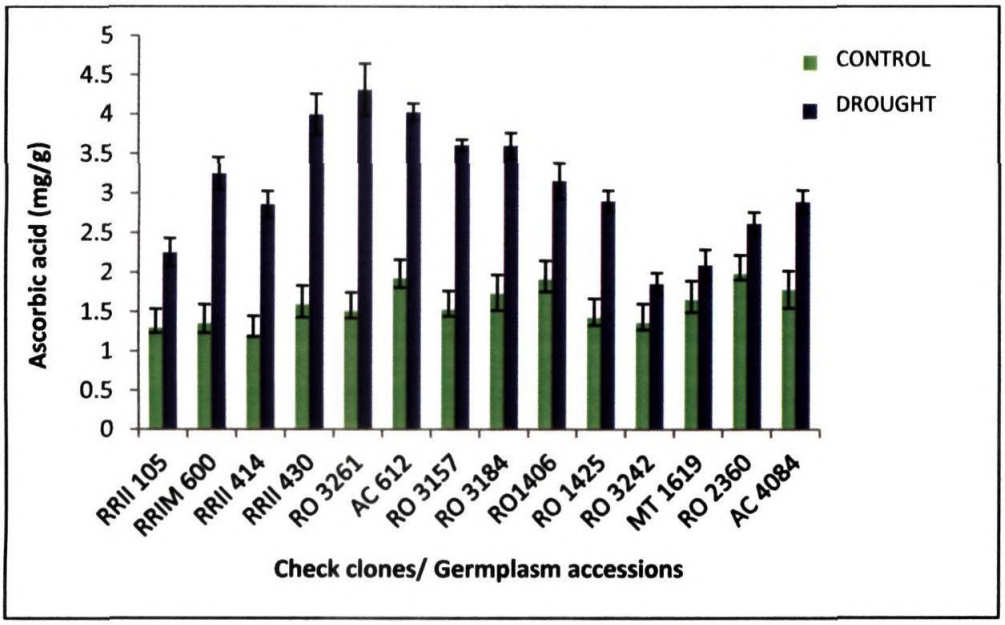

Fig. 4.15. Ascorbic acid content in different *Hevea* genotypes after seven days drought imposition in glass house

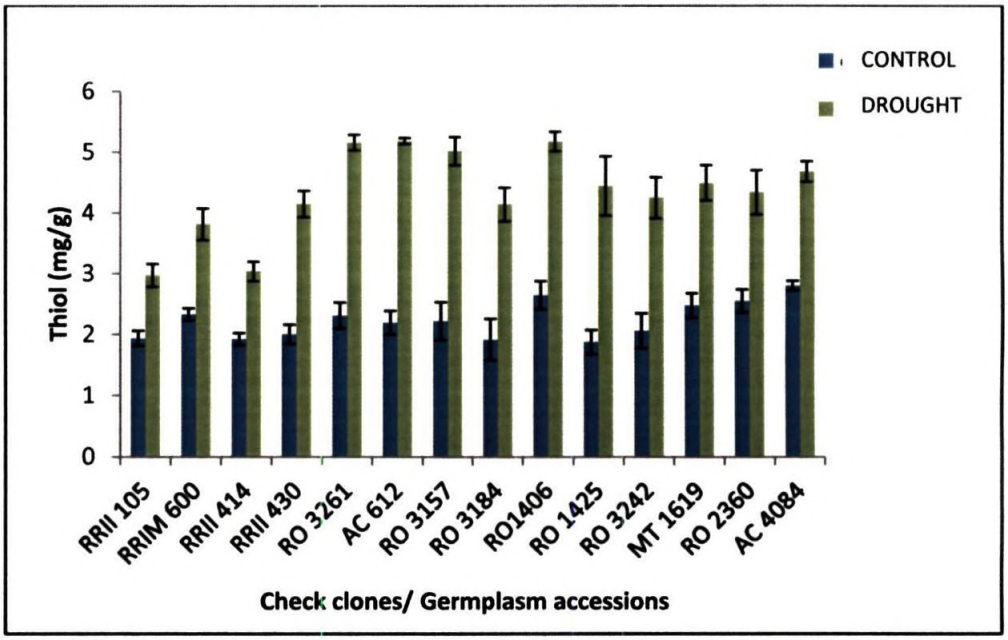

Fig. 4.16. Thiol content in different *Hevea* genotypes after seven days drought imposition in glass house

The chloroplast protein profiling by SDS-PAGE was performed in tolerant as well as susceptible germplasm accessions along with elite clones, RRII 430 and RRII 414 (Fig. 4.17 &4.18). The level of expression of a chloroplast stress protein in the molecular weight of 23.8 kDa was found prominent in drought stressed plants of tolerant clone RRII 430 as well as in tolerant accessions RO 3261, AC 612 and RO 3157, when compared to irrigated control plants. However, the expression of this stress protein was not completely absent in control plants. In susceptible clone RRII 414 and in susceptible accessions RO 3242 and MT 1619, the expression of 23.8 kDa chloroplast stress protein was found almost absent. The western blot profiling of the same stress protein using antibodies raised by polyclonal antibodies also showed the presence of 23.8 kDa chloroplast stress protein in tolerant clones/accessions (Fig. 4.19). Its expression level was prominent in drought stressed plants. However, in western blot profile, the chloroplast stress protein was noticed in susceptible accessions also. But the level of

expression showed considerable variation among tolerant and susceptible clones/accessions, as well as in plants under irrigation and drought stress. The susceptible clones/accessions showed a low level of expression under drought and high light conditions.

