Assessment of variability in Acquired Thermotolerance in *Hevea brasiliensis* genotypes -In vitro studies

Dissertation submitted to CMS College Kottayam (Autonomous)

in partial fulfilment of the requirements for the award of **Degree**

Master of Science in Biotechnology

By

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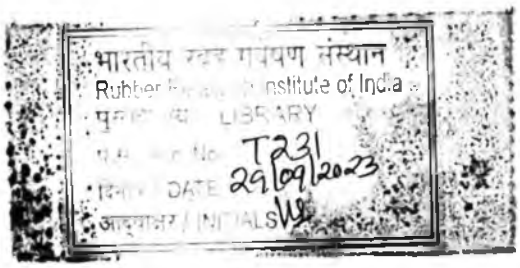
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CERTIFICATE

This is to certify that Ms **Megha S.** is a bonafide student of MSc Biotechnology, Department of Biotechnology, CMS College Kottayam.She has completed her dissertation entitled **"Assessment of Variability in Acquired Thermotolerance in *Hevea brasiliensis* genotypes-In Vitro studies."**under the guidance of **Dr. JayasreeGopalakrishnan** at **RRII**(institution) durmg April2023-June 2023. This report is submitted for the partial fulfillment of Masters degree in Biotechnology to CMS College (Autonomous) Kottayam.

Kottayam

16.05.2023

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This is to certify that this Dissertation entitled "**Assessment of variability in Acquired thermotolerance in *Hevea brasiliensis* genotypes –In vitro studies"** is an authentic record of research work carried out by Ms **Megha S.** under my supervision and is submitted in partial fulfillment for the award of degree of Master of Science in Biotechnology to the C.M.S College (Autonomous) Kottayam

Place: Kottayam

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DECLARATION

I hereby declare that the dissertation entitled "Assessment of Variability in Acquired Thermotolerance in *Hevea brasiliensis* genotypes -In Vitro studies." Submitted in partial fulfilment for the award of the Degree of Master of Science in Biotechnology of Mahatma Gandhi University is a bonafide work carried out by me under the guidance and supervision of Dr. Jayasree Gopalakrishnan, Scientist D, Crop Physiology Division, Rubber Research Institute of India, Kottayam and that no part of this dissertation has been presented earlier for any other degree/diploma/associateship/fellowship or other similar title for any other University/Institution.

Megha S.

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Megha S.

Contents

Abstract

Prevalence of high temperature is the major limitation for the cultivation of crops in tropical conditions. The day temperatures to which the plants are exposed in many tropical areas are often above their optimal growth temperature and a small increase above optimum has large effect on growth rate. In current ever changing environment, development of genotypes that are capable to survive better under high temperature stress is paramount important and inevitable. In this context a protocol called Temperature Induction Response (TIR) technique has utilized to assess the Acquired Thermos Tolerance(ATT) of *Hevea* genotypes. Acquired tolerance on temperature means the level of protection beyond the inherent thermotolerance that result from prior exposure to elevated lethal temperature. The in-vitro study on acquired thermotolerance of *Hevea* clones, RRII 414, RRII 417,RRII 422, RRII 429, RRII 430, RRIC 100, RRII 105 and RRIM 600 by TIR have shown that , the cellular activity was high in clone RRII 430 followed by RRII 414, RRII 422, RRII 417 and RRIM 600 after acclimatization treatment (35°C, 2h→50°C, 1h). It was also observed that with respect to total chlorophyll and it`s percentage of decrease by temperature treatment, a high basal thermotolernance (BTT) in clones, RRII422 (1.26% decrease), RRII 417 (2.88% decrease) and RRII 414 (5.77% decrease). ATT also showed a same trend that % decrease in RRII422 (4.57%), 7.87% in RRII 417 and 9.18% in RRII 414. In the CMS assay direct exposure to high temperature both 35°C and 50°C showed membrane injury to a larger extent than 35°C→50°Cor acclimatized condition. Results of the study revealed that changes in responses of genotypes to induction was prominent. It is expected that with a whole plant level treatment may get clearer picture of the induction responses. However for a rapid screening of a large number of diverse genotypes this methodology can be employed to determine the relative heat tolerance.

Key Words: Heat tolerance, Acclimatization, Temperature Induction Response, Acquired Thermotolerance ,Physiological Assay

Introduction

Hevea brasiliensis generally known as Para rubber tree of Euphorbiaceae family is the most important commercial source of natural rubber, a product derived from its latex, that has great significance(Charles,1734). This tropical tree is native to the Amazon basin in Brazil and the most economically important member of the genus *Hevea*. It was introduce to Asia by Henry Wickham in the year 1876, through Kew gardens in England. Following species are recognised of *Hevea*., *Hevea benthamiana, Hevea brasiliensis, Hevea camargoana, Hevea camporum Hevea guianensis, Hevea microphylla, Hevea nitida, Hevea pauciflora, Hevea rigidifolia, Hevea spruceana*. Covering 2 third of the geographical area, it has wide distribution over fair range of habitat and is the only species grown for commercial source of natural rubber among others in India.

Hevea brasiliensis is a quick growing sturdy tree, with its bark greyish and the leaves alternate trifoliate.It is successfully cultivated under humid law land tropical condition roughly between 15 degree N and 10 degree S at an altitude between 300-500m.A warm humid equable climate between 21-35°Cand fairly distributed annual rainfall not less than 200cmare required for optimum growth. The requirement of atmospheric humidity is high of about 80 percent, with moderate wind, bright sunshine of about 2000 hours per year and well drained, fairly deep loamy soil with pH 4.0-6.0 and it's nutrient requirement compared to other crops is minimum.

The rubber growing regions in India can be classified under 2 main zones, traditional and non - traditional on the basis of agro-climatic condition .Former include Kanyakumari, Whole Kerala, Dakshina Kannada and Coorg. Rubber is now being grown in North eastern states, West Bengal, Konkan region of Goa and Maharashtra, Parts of Andhra Pradesh, Madhya Pradesh, and Orissa states, which are under non- traditional zone.

The yield of rubber is influenced by various factors such as genetic nature, nutrition, rainfall, tapping nature and climate (Jacob *et al.*,1999).They are also susceptible to biotic factors like bacteria, fungus etc. And abiotic stress such as of drought, cold, wounding etc.

Of the major forms of abiotic stress plants are exposed to in nature, heat stress has an independent mode of action on the physiology and metabolism of plant cells. The susceptibility to high temperature in plants varies with the stage of plant development. The observed effects depend on species and genotypes, with abundant inter and intra specific variation(Barnabar *et al.*,2008;Sakata and Higashitani,2008).

Various physiological injuries, that have been observed under elevated temperature, include, leaves scorching, leaf abscission, senescence, shoot and root growth inhibition, fruit damage, decrease plant productivity(Vollenweider and Gunthardt -George,2005). Heat stress induce changes in photosynthesis and respiration and can lead to structural alterations in chloroplast protein complexes and reduced activity of enzymes (Ahmad *et al.*, 2010). In addition, by causing injuries to the cell membrane, organization of microtubules and ultimately to the cytoskeleton, heat stress changes membrane permeability and alters cell differentiation, elongation, and expansion (Smertenko *et al.*, 1997; Potters *et al.*, 2008; Rasheed, 2009). It has been observed that the photochemical modifications in the carbon flux of the chloroplast stroma and those of the thylakoid membrane system are considered the primary sites of heat injury (Wise *et al.*, 2004). A specific effect of high temperature on photosynthetic membrane is the accompanying of ion leakage from leaf cells(Wahid and Shabbir, 2005; Allakhverdiev *et al.*, 2008).The detrimental effects of heat on chlorophyll and the photosynthetic apparatus are also associated with the production of injurious reactive oxygen species (Camejo *et al.*, 2006; Guo *et al.*, 2007). High temperature modifies the activities of carbon metabolism enzymes, starch accumulation, and sucrose synthesis, by down-regulating specific genes in carbohydrate metabolism (Ruan *et al.*, 2010). Sexual reproduction and flowering in particular have been long recognized as extremely sensitive to heat stress, which often results in reduced crop plant productivity(Hedhly *et al.*, 2009; Thakur *et al.*, 2010). Heat stress during seed development may result in reduced germination and loss of vigour, leading to reduced emergence and seedling establishment (Akman, 2009; Ren *et al.*, 2009)and also Quality reductions in starch, protein, and total oil yield have been associated with heat stress during seed development (Banowetz *et al.*, 1999).

Plants have evolved various mechanisms to ensure survival under elevated temperatures. Among general stress tolerance mechanisms, stress proteins, osmo-protectants, free-radical

scavengers, ion transporters and factors involved in signaling cascades and transcriptional control are essential to counteract stress effects (Wang *et al.*, 2004).Heat tolerance is a multigenic character with numerous biochemical and metabolic traits (Kaya *et al.*, 2001)and plants are capable of adapting to a wide range of temperatures by reprogramming their transcriptome, proteome, and metabolome and even by activating cell death mechanisms leading to organ abortion or entire plant death(Qi *et al.*, 2011; Sánchez-Rodríguez *et al.*, 2011).