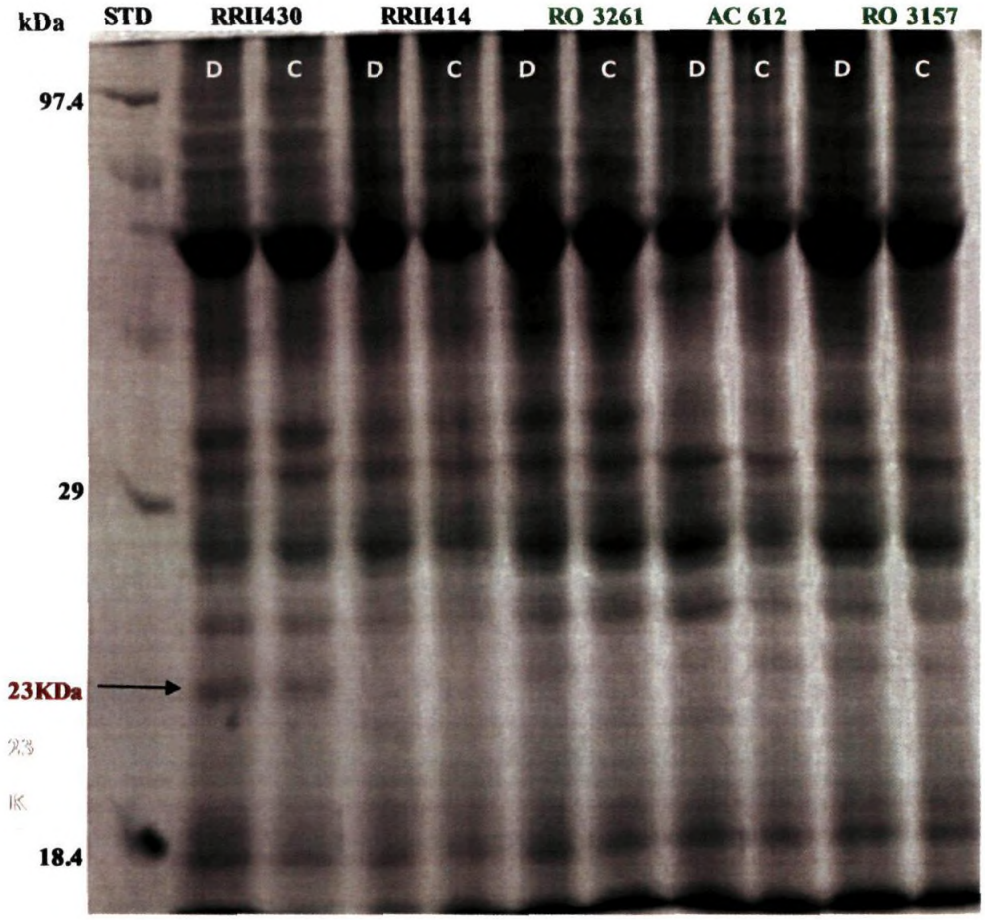

Fig. 4.17. SDS-PAGE profile of chloroplast proteins in tolerant *Hevea* germplasm accessions and check clones

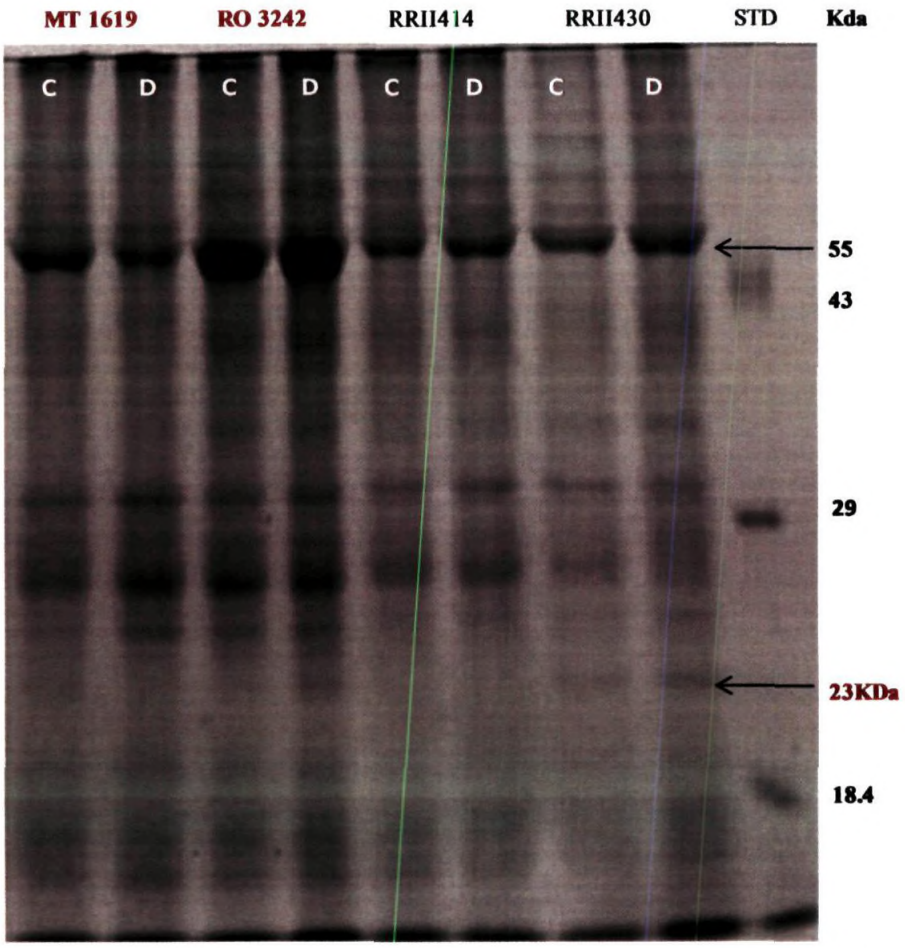

Fig. 4.18. SDS-PAGE profile of chloroplast proteins in susceptible *Hevea* germplasm accessions and check clones

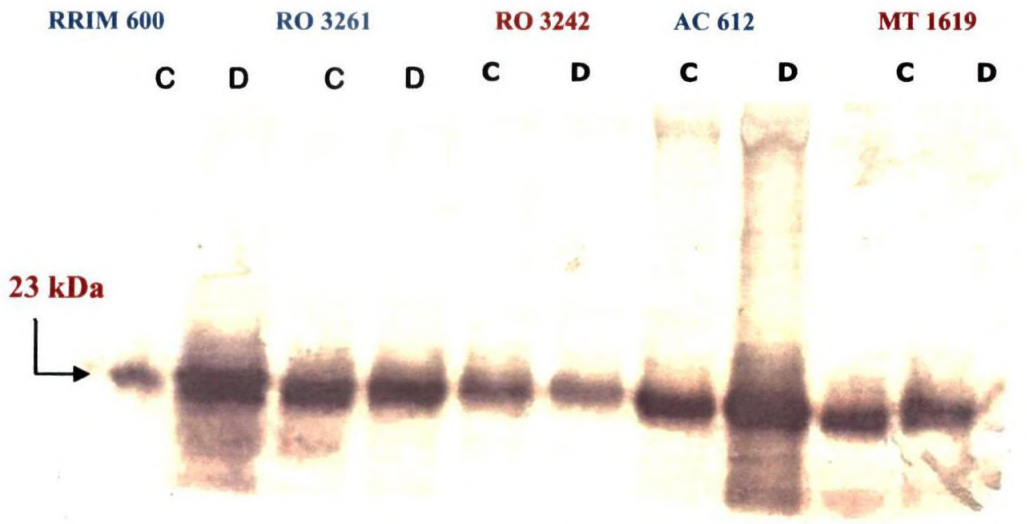

Fig. 4.19. Western blot profile of 23 kDa protein in tolerant / susceptible *Hevea* germplasm accessions/check clones

The results of DNA fragmentation analysis conducted in two *Hevea* clones, RRII 430 (relatively drought tolerant) and RRII 414 (relatively drought susceptible) showed that PEG stress under low light doesn't inflict any significant damage to genomic DNA, however clear signs of fragmentation were observed under high light in both samples, incubated in PEG as well as in distilled water in the susceptible clone, RRII 414. Under high light conditions in the open field, serious damage to DNA was observed in both clones RRII 414 and RRII 430, irrespective of tolerant or susceptible (Xavier *et al.*, 2017).

On the basis of preliminary investigation done in two *Hevea* clones, the study was extended to tolerant as well as susceptible germplasm accessions. The leaf discs were incubated in distilled water and exposed to low and high light conditions inside a growth chamber, and also in open field conditions (Fig. 4.20 & 4.21). Under high light conditions in the growth chamber the DNA showed fragmentation in the susceptible accessions RO3242 and MT 1619. However, there were no visible signs of

fragmentation in tolerant accessions RO 3261, AC 612 and RO 3157. In the open field conditions, under high light intensity all the germplasm accessions showed significant fragmentation indicating that high light can inflict serious damages to DNA, irrespective of tolerant or susceptible.

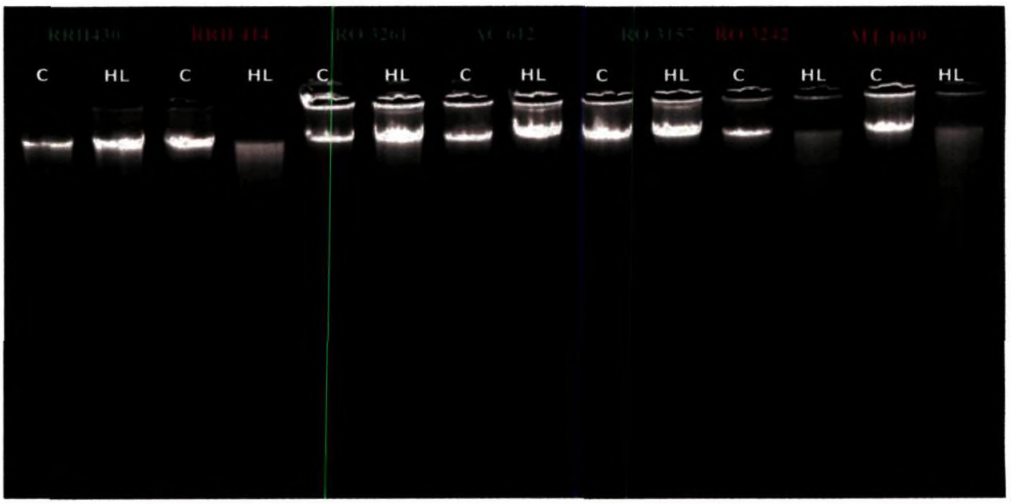

Fig. 4.20. Visualization of genomic DNA (2µg) from *Hevea* leaf tissue on 1% agarose gel. The leaf discs were incubated in distilled water at low light at room temperature and high light in the growth chamber.

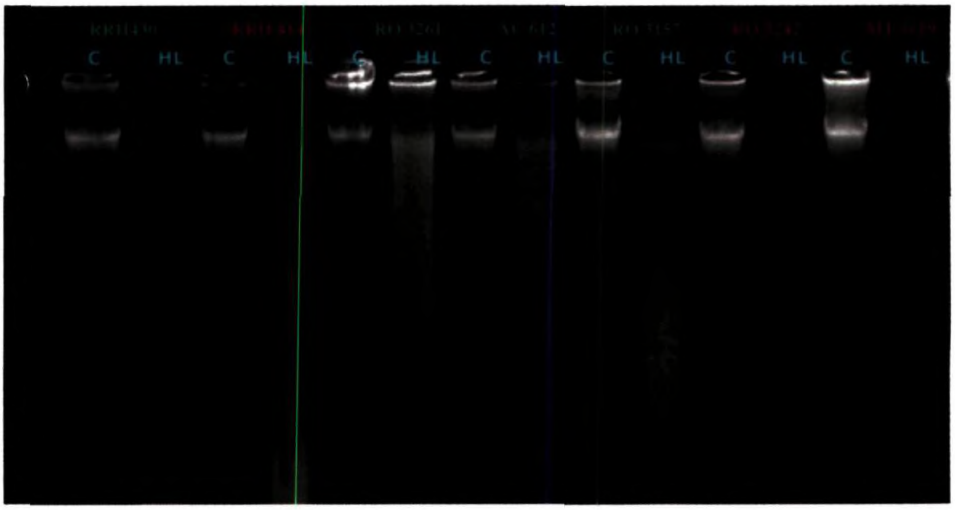

Fig.4. 21. Visualization of genomic DNA (2µg) from *Hevea* leaf tissue on 1% agarose gel. The leaf discs were incubated in distilled water at low light at room temperature and high light in the open field.

4.4. Discussion

Soil moisture content indicates the water resource which is available for growth and proper functioning of plants (Bahrun *et al.,* 2002). Increased soil moisture deficit leads to changes inwater potential (Ψw) of roots and leaves in plants (Fghire *et al.,* 2017). Maintaining proper soil moisture is very essential as it is closely related to crop yield (Kramer, 1983). Soil moisture deficit stress due to drought occurrences causes inhibition of growth as well as plant wilting which ultimately results in plant death (Tesfaye *et al.,* 2013). In this study, soil moisture is found decreased under drought stress. Earlier studies indicated that in rubber plants, soil water deficit causes a progressive decrease in leaf water potential and osmotic potential (Samarappuli *et al.,* 1993).

The most important physiological parameter being affected by water deficit is photosynthesis; causing a progressive and severe reduction in CO_2 assimilation capacity. The reduction in photosynthesis may occur due to restricted leaf expansion, chlorophyll bleaching and premature leaf senescence and also due to impaired photosynthetic machinery (Wahid and Rasul, 2005; Farooq *et al.,* 2009). The decrease in net photosynthetic rate is associated with stomatal closure induced by a reduction in leaf turgor which limits diffusion of CO_2 into the sub-stomatal chamber. Under such conditions, photoinhibition may occur, a process that reduces quantum yield of PSII and also it induces photorespiration and H_2O_2 production (Hossain *et al.,* 2009). Relative water content (RWC) is the measure of water present in leaves and also itinfluences plant waterrelations.In this study RWC showed a significant reduction under drought stress in all the clones and accessions compared to irrigated control plants, however the reduction was less in tolerant clones and accessions. Higher RWC is reported as a major characterstic which helps in maintaining water balance and contributes to

drought tolerance in plants (Abdel-Kader *et al.,* 2015). Anjum *et al.* (2011) reported high RWC in early stages of leaf development in plants, which may further decreases as the leaf matures and dry matter accumulates. Also plants which are exposed to dehydration stress exhibit an increased leaf temperature (Siddique *et al.,* 2001). Water deficit stress is reported to have adverse effects on plant water relations and rate of photosynthesis in groundnut (Kiranmai *et al.,* 2018).