Acquired thermo-tolerance in plants refers to the ability to cope with lethal high temperatures following acclimatization at sub-lethal high temperatures. It reflects an actual thermo-tolerance mechanism naturally occurring in plants and has been extensively used in thermotolerant line identification. Acquired thermotolerance lead to enhanced protection of plant cells from subsequent heat induced injury. In recent years, great progress has been achieved in the elucidation of biochemical, physiological, and molecular mechanisms of thermotolerance acquisition by using genomic approaches, including microarray analysis and mutation, knockout, and over expression of related genes(Song *et al.*,2012).

Involvement of Heat shock proteins (HSPs), such as Hsp101, BOBBER1 and Hsa32(Song *et al.*,2012)and up regulation of genes related to heat shock factor, stress associated proteins, ROS scavenging, fatty acid metabolism, are some of the mechanism for acquired thermos tolerance. The significant enrichment of KEGG pathways involved in protein processing, MAPK signalling and HSPs are also involved in acquired thermo-tolerance (Kumar *et al.*,2018).Acquired thermotolerance was been associated with induction of Peroxidase, Ascorbic Peroxidase and Catalase Activities(Sharma *et al.*,2014).The induction of chloroplast LeHSP100/ClpB contributes to the acquisition of thermotolerance in higher plants and involvement of choloroplast HSP100Clpb in the acquired thermotolerance is also an ATT mechanism (Jin-Ying *et al.*,2006).In land plant thermal sensing and acquired thermotolerance are been controlled by Plasma membrane cyclic nucleotide gated calcium channel(Andrija *et al.*,2012).

ATT have been reported from many crop species including Rice, Wheat, Tomato, mustard and legume family etc. Prevalence of high temperature and the day temperatures exposure above their optimal growth temperature and a small increase above optimum has large effect on growth rate and plant cultivation (Howarth, 1996). In current ever changing

environment, development of genotypes that are capable to survive better under high temperature stress is paramount important and inevitable. And here Acquired thermotolerance have a prominent role in plant protection and it's optimal growth as well good cultivation under the heat stress condition.

Most of the abiotic stress studies in *Hevea* is related to drought, cold and high light conditions. Studies, related to adaptations or resistance to high temperature was limited in the case of *Hevea*. In the current scenario of raising global temperatures, investigations into the heat tolerance of plants have gained renewed high interest in recent years. Temperature Induction Response(TIR) technique, which widely used for rapid screening of genotypes for high temperature tolerance. Hence present study is to develop a rapid screening method for high temperature tolerance by adopting this technique and assessment of some elite *Hevea* varieties. Rubber plant genotypes that has been evaluated by ATT is expected to have a good Heat tolerance response in its cultivation, growth and yield. In this study the genotypes included are RRII 414,RRII 417,RRII 422, RRII 429,RRII 430,RRIC 100,RRII 105 and RRIM 600.Physiological assay with respect to cell viability, chlorophyll content, membrane damage etc., besides TIR technique that would promote a good screening program for heat stress and can be implemented as part of breeding programme for the detection of heat tolerant genotypes, as well a tool to select relative heat tolerant onesfor promoting their propagation.

Aim and Objective

Aim

Evaluation of the AcquiredThermotolerance in *Hevea* genotypes by in vitro studies

Objective

- To determine the Acquired Thermotolerance using Temperature Induction Response(TIR) technique in *Hevea brasiliensis*.

- Identification of traits related to acquired thermotolerance to employ them in screening for tolerant varieties or to use them in crop improvement programmes.

Review of Literature

In the present scenario, we can see a situation in our climatic condition, with simultaneous change of temperature by its high rate of increase ,as a result of global warming, causing serious threat to the life forms. Global warming is predicted to have a general negative effect on plant growth, due to the damaging effect of high temperature on their development. The increasing threat of climatological extremes, including very high temperature might lead to catastrophic loss of crop productivity and result in widespread of famine.

Thus the situation demands for the generation of those varieties, with higher average temperature and larger temperature fluctuation tolerance, besides sustainable yield production. Acquired tolerance on temperature means the level of protection beyond the inherent thermotolerance that result from prior exposure to elevated lethal temperature. Assessment of varieties that have ability to acquire thermotolerance to lethal temperature paves much importance in their selection and propagation besides other technique for the crop improvement to heat stress tolerance.

Of the major forms of abiotic stress plants are exposed to in nature, heat stress has an independent mode of action on the physiology and metabolism of plant cells. The susceptibility to high temperature in plants varies with the stage of plant development, the observed effects depend on species and genotypes, with abundant inter and intra specific variation (Barnabar *et al*.,2008;Sakata and Higashitani,2008)

Various physiological injuries, that have been observed under elevated temperature, include, leaves scorching, leaf abscission, senescence, shoot and root growth inhibition, fruit damage, decrease plant productivity(Vollenweider and Gunthardt George,2005). Heat stress induce changes in photosynthesis and respiration and can lead to structural alterations in chloroplast protein complexes and reduced activity of enzymes (Ahmad *et al*., 2010). In addition, by causing injuries to the cell membrane, organization of microtubules and ultimately to the cytoskeleton, heat stress changes membrane permeability and alters cell differentiation, elongation, and expansion (Smertenko *et al*., 1997; Potters *et al*., 2008; Rasheed, 2009). It has been observed that the photochemical modifications in the carbon flux of the chloroplast stroma and those of the thylakoid membrane system are considered the primary sites of heat injury

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Sexual reproduction and flowering in particular have been long recognized as extremely sensitive to heat stress, which often results in reduced crop plant productivity (Hedhly *et al.*, 2009; Thakur *et al.*, 2010).The male gametophyte is particularly sensitive to high temperatures at all stages of development, while the pistil and the female gametophyte are considered to be more tolerant (Hedhly, 2011). Male sterility as a consequence of heat stress can be widely observed among many sensitive crop plants (Sakata and Higashitani, 2008; Wassmann *et al.*, 2009) Heat stress during seed development may result in reduced germination and loss of vigour, leading to reduced emergence and seedling establishment as has been shown for several crop plants (Akman, 2009; Ren *et al.*, 2009) Quality reductions in starch, protein, and total oil yield in several crop species have been also associated with heat stress during seed development (Banowetz *et al.*, 1999).

Plants have evolved various mechanisms to ensure survival under elevated temperatures. Among general stress tolerance mechanisms, stress proteins, osmo-protectants, free-radical scavengers, ion transporters and factors involved in signaling cascades and transcriptional control are essential to counteract stress effects (Wang *et al.*, 2004).Heat tolerance is a multigenic character with numerous biochemical and metabolic traits (Kaya *et al.*, 2001)and plants are capable of adapting to a wide range of temperatures by reprogramming their transcriptome, proteome, and metabolome and even by activating cell death mechanisms leading to organ abortion or entire plant death (Qi *et al.*, 2011; Sánchez-Rodríguez *et al.*, 2011).

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cells from subsequent heat induced injury .In recent years, great progress has been achieved in the elucidation of biochemical, physiological, and molecular mechanisms of thermo-tolerance acquisition by using genomic approaches, including microarray analysis and mutation, knockout, and over expression of related genes(Song *et al.*,2012).

Heat shock proteins (HSPs) such as, Hsp101, BOBBER1 and Hsa32, have been shown to be important for inducement and maintenance of acquired thermo-tolerance (ATT).Their appearance, correlated with the development of acquired thermo-tolerance is strong(Song *etal.*,2012). Downstream target genes and upstream regulation factors of HsfA2, including Hsa32, Apx2, small ubiquitin-like modifier proteins, FK506-binding proteins ROF1 (FKBP62) and ROF2 (FKBP65), and heat shock transcription factor binding protein, have been revealed to be involved in thermo-tolerance acquisition regulation. Hsa32 a novel Hsp is required for not only the induction but also maintenance of ATT(Yee-Yung Charng *et al.*, 2006).Moreover, the role of abscisic acid, ethylene, hydrogen peroxide, and salicylic acid in acquired thermo-tolerance has been demonstrated by molecular evidence from *Arabidopsis* mutants and transgenic lines(Song *et al.*,2012).

Acquired thermo-tolerance at the molecular level, the RNA sequence approach was employed by adapting TIR method analysis showed that the genes related to heat shock factor, heat shock proteins, stress associated proteins, ROS scavenging, fatty acid metabolism, protein modification were significantly up regulated during induction, thus preparing the organism or tissue at molecular and cellular level for Acquired Thermo-tolerance. KEGG pathway analysis revealed, the significant enrichment of pathways involved in protein processing, MAPK signaling and HSPs which indicate that these process are conserved and involved in Thermo-tolerance (Kumar *et al.*,2018).