Cuticular wax deposition is another major physical adaptation in plants that provides an external barrier against various biotic and abiotic stresses such as drought, salinity, high and low temperatures, bacterial and fungal pathogens invasion *etc.* (Lee and Suh, 2015). It is an outermost hydrophobic layer that was reported to accumulate under drought in plants like rice, alfalfa, tobacco and wheat (Guo *et al.,* 2016). Enhanced accumulation of epicuticular wax was reported in drought tolerant genotypes of peanuts, which also helped in reducing leaf temperature under water deficit stress (Solanki and Sarangi, 2015). Previous reports in *Hevea* showed that enhanced epicuticular wax on leaf surface helps in reducing stomatal transpiration and also it helps in reflection of radiant energy (Rajagopal *et al.*, 1990; Thomas *et al.,* 2015). This study also revealed the increased accumulation of epicuticular wax under drought stress in tolerant clones and accessions.

Chlorophyll, which is a major chloroplast component for photosynthesis, influences photosynthetic rate (Anjum *et al.,* 2011). Decreased chlorophyll under water stress generally occurs due to damage of chloroplasts caused by oxidative bursts or due to changed ratios of lipid protein complexes or elevated chlorophyllase activity which degrades chlorophyll and damages light harvesting machinery (Kaya *et al.,* 2006). Decreased or unchanged chlorophyll level under water deficit stress has been

reported in many plants as they depends on the duration and severity of drought (Kpyoarissis *et al.,* 1995; Zhang and Kirkham, 1996). In this study, the level of chlorophyll content was only slightly reduced under drought stress, maintaining values near to the control level. This indicates that chlorophyll degradation is a delayed process under drought stress in *Hevea* compared to rate of photosynthesis and stomatal conductance (g_s) (Sumesh *et al.,* 2011). Carotenoids are accessory pigments in the photosystems and they are powerful antioxidants. In this study, the level of carotenoids were also found declined, but the reduction was found negligible in most cases. Dehydration stress was reported to reduce the level of chlorophylls and carotenoids in tissues (Havaux, 1998; Kiani *et al.,* 2008). Anthocyanins were reported to have photoprotective functions as well as antioxidant potential (Edreva *et al.,* 2008) and thereby providing protection to cell membranes, organelles and DNA (Close *et al.,* 2002). Anthocyanin contents showed an increased accumulation under drought stress and significant genotypic variation was observed among tolerant and susceptible clones/accessions of *Hevea*. Increased accumulation of anthocyanins under water deficit stress was reported in tolerant genotypes of tree crops like almonds (Karimi *et al.,* 2013) and apple (Bolat *et al.,* 2014).

Proline is an important parameter to determine the stress tolerance capacity of plants and its accumulation is considered as an early response to drought stress (Ramanjulu and Sudhakar, 2000). The accumulation of proline and its osmoprotective function under various environmental constraints has been reported in numerous plants (Verbruggen and Hermans, 2008). It protects the plants from different stress conditions and also helps plants to recover from stress more rapidly (Hayat *et al.*, 2012). Proline accumulation normally occurs in cytoplasm where it functions as molecular chaperone stabilizing the structure of proteins and buffers cytosolic pH and maintains cell redox status.

It has been suggested that it may be a part of stress signal influencing adaptive responses (Ashraf and Foolad, 2007; Hayat *et al.*, 2012). In this study, low level of proline was observed in irrigated control plants. However, seven days drought stress caused tremendous increase in the level of proline in tolerant as well as in susceptible germplasm accessions. The level of proline accumulation under stress in plants can be 100 times greater than in control condition and it differs from species to species (Verbruggen and Hermans, 2008). Wu *et al.,* (2016) reported that osmotic stress increased the accumulation of proline in *Beta vulgaris.* High level of proline was also reported in drought tolerant alfalfa varities under drought stress, whereas its level was low in irrigated plants (Quan *et al.,* 2016).

In plants another osmolyte, glycine betaine (GB), which is a quaternary ammonium compound, is abundant in chloroplast and plays a vital role in protection of thylakoid membrane, thereby maintaining photosynthetic efficiency under water deficit stress (Genard *et al.*, 1991). Plants of different species contain low level of GB under normal conditions; however, they accumulate larger amounts of GB when subjected to various abiotic stress conditions (Chen and Murata, 2011). The glycine betaine content reported to be increased under drought stress in barley, spinach, maize, sugar beet, and in higher plants (Bohnert *et al.*, 1995; Nakamura *et al.,* 2001). In this study also, an increased level ofglycine betaine was observed under drought stress in *Hevea* clones and accessions. Reactive oxygen species (ROS) production is enhanced under abiotic stresses which lead to photoinhibition of PSII in chloroplast. Glycine betaine protects the photosynthesis machinery by stabilizing the activity of proteins and thus provides tolerance to abiotic stresses even at low concentration (Takahashi *et al.,* 2008).

Superoxide dismutase (SOD) and peroxidase (POD) are two major antioxidant enzymes involved in enzymatic defence mechanisms against ROS production during abiotic stresses (Apel and Hirt, 2004). SOD catalyzes the dismutation of superoxide ($O_2^{\cdot}$) anions into H_2O_2 and O_2 and POD reduces H_2O_2 produced to water using different substrates as electron donors and thus protects the plants from increased H_2O_2 accumulation (Carrasco-Rios and Pinto, 2014). Enhanced activity of the antioxidant enzymes under drought stress had been reported in several plant species (Sharma and Dubey, 2005; Lee *et al.,* 2009; Chugh *et al.,* 2011; Chakraborty and Pradhan, 2012; Marok *et al.,* 2013) and also suggested that it has a strict relation with drought tolerance (Turkan *et al.,* 2005). Tolerant genotypes usually have a better antioxidant scavenging system to protect the plants from oxidative stress during various stress conditions (Chang-Quan and Rui-Chang, 2008). Researches in different plant species have reported that water deficit stress increases SOD activity in the tolerant plants whereas in the sensitive varieties a decrease of enzyme activity occurs (Arabzadeh and Khavari-Nejad, 2013). Increased SOD activity had been reported in higher plants under drought stress (Ramachandra Reddy *et al.,* 2004). It has been shown that drought increased SOD activity in drought tolerant maize genotype and decreased in drought sensitive genotypes (Moussa and Abdel-Aziz, 2008). Enhanced SOD activity was also reported by Manivannan *et al.,* (2007) in *Vigna* plants under water deficit stress.Enhanced SOD activity was reported in *Radix astragali* under drought stress, which were found, varied among three different genotypes (Tan *et al.*, 2006). The activity of both SOD and POD has been reported in *Camptotheca acuminate* (Ying *et al.*, 2015) under drought conditions. Significant accumulation of SOD and POD under drought stress was observed in *Hordeum vulgare.* In transgenic plums and tobacco, the tolerance to drought stress was enhanced with the overexpression of isoenzyme CuZnSOD, and also it alleviated the oxidative

stress damage caused by water deficit (Faize *et al.,* 2013). Enhanced POD activity was observed in chickpea cultivars under drought stress (Oberoi *et al.,* 2014). Increased activity for POD under water deficit stress was also observed in *Arabidopsis thaliana, Myrtus communis* and *Phillyrea angustifolia* (Jung., 2004; Caravaca *et al.,* 2005). The same has been reported in poplar (Xiao *et al.,* 2008) and *Brassica napus* (Abedi and Pakniyat, 2010). Sreelatha *et al.,* (2003) reported significant increase in POD activity in high yielding *Hevea* clones under drought stress. In sunflower plants, dehydration stress caused an increase in POD activity in all parts of the plants (Manivannan *et al.,* 2014). Increased activity of SOD and POD was reported by Thomas *et al.,* (2014) in tolerant clones of *Hevea* under drought stress. The results obtained in the present study are also in agreement with the above findings reported in other crops. The activity of both SOD and POD increased significantly after seven days of drought stress compared to irrigated control plants and the increase was significantly high in tolerant clones and accessions compared to relatively susceptible ones.