Plant Hsfs, paly vital role in plant response to heat shock. *Arabidopsis* seedling of ZmHsf05 isolated from maize, over expressing increased both the basal and acquired thermo-tolerance. After heat stress, the ZmHsf05 over expressing lines showed enhanced survival rate and chlorophyll content compared with WT seedling. The expression of Hsps was up regulated in the ZmHf05 over expressing *Arabidopsis* lines after heat stress treatment (Li *et al.*,2019). HsfA2 as a heat inducible trans-activator sustain the expression of Hsp gene and extend the duration of AT in *Arabidopsis* (Charng*et al.*, 2007).HsfB1 and HsfB2b appear to be necessary

for the expression of heat –inducible Hsp genes under heat stress condition which is required for the AT (Ikeda *et al*.,2011). In potato acquired thermo-tolerance response whereby treatment at a mildly elevated temperature primes the plant for more severe heat stress. Physiological, transcriptomic and metabolomics approach were employed to elucidate potential mechanism that underpin the acquisition of heat tolerance which indicate the role of cell wall modification, auxin and ethylene-signaling, and chromatin re-modeling in acclamatory priming (Mozos*et al*., 2018). In the study on bread wheat, heat tolerance in relation to acquired thermo-tolerance for membrane lipid was observed and the correlation coefficient revealed that the Heat Response Index was the most important trait followed by Tetrazolium Triphenyl Chloride test (Dhanda and Munjal,2012). Wheat plant is also been studied for presence of plastid- localized HSP26 gene family, that are closely associated with acquired thermo-tolerance in them(Joshi *et al*.,1997). Acquired-thermo-tolerance, had been studied as a mechanism in heat stress tolerance in peanut seedling (Selvaraj *et al*.,2011).

Of a screening procedure for the isolation and characterization of acquired-thermo-tolerance mutants, inhibition of chlorophyll accumulation in etiolated tissue following challenges at lethal temperature and the prevention of this inhibition by pre incubation at a non-lethal elevated temperature is been employed for their identification. *Arabidopsis thaliana* mutant deficient in varying level of acquired thermo-tolerance have been identified from both the RLD and Columbia ecotypes (Burke*et al*.,2001). The physiological basis of thermo-tolerance is referred with respect to higher chlorophyll stability index and a strong antioxidant enzyme system with lesser lipid peroxidation in terms of Malondialdehyde content values in the Rice plant (Vijayalakshmi *et al*.,2015). TIR studies is a rapid screening protocol to dissect the genetic variability in acquired thermo-tolerance and identify novel donor for high temperature stress tolerance, that has been employed in rice plant(Vijayalakshmi *et al*.,2015). Heat acclimation in rice seedling showed positive feedback loop formed by two heat – inducible genes, Heat Shock Protein101(HSP101) and Heat Stress Associated 32-KD Protein (HSA32), at the post transcriptional level that prolongs the effect of acclimation. Molecular mechanism underlying heat acclimation memory confers long term acquired thermo-tolerance (Lin *et al*.,2014). Different *Triticum durum* cultivars were characterized for their response to high temperature at the physiological and molecular level; in which the accumulation of mitochondrial HSP transcript appear to be related to the acquisition of thermal tolerance

(Rampino *et al*.,2009).Acquired thermo-tolerance also had been evaluated in *Triticum aestivum* and *Triticum durum* cultivars grown in Turkey (Yildiz and Terzi.2008).Exposure of 24-40 hour old sorghum bicolour seedlings to heat shock resulted in depression of normal labelled amino acid incorporation into protein and rapid production of a small HSPs. were it was able to acquire tolerance to elevated temperature as a consequences to brief exposure to 45°C-50°C(Ougham and Stoddart,1986).

The induction of thermo-tolerance using the efficacy of heat acclimatization was analysed in nine varieties of moth bean, where Acquired thermo-tolerance was been associated with induction of Peroxidase, Ascorbic Peroxidase and Catalase Activities, among them CAT has the greater activity under lethal temperature(Sharma *et al*.,2014). The induction of chloroplast LeHSP100/ClpB contributes to the acquisition of thermo-tolerance in higher plants and involvement of chloroplast HSP100Clpb in the acquired thermo-tolerance have been found in tomato (Yang*et al*.,2006).In land plant thermal sensing and acquired thermotolerance are been controlled by Plasma membrane cyclic nucleotide gated calcium channel(Finka*et al*.,2012).

Clonal difference in low temperature response has been reported in *Hevea* based on physiological traits like loss of membrane stability (Sathik *et al*., 1998a).High light during day time combined with cold stress in the previous nights during winter seasons leads to severe inhibition of photosynthesis and chloroplyll bleaching (Jacob *et al*., 1999) in cold susceptible clones like RRII 105 whereas the cold tolerant clones like RRIM 600 showed better photosynthesis and lesser membrane permeability. In *Hevea brasiliensis* 30 Hsf genes were identified using genome wide analysis, that possess a structurally conserved DNA binding domain and an oligomerization domain (Yan Li *et al*..2019).q RT-PCR analysis showed that the 7 HbHSP90 genes responded in different degree to temperature, also HbHSP90. 1 has been characterized as candidate gene for stress response in rubber tree(Lin*et al*.,2022). Down regulation of auxin and ethylene signalling and activation of heat shock module and ROS scavenger is a primary strategy for *H.brasilensis* to cope with low temperature tolerance stress. Cope with cold stress, the RRIM600 clone up regulates genes promoting stomata closure, photosynthetic inhibition and a more efficient reactive oxygen species scavenging system. Deep expression analysis reveals distinct cold response strategies in rubber tree(Mantello*et al*.,2019). ICE, an inducer of CBF expression is a positive regulator of cold signalling pathway in plants

and they mainly include HbICE1,HbICE 2 and HbICE4 (Li *et al.*, 2022). Increased temperature causes loss of soil moisture content. *Hevea brasiliensis* that analysed under soil moisture deficit condition showed chloroplast stress protein by western blot technique that indicate its consistent over expression in the drought tolerant clones. abundance of this protein was associated with lesser inhibition in photosynthesis that are subjected to water deficit stress (Annamalainathan *et al.*,2017). RRIC 100 are more tolerant to drought than RRII 105(Chandrashekar *et al.*,1998). Peroxidase and WRKY transcription factor were found to be strongly associated with drought tolerance in rubber plant. Water use efficiency under water deficit condition was the highest in RRIM 600 and the least in RRII 105 (Thomas *et al.*,2012). However the contents of Malondialdehyde (MDA) and Proline were significantly increased under water deficit stress. Peroxidase and superoxide dismutase activities were enhanced at 1 and 3dww, respectively. Meanwhile, the soluble sugar content was constant under the water deficit stress. Energy biosynthesis and ROS scavenging system related genes including HbCuZnSOD, HbMnSOD, HbAPX, HbCAT, HbCOA, HbATP and HbACAT were significantly up regulated by water deficit stress, indicating the adaptation of rubber tree governing by energy biosynthesis, antioxidative enzymes and also osmoregulation, during drought stress that could be arise due to increase temperature(Wang,2014).

Temperature extremes are the major factors limiting the productivity and geographical distribution of this crop. Shortage of available land ensuing from competition with other crops and increasing national and international demand for NR led rubber cultivation to be extended to marginal and subtropical environments. Hence crop improvement programme for agroclimatically stressful areas in which *Hevea* is expected to thrive needs to take the account of inherent or intrinsic capabilities to adapt the limiting conditions.

Materials and Methods

Plant material:

The present work was conducted at Rubber Research Institute of India experimental farm. The study was carried out with eight *Hevea* clones Viz. RRII 414, RRII 417, RRII 422, RRII 429, RRII 430, RRIC 100, RRII 105 and RRIM 600. They were grown under a well-managed condition in the nursery. These plants were evaluated for acquired thermotolerance. Youngest fully developed leaves from these plants were sample at morning hours, packed in a polythene bag and quickly transported directly to the laboratory from the nursery so that the leaves were available for the bioassay in less than 4 hours. Leaf disks were then cut from the disinfested leaves using a 15mm cork borer and placed in moisten petri plate. The temperature treatment of the study is based on temperature induction response.

Temperature Induction Response (TIR) Method:

Leaf discs of 15mm were excised from all the clones. These leaf tissues were exposed to the following treatments,

Control treatment – 25°C under normal condition

Sublethal Temperature (35°C) treatment –T1.

Leaf discs were placed in petri plate and kept in an incubator, subjected to 35°C for 1hr imposed

Sub lethal Temperature to high temperature (lethal temperature, 50°C) treatment –T2

Leaf disc were placed in petridish and kept in an incubator at first exposed to 35°C for 2hrs after that exposed to lethal temperature 50°C for two hours.

High temperature (lethal temperature,50°C) treatment- T3

Leaf discs were placed in petri plate and kept in an incubator, lethal temperature 50°C for was imposed one hour.

At the end of the treatments a. following parameters were studied.

Heat stress assay (2,3,5-Triphenyl Tetrazolium Chloride reduction assay)

Temperature tolerance of *Hevea* genotypes was measured using a 2,3,5 triphenyl tetrazolium chloride (TTC) salt solution method that is Heat stress assay (HSA) (Cottee *et al.*, 2010). Leaf discs 15 mm in diameter were excised and treatments were imposed and TTC reduction is performed. Preparation of reagents are as follows.