The concentrations of non enzymatic antioxidants ascorbic acid and glutathione were reported to be high after exposure to drought stress in many plants (Anjum *et al.,* 2017). Ascorbic acid, being an ubiquitous antioxidant present in plant system, plays several important roles such as scavenging of harmful ROS produced during abiotic as well as biotic stresses (Teixeira *et al.,* 2004). It is a major participant in detoxification mechanisms and plays a central role in photosynthesis (Smirnoff, 1996). The H_2O_2 and $O_2^{\cdot}$ (superoxide) radicals generated under stress conditions were reduced to water by AsA in the presence of ascorbate peroxidase (APX). Thus AsA guard cells and organelles against oxidative damage by eliminating ROS produced by environmental stresses like drought, cold, salinity and excess light (Kapoor *et al.,* 2015). During drought stress plants over accumulate

ascorbate to counter fall of physiological parameters and for plant survival (Dolatabadian *et al.,* 2009). AsA levels have been reported in plants subjected to high light and oxidative stress (Yabuta *et al.,* 2007). In *Brassica napus* and *Picea asperata,* AsA was reported to accumulate under drought stress (Yang *et al.,* 2008; Shafiq *et al.*, 2014). Increased AsA concentration under water stress was also reported in turfgrass (Jaleel *et al.*, 2007). Enhanced AsA content was observed under drought stress in tolerant *Hevea* clones (Thomas *et al.,* 2014). The same trend was observed in germplasm accessions also. The tolerant accessions maintained a significantly higher level of ascorbic acid compared to susceptible ones.

Glutathione (GSH), a low molecular weight tripeptide thiol (γ-glutamyl cysteinyl glycine) is another important metabolite which is widely distributed throughout the living systems (Gill and Tuteja, 2010; Noctor *et al.,* 2012). It plays major role in cellular metabolism and it also functions as a reductant in scavenging of radicals in intracellular environment (Gill and Tuteja, 2010) and helps in detoxification of toxins. GSH along with ascorbate (AsA) and related enzymes plays a central role in scavenging ROS and thus maintaining the homeostasis of cellular environment and protects the plant system from adverse effects of different stresses (Kapoor *et al.,* 2015). In this study elevated levels of thiol was observed under drought stress in both tolerant as well as susceptible clones implicating their role in scavenging free radicals produced due to drought and high light stresses.

Heat shock proteins (HSPs), initially identified in response to high temperatures, play major roles in maintaining cellular homeostasis under optimal growth conditions as well as under stress and are also responsible for maintaining folding, translocation and degradation of proteins (Rodziewicz *et al.*, 2014). Small heat shock proteins (sHSPs) are ubiquitous stress proteins which are induced under abiotic stresses including drought, salinity,

cold *etc.* (Hamilton and Heckathorn, 2001). In plants, presence of a chloroplast protein, HSP70B was found associated with oxidative stress response and defined as marker of oxidative stress (Chankova and Yurina, 2016). In wheat, a chloroplastic sHSP (HSP26) was found highly induced by heat stress (Chauhan *et al.,* 2012). Annamalinathan *et al.,* (2006; 2010) have reported that drought stress induced a 23.8 kDa chloroplast protein associated with chloroplast thylakoid membrane in *Hevea.* In this study also, the SDS PAGE profile showed consistent expression of a 23.8 kDa chloroplast stress protein in tolerant clones/ accessions under drought stress. Western blot profile of the same also produced a prominent expression of 23.8 kDa under drought stress in tolerant clones/ accessions, however the accumulation of this protein at a lesser extent was also observed in germplasm control plants. The presence of stress protein in control irrigated plants in the case of wild germplasm needs to be further investigated.

In the present study visible signs of DNA fragmentation was observed in young plants of *Hevea* under high light induced osmotic stress. Plant nuclear DNA being an unstable molecule can be damaged by a number of stress factors including drought, extreme temperatures and pathogen attack (Ryerson and Heath, 1996). In plants, cell death is accompanied by DNA cleavage into oligonucleosomal fragments that form a "ladder" on agarose gels (Ryerson and Heath, 1996). DNA fragmentation has been observed in plants under high light as well as drought stress (Danon and Gollois, 1998; Wituszynska and Karpinski, 2013). In plants, high light stress can damage DNA *via* indirect photosensitizing reactions mediated ROS generation especially singlet oxygen ($^1O_2$) (Alscher*et al.,* 1997). In excessively light-stressed plants H_2O_2 has also been reported to initiate PCD (Mullineaux *et al.,* 2006). Drought stress induced oligonucleosomal DNA fragmentation or DNA ladder formation was observed in susceptible

genotype of wheat (Hameed *et al.,* 2013). DNA fragmentation was also reported in plants undervarious environmental stresses such as cold (Koukalova *et al.,* 1997), salinity (Katsuhara 1997, Ling *et al.*, 2009) and heat stress (Balk *et al.,* 1999).

4.5. Conclusions

After seven days of water deficit stress, the RWC was found decreased in all the *Hevea* clones and accessions, however the reduction was lesser in tolerant accessions and check clones compared to susceptible ones. The gas exchange parmeters, CO_2 assimilation rate (A) and stomatal conductance (g_s) were reduced in all the clones and accessions after exposure to drought conditions and the reduction was less in tolerant accessions. The levels of photosynthetic pigments were reduced under drought in all the clones and accessions compared to irrigated control plants. Anthocyanins showed an increase under drought stress and their accumulation was found high in tolerant accessions. The antioxidant enzymes, SOD and total peroxidase activities increased significantly during dehydration stress and the increase was found associated with drought tolerance in germplasm accessions/check clones. Both thiols and ascorbic acid which are the non enzymatic antioxidant metabolites were also found increased in stressed plants compared to irrigated control plants. Osmolyte, proline showed an increase under drought stress in all the clones and accessions and the level of increase was more in the germplasm accessions compared to clones. Glycine betaine content was also found increased under water deficit stress. The expression of 23.8 kDa chloroplast stress protein was observed in all the tolerant accessions and check clones. The enzyme activities as well as other stress components were found increased under drought stress especially in a higher magnitude in tolerant germplasm accessions. The western blot profile of the 23.8 kDa protein showed its presence in both tolerant and susceptible

accessions, but the level of expression was observed prominent in tolerant accessions. The genomic DNA was found intact and did not show any signs of fragmentation in relatively drought tolerant clone RRII 430 and a few other tolerant germplasm accessions, RO 3261, AC 612 and RO 3157 under high light conditions.