Reagents

1. Buffer: 0.05M KPO4 buffer is prepared by dissolving 8.7g K2PO4 and 6.8g KH2PO4 in ddw l with pH 7.5 and adjust the pH with KOH/NaOH
2. 2,3,5-triphenyl tetrazolium chloride: TTC reagent is prepared by dissolving 0.6g TTC 100ml distilled water and the solution should be covered/placed in dark
3. 95% ethanol

Procedure

Leaf discs of 15 mm in diameter were excised from two replica of each genotypes RRII 414, RRII 417, RRII 422, RRII 429, RRII 430, RRIC 100, RRII 105 and RRIM 600,and are placed in moisten petri plate, for temperature treatment by TIR technique. A phosphate buffer solution containing 0.05 M phosphate-buffered saline and 0.6% w/v 2,3,5-triphenyl tetrazolium chloride (TTC) was prepared and 5 mL was added to each vial. And left to incubate at 25 °C in the dark for 24 h. Discs were triple rinsed with Millipore water, submerged in 5 mL of 95% (v/v) ethanol, and incubated for a further 24 h in the dark. Enzyme viability was determined by spectrophotometric absorbance at 530 nm using 95% ethanol as a reference (Spectro UV-VIS Dual Beam spectrophotometer, Labomed.Inc).

Data is presented as absorbance values for samples incubated at 25 °C (Abs 25) and 35 °C (Abs 35). 35°C →50°C (Abs 35 →50) and 50°C (Abs 50). The relative absorbance (Rel Abs) is calculated as the quotient of absorbance values at 530 nm for samples incubated at 35°C, 35°C 50°C, 50°C 40 °C to that of 25 °C.

Chlorophyll bioassay

Reagents

1. Acetone.100% - Solvent A
2. Dimethyl Sulfoxide: 100% - Solvent B
3. Extraction solvent: A:B in 1:1 ratio

Procedure

The chlorophyll content was quantified in the leaf discs following leaf bioassay, for that Leaf discs of 15 mm in diameter were excised from two replica of each genotypes RRII 414, RRII 417, RRII 422,RRII 429,RRII 430, RRIC 100, RRII 105 and RRIM 600 and are placed in moisten petri plate, for temperature treatment by TIR technique. Chlorophyll was extracted from leaf tissue in Acetone: Dimethyl sulfoxide (1:1)mix and centrifuged and the supernatant was made up to a known volume (triplicate was maintained). The absorbance was recorded at 470, 663, 665 and 645 nm using a UV-VIS Dual Beam spectrophotometer(Labomed. Inc) to estimate the chlorophyll content (Hiscox & Israelstam, 1979).

Chlorophyll content was calculated as

Total chlorophyll = [20.2 x A645 + 8.02 x A663]

The values thus obtained are in µg/ml of extract (solvent). Values in mg/g fresh weight are obtained by multiplying the above values with "V/(W or A x 1000)", where V is volume of extract (ml); W is fresh weight of sample (g); A is area of the sample.

Cell membrane stability (CMS)

Leaf electrolyte leakage as a measure of CMS was determined according to the method of Deshmukh *et al*., (1991).Leaf discs of 15 mm in diameter were excised from two replica of each genotypes RRII 414, RRII 417, RRII 422, RRII 429, RRII 430, RRIC 100, RRII 105 and RRIM 600 and are placed in moisten petri plate, for temperature treatment by TIR technique. Leaf samples (three 15mm leaf discs) were then placed in test tubes filled with 25 ml double distilled water and kept at room temperature for 24hs. The electric conductivity (EC1), the initial reading was measured by using an electric conductivity meter (CON 2700, EUTECH INSTRUMENTS). Then, test tubes were heated in the boiling water bath for 30minutes, 1hr and cooled to room temperature and the final electrical conductivity (EC2) was recorded. The relative injury or electrolyte leakage was calculated based on the formula by Karlidag *et al*., (2010).

MSI = (C1/C2) x 100.

Results and Discussion

Prevalence of high temperature is the major limitation for the cultivation of crops in tropical conditions. The day temperatures to which the plants are exposed in many tropical areas are often above their optimal growth temperature and a small increase above optimum has large effect on growth rate (Howarth, 1996). In current ever changing environment, development of genotypes that are capable to survive better under high temperature stress is paramount important and inevitable. In this context a protocol called temperature induction response (TIR) technique has utilized to assess the acquired thermo-tolerance of *Hevea* genotypes. Fig 1-3 shows the temperature induction in leaf tissue.