Chapter 5

Expression analysis of drought responsive genes in wild *Hevea* germplasm accessions

5.1. Introduction

Drought is one of the major abiotic stress that negatively influences plant growth and development and also it causes serious loss to crop yield worldwide. Plants have developed diverse mechanisms that lead to adaptation and survival during periods of water deprivation. In response to water deficit stress, plants exhibit the following adaptation strategies: (i) escape, (ii) avoidance, and (iii) tolerance (Jarzyniak and Jasinski, 2014). Drought stress triggers a set of physiological and biochemical responses in plants which include stomatal closure, repression of cell growth and photosynthesis *etc.* (Shinozaki *et al.,* 2003). Drought tolerance is associated with the ability of tissues to withstand low water potential; this is achieved *via* osmotic adjustment and the synthesis of low-molecular weight proteins that protect plants from damage caused due to water deficiency (Bacelar *et al.,* 2012).

The cultivation of natural rubber (*Hevea brasiliensis),* is being extended to drought prone regions which experience high atmospheric temperatures along with high solar light, soil and atmospheric drought and low relative humidity (RH) (Devakumar *et al.,* 1998). In the field, water deficit stress usually occurs in combination with high light and heat and the simultaneous action has a synergistic detrimental effect on plant productivity (Rampino *et al.,* 2012). Prolonged drought stress is often associated with ROS accumulation and hence functional ROS detoxifying

systems are essential for the tolerance of plants towards drought and desiccation (Kranner *et al.,* 2002).

It is need of the hour to develop stress tolerant and climate resilient crops with improved qualities against multiple environmental stresses and high yield. The conventional breeding approaches had only marginal success due to the complexity of stress tolerance traits. For crop improvement programmes, there is a need to understand the function of genes in response to various stresses and during stages of growth and development, thereby necessitating gene expression profiling to identify the candidate genes. So identifying and characterizing critical genes involved in plant stress responses is an essential prerequisite for engineering stress-tolerant crops (Wang *et al.,* 2016).

A number of critical genes involved in enhancing abiotic stress tolerance have been identified and validated in other crops, which are generally classified into two types: functional genes and regulatory genes (Shinozaki *et al.,* 2003). The former encodes important enzymes and metabolic proteins (functional proteins), such as detoxification enzymes, heat shock proteins (HSPs), water channel ion transporters and late embryogenesis abundant (LEA) proteins, which directly functions to protect cells from stresses. The latter encodes protein kinases, protein phosphatases and various regulatory proteins including transcription factors (TFs) which regulate signal transduction and gene expression in response to stress. Although there have been numerous studies on functional genes, most of these studies paid more attention to single gene or several genes encoding enzymes and protective proteins by imposing a given stress. Due to the complexity of stress responses, as they are regulated by multi-genes, little success has been achieved by a single functional gene approach to

enhance plant stress tolerance significantly (Mittler and Blumwald, 2010; Varshney *et al.,* 2011).

Recent progress in plant functional genomics has enabled the identification and characterization of genes involved in various important steps of the drought stress response. Identifying the expression of candidate genes is essential for elucidating the molecular adaptations of plants to drought stress. Furthermore, the identification of the key candidate genes can be used for selection of drought tolerant varieties through marker assisted selection (MAS) (Jiang *et al.,* 2010). Quantitative Real-Time PCR (qPCR) is a reliable method for accurate quantification of gene expression to understand precise gene functions (Reddy *et al.,* 2016).

Studies revealed the occurrence of altered level of expression of various abiotic stress responsive genes in *Hevea* clones in imparting stress tolerance (Thomas *et al.,* 2011; 2012; Sathik *et al.,* 2012; 2018; Luke *et al.,* 2015; 2017) and have identified a number of genes associated with drought tolerance. In this study attempts have been made to identify drought responsive genes in *Hevea* germplasm accessions and to validate their association with drought tolerance. The list of genes studied and their functions are given in Table.5.1.

Table 5.1. List of genes studied and their functions

Genes	Functions
LEA 5 protein	Provides tolerance to plants under water deficit stress (Battaglia *et al.,* 2008).
CRT/DRE bf	*Cis* - acting element involved in ABA dependent and ABA-independent gene expression in response to abiotic stress (Shinozaki *et al.,* 2005).
WRKY tf	Transcription factors that regulate pathways in response to biotic and abiotic stresses in plants (Mingyu *et al.,* 2012).
ABCT protein	Involved in active transport of substances like alkaloids, amino acids, heavy metal chelates, inorganic ions, lipids, peptides and sugars (Jasinski *et al.,* 2003).
Caspase	Involved in Programmed cell death (PCD) that removes unwanted or damaged cells under oxidative stress in both biotic and abiotic stress, has role in defense to environmental stresses and pathogen invasion (Fagundes *et al.,* 2015).
TIP	Vital role in water transport across membranes and improve drought and salt tolerance (Peng *et al.,* 2007; Khan *et al.,* 2015).
MAPK	Involved in signal transduction and also in drought and salt adaptation in different plant species (Golldack *et al.,* 2014).
NAC tf	Roles in plant development and growth, involved in ABA-dependent gene expression under various stresses, including drought and cold (Nakashima *et al.,* 2007; 2012).
ERF	Involved in vegetative and reproductive development. Also expressed in various biotic and abiotic stress responses (Sharoni *et al.,* 2011; Xu *et al.,* 2011).
Ferritin	Iron storage protein, important in sequestering or releasing iron upon demand. Gene expression occurs in pathogen infection, drought, salt, cold *etc.* (Briat *et al.,* 2010; Zang *et al.,* 2017).
MYB tf	Regulates defense responses to various abiotic stress responses, hormone signalling and also involved in many metabolic and developmental processes in plants (Cominelli *et al.,* 2005; Smita *et al.,* 2015).

PP2C	Role in various abiotic stress signal transduction pathways and plant development (Singh *et al.*, 2010).
DNA bp	Regulate leaf maturation and senescence, control flowering, increase plant tolerance to drought, cold responses and salt stresses (Sohn *et al.*, 2006).
HbsHSP23.8	Act as chaperones and play significant role in drought tolerance in *Hevea* (Jun *et al.*, 2015)

5.2. Materials and Methods

5.2.1. Plant material and stress induction

Six month old polybag plants of six germplasm accessions (3 relatively drought tolerant - RO 3261, AC 612, RO 3157 and 3 susceptible - RO 3242, MT 1619, RO 2360) were selected for this study along with relatively drought tolerant (RRIM 600 and RRII 430) and susceptible (RRII 105 and RRII 414) check clones (Sumesh *et al.*, 2011).The plants were grown in open field of RRII under direct sunlight. The plants were subjected to water deficit stress by withholding irrigation for nine days during summer season. A set of control plants was also maintained with saturated level of soil moisture by irrigating on alternate days. The magnitude of impact of water stress was assessed by gas exchange parameters and the leaf samples were collected in liquid nitrogen for gene expression analyses.

5.2.2. Physiological parameters

The degree of impact of drought stress was assessed by measuring the physiological parameters, CO_2 assimilation rate (A) and stomatal conductance (g_s) using a portable photosynthesis system (LI-6400, LI-COR, USA) as mentioned in section 3.2.1.

5.2.3. Total RNA isolation

Leaf total RNA was isolated according to Chang *et al.* (1993).

- **RNA Extraction buffer:** 2% CTAB (hexadecyl trimethylammonium bromide); 2% PVP (polyvinyl pyrrolidone K 30); 100 mM Tris-HCl (pH 8.0); 25 mM EDTA; 2.0 M NaCl; 0.05% spermidine; 2% v/v β-mercaptoethanol (added just prior to use).
- Chloroform: Isoamyl alchol (24:1)
- 10 M Lithidium chloride
- **SSTE:** 1.0M NaCl; 0.5% SDS; 10 mM Tris HCl (pH 8.0); 1.0 mM EDTA (pH 8.0)

Leaf tissues were ground to fine powder in liquid nitrogen using mortar and pestle. Prewarmed 15 ml of extraction buffer to 65°C and then added approximately 2 to 3 grams of ground tissue and mixed well by inverting the tube. The samples were centrifuged at 7000 rpm at 4^0C for 20 min. The top aqueous layer was collected and mixed with equal volume of chloroform and isoamyl alchohol. The tubes were spun at 7000 rpm for 5 min at room temperature. The top aqueous phase was transferred into fresh centrifuge tube and the extraction repeated using chloroform: isoamyl alcohol mixture. The top aqueous phase was again transferred to another tube and mixed with 0.3 volume of 8 M lithium chloride and kept the tubes at 4^0C overnight. The tubes were spun at 7000 rpm for 20 min at 4°C. The pellet was washed with 2 ml of 2M lithium chloride. The tubes were spun at 7000 rpm for 20 min at 4°C. The pellet was resuspended in 500 µl SSTE and mixed with equal volume chloroform: isoamyl alchol (24:1). The tubes were spun at 10,000 rpm for 10 min at 4°C. The upper phase was transferred to another tube and mixed with double volume of absolute ethanol. The tubes were kept at -20°C for 2 hours. The tubes were spun at

10,000 rpm for 20 min to pellet the RNA. The pellet was then dried using vacuum and dissolved in autoclaved DEPC treated water. The concentration and quality of total RNA prepared was determined by spectrophotometer (Nanodrop ND 1000, USA) and formaldehyde denaturing (1.4%) gel electrophoresis.

5.2.4. Formaldehyde gel electrophoresis

Formaldehyde gel (1.4%) was prepared by adding required amount of agarose in 10X MOPS buffer and DEPC water. It was melted to homogeneity and allowed to cool down to about 50^0C. After adding required quantity of formaldehyde and proper mixing, it was poured into casting tray with appropriate sized comb. After 30 min the gel was run in an electrophoretic tank containing 1X MOPS buffer after loading RNA sample plus loading dye. Electrophoretic separation was carried out at 80V till the dye front reached the end of the gel and RNA was visualized and photographed using gel documentation system.

5.2.5. cDNA synthesis

cDNA was synthesized from total RNA using Superscript™ III first strand synthesis system (Invitrogen). 3µg of total RNA was combined with 1µl of oligo dT primer (50 µM) and 1µl 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 5 min and immediately chilled on ice for a min. The cDNA synthesis system mix (10 µl) was prepared by combining 2 µl of 10X RT buffer, 4 µl of $MgCl_2$ (25mM), 2 µl 0.1M DTT, 1 µl of RNase OUT (40 U/µl) and 1 µl of Superscript III reverse transcriptase (200 U/µl). This reaction mix was added to 10 µl of prepared RNA-primer mixture RNA mix (10µl) after incubation at 65^0C for 5 min followed by incubation in ice for a min, then it was mixed with cDNA mix (10µl). The mixture was incubated at 50^0C for 50 min (oligo dT) followed by incubation at 85^0C for 5 min. After a brief incubation in ice,

1 µl of RNase H was added to each tube and kept at 37^0C for 20 min. The cDNA was quantified and stored at -20^0C for further use.

5.2.6. Quantitative PCR (qPCR)

The primers were designed for 14 selected genes which are listed in Table 5.2. Quantitative real-time PCR was performed in triplicate, using specific primers and generated cDNA astemplate in real- time PCR machine (Light cycler 480 II, Roche, Germany).

The primers were standardized for

- Template concentration.
- Primer concentration
- Primer specificity and primer efficiency

Standardisation of template concentration

Different template DNA concentrations like 200 ng, 100 ng and 50 ng were used for standardisation. Template concentration for relative quantification (RQ) analysis was decided based on C_T value in the optimum range.

Standardisation of primer concentration

Various combinations of forward and reverse primer in the range of 50/50, 50/25, 25/50, 25/25, 12.5/12.5, 6.25/6.25 nM were attempted to find out the optimum primer concentration. Primer combinations that did not produce a fluorescence peak in no template control (NTC) were selected for further use in qPCR.

Quantitative gene expression analysis was eventually carried out using Light Cycler 480 II, Roche Real Time PCR System. qPCR was performed in a 20 µl reaction mixture containing 1 µl of 1/10 dilution of first-strand cDNA reaction, 125 nM of each primer and 10 µl of Lightcycler 480 SYBR Green I

Master (Roche Diagnostics Gmbh, Germany). qPCR was performed by incubating the mix at 95° C for 7 min, followed by 40 cycles of 95 °C for 20 seconds and 60 °C for 30 seconds. This was followed by a melt curve analysis (95 °C for 20 seconds, 60 °C for one minute, 95 °C for 5 minutes). Each experiment was repeated two to three times and each PCR reaction was performed in triplicate with no template controls (NTC). Reaction efficiency of both the target genes and the endogenous control was calculated based on the formula, Efficiency = $10^{(-1/slope)}$ -1). Primers with slope values between −3.2 and −3.5 only were employed for these reactions. GAPDH was used as the endogenous control for qPCR analysis. The Relative Quantification (RQ) values were analyzed using the software of Light Cycler 480 (release 1.5.0.) and the rate of gene expression was represented as fold change.

Data analysis

The $2^{-\Delta\Delta ct}$ method was adopted to analyse the relative changes in gene expression from qPCR experiments (Livak and Schmittgen, 2001) and the data were presented as fold change. Three biological replications were included in the qPCR analysis for each experiment.

Table 5.2. List of genes and the corresponding primers used for qPCR analyses

Sl.No	Gene	Forward primer	Reverse primer
1	LEA5	CTCGCTTTCCCTCCAATG	TTCCTCACCATACCACTCC
2	WRKY tf	AGGGAATGGAGATGAGGGAAA	GGGACATAACCAGGTGGCTAGA
3	MAPK	CTGTTGTGTGCAAGCAGGTTTT	CCCTATGTATGACATGTCGCTCAT
4	CRT/DRE	AGTCCCGGCATTGCAAAA	GAGTCAGCGCCGGAGGAT
5	NACtf	TTCAATGGTGGCTTACTCT	CAAGACTGACTGGATTATGC
6	MYB	TGTGACCACTAGAACACCAACTCA	TCCTGTGCTCTGCCTGATAAAA
7	pp2c	AAACGAAACAGAAGGAGAGATTAC	AGAGACGATGAAGGAGAG
8	Ferretin	CTAAACGAGAATAGAAAGCCCAAA	CAGAGCCACCATCCTTCAT
9	ABCT	AGGACTGTTATTGCTTCA	AATAGACTGTTCTGCCATA
10	DNAbp	CTACGAGAAGAGAACAGA	AATGGATATGGAGTCACTA
11	ERF	AGGATTATAGAGTCTTTGAGATTGA	GATAGTTCTTGTGGCTTGTAG
12	Caspase	GTATGGAAAGGAACAAATGGT	GAAGTGTCAGCAGAGGTT
13	TIP	CCCACTGGTCTCATGCCATTA	TGCCGCTATTGTGGCTTCTC
14	HbsHSP23.8	GATGTGGTCGACTCATTTTCTCCA	CTTTGACGTCCTGTTTGCTTAGCC
15	GAPDH	GCCTGTGATAGTCTTCGGTGTTAG	GCAGCCTTATCCTTGTCAGTGAAC

5.3. Results

The study comprises of six germplasm accessions (3 relatively drought tolerant; RO 3261, AC 612, RO 3157 and 3 susceptible; RO 3242, MT 1619, RO 2360accessions) along with elite check clones; relatively drought tolerant (RRIM 600 and RRII 430) and susceptible (RRII 105 and RRII 414). The impact of drought stress on plants was assessed by gas exchange parameters (Figs. 5.1 & 5.2). Stomatal conductance was found to be decreased in all the germplasm accessions and check clones on

drought tolerance as well as in defence mechanisms against high temperature showed a significant increase during drought stress. Among the biochemical factors investigated, the antioxidant enzymes superoxide dismutase and peroxidase as well as non enzymatic antioxidants ascorbic acid and thiols showed an increase under drought stress. Enhanced activity of antioxidant enzymes SOD and POD were found in tolerant clones and accessions. The level of ascorbic acid also followed the same trend. But the level of thiols showed an increase in all the clones and accessions under drought stress, irrespective of tolerant or susceptible. This reveals the presence of an active free radical scavenging system especially in drought tolerant clones/accessions. The compatible solutes glycine betaine and proline also showed significant accumulation under drought stress. Among which proline showed a tremendous increase in germplasm accessions compared to clones. Glycine betaine also showed an increase under drought stress in tolerant clones and accessions. Water deficit stress slightly reduced the level of plant pigments chlorophyll and carotenoids, whereas anthocyanins showed an increase under drought stress. An analysis of biochemical traits indicated a strong association of these factors in enhancing drought tolerance.

The SDS-PAGE profile showed differential expression of a chloroplast stress protein of molecular weight 23.8 kDa under drought stress. The expression was found prominent in tolerant clones/accessions under drought stress compared to irrigated control plants. Western blot analysis using the antibody raised against 23.8 kDa chloroplast stress protein showed their expression in susceptible accessions also, but the level of expression was found less compared to tolerant accessions. The results showed the association of chloroplast stress protein expression in enhancing drought tolerance in *Hevea*.

The *Hevea* genomic DNA showed considerable fragmentation in susceptible clones and germplasm accessions under high light in growth chamber conditions where as in tolerant accessions the DNA remained more intact indicating their drought tolerance potential. However, in the open field conditions, under high light intensity all the germplasm accessions showed significant fragmentation indicating that high light can inflict serious damages to DNA, irrespective of tolerant or susceptible.

The expression analysis of drought related genes showed that the genes such as *NAC tf, LEA5, WRKY tf, DNA bp, ERF, MAPK, TIP* and *HbsHSP23.8* were drought responsive and were found upregulated under drought stress in *Hevea* clones and germplasm accessions. *TIP* showed an upregulation in all the clones and accessions studied, whereas *HbsHSP23.8* was found upregulated almost in all the clones and accessions except in susceptible clone RRII 414 and accession MT 1619. *MAPK* was found upregulated in all the clones and accessions except in clones RRII 105, RRII 414 and accession MT 1619. The transcripts *NAC tf, LEA 5, WRKY tf, DNA bp* and *ERF* were significantly upregulated in tolerant clones and accessions alone, showed a clear trend in their pattern of expression and thus reveal their association in enhancing drought tolerance.

On the basis of physiological, biochemical and molecular investigations, the accessions RO 3261 and AC 612 are the top drought tolerant accessions among the lines studied and can be further used for crop improvement programmes in *Hevea*. The transcripts *viz. NAC tf, LEA 5, WRKY tf, DNA bp* and *ERF* showed strong association with drought tolerance and exhibited a clear trend in their expression pattern, hence can be further used as molecular markers for early screening of *Hevea* genotypes for drought tolerance.

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List of Publications

Journals

Smitha M. Xavier, Molly Thomas, K.V. Sumesh and K. Annamalainathan. (2018). Drought Induced Changes In Leaf Pigments And Osmolyte Contents In *Hevea* Germplasm Accessions. *Indian Journal of Scientific Research,* 19(1): 61 – 67.

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Seminars

Smitha M. Xavier, Molly Thomas, K.V. Sumesh, K. Annamalainathan and James Jacob. (2018). Quantitative expression analysis of selected drought responsive genes in *Hevea* germplasm accessions. *International Conference on Science, Engineering, Technology and Social sciences (ICSETS-2018), Kuriakose Elias College, Mannanam. Abstracts pp. 23-2.*

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Molly Thomas, **Smitha M. Xavier**, K.V. Sumesh, K. Annamalainathan, D.B. Nair and M.A. Mercy. (2014). Identification of potential drought tolerant *Hevea* germplasm accessions using physiological and biochemical parameters. *International Symposium on Plantation Crops (PLACROSYM XXI) at Indian Institute of Spices Research, Kozhikode. Abstracts. pp. 27.*

Molly Thomas, James Jacob and **Smitha M. Xavier** (2016). High light and osmotic stress Induced fragmentation of genomic DNA in *Hevea Brasiliensis. National Conference of Plant physiology – 2016, University of Agricultural Sciences, GKVK, Bengaluru. Souvenir and Abstracts. pp – 110*