Fig.1.Temperature induction at 35°C (1hr) sub lethal temperature (T1) of *Hevea* genotypes:RRII414, RRII 417, RRII 422,RRII 429,RRII 430, RRIC 100, RRII 105,RRIM 600

Fig.2.Temperature induction at 35°Cfor 2hrs and 50°Cfor 1hr Acclimatization treatment (35°C 2hrs→50°C1hr - T2) of genotypes: RRII414, RRII 417, RRII 422, RRII 429, RRII 430, RRIC 100, RRII 105,RRIM 600

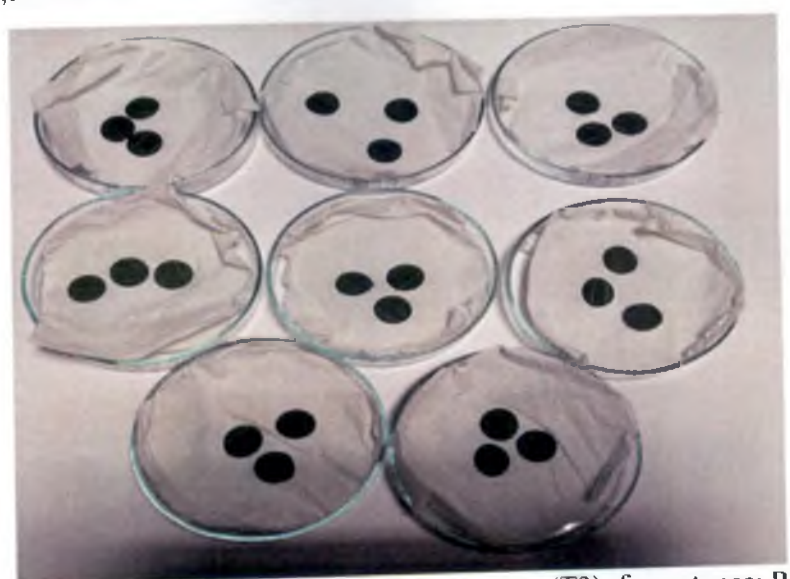

Fig.3.Temperature induction 50°C(1hr)- lethal temperature (T3)of genotypes: RRII414, RRII 417, RRII 422, RRII 429,RRII 430, RRIC 100, RRII 105,RRIM 600

Fig .4. TTC reduction in leaf discs of *Hevea* genotypes in control and temperature treatments

Fig. 4.1 Control RRII 414

Fig. 4.2 Control RRII 417

Fig.4.3 Control RRII 422

Fig.4.4 Control RRII 429

Fig.4.5 Control RRII 430

Fig.4.6 Control RRIC100

Fig.4.7 Control RRII 105

Fig.4.8 Control RRIM 600

Fig.4.a. T1- RRII 414

Fig.4.b.T1-RRII 417

Fig.4.c T1-RRII 422.

Fig.4.d T1-RRII 429

Fig.4.eT1-RRII 430.

Fig.4.fT1- RRIC 100

Fig.4.g.T1-RRII 105.

Fig.4.h.T1-RRIM 600

Fig.4.T2 (1)-RRII 414.

Fig.4.T2 (2)-RRII 417

Fig.4.T2 (3)-RRII 422

Fig.4. T2(4)-RRII 429

Fig. 4. T2(5)- RRII 430.

Fig.4.T2(6)-RRIC 100

Fig.4.T2(7)-RRII 105.

Fig.4. T2(8)- RRIM 600

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Fig.4. T3 (a)-RRII 414

Fig.4. T3(b)-RRII 417

Fig.4. T3(c)RRII 422.

Fig.4.T3(d)-RRII 429

Fig.4.T3(e)- RRII 430

Fig.4.T3(f)- RRIC 100

Fig.4.T3(g) -RRII 105.

Fig.4. T3(h)- RRIM 600

Heat stress assay (TTC Assay)

Heat tolerance is quantified by mitochondrial reduction of 2, 3, 5-triphenyl-2H-tetrazolium chloride (TTC). The method is based on the reduction of the colourless and water soluble TTC to an insoluble red compound (formazan). This reduction occurs as a consequence of hydrogen ions donated to the TTC upon dehydrogenase activity in metabolically active tissues such as the seed embryo (Junillon *et al.*, 2014; Lopez *et al.*, 2017). This method is used to determine the viability and status of metabolic activity in other types of plant parts, leaves stems fine roots (Ruf and Brunner, 2003). Towili and Manzur (1975) reported that the relative level of TTC reduction to formazan quantifies the cell viability by spectrophotometric assay of the red formazan.

The TTC reduction in *Hevea* under control and temperature treatments in leaf tissue was shown in Fig. 4. In the present study the mean OD values at 35°C and acclimatized condition (35°C, 2h→50°C, 1h) ranges from 0.438 to 0.665 and 0.352 to 0.654 respectively. It was observed that at sub lethal temperature the OD was low in RRIM 600 and high in RRII 105. The optical density (OD) values ranged from 0.321 (low) in RRIM 600 to 0.646 (high) in clone RRII 430 at lethal temperature (Fig.5). TTC reduction was decreased significantly by temperature treatment compared to control ($t \leq 0.05$).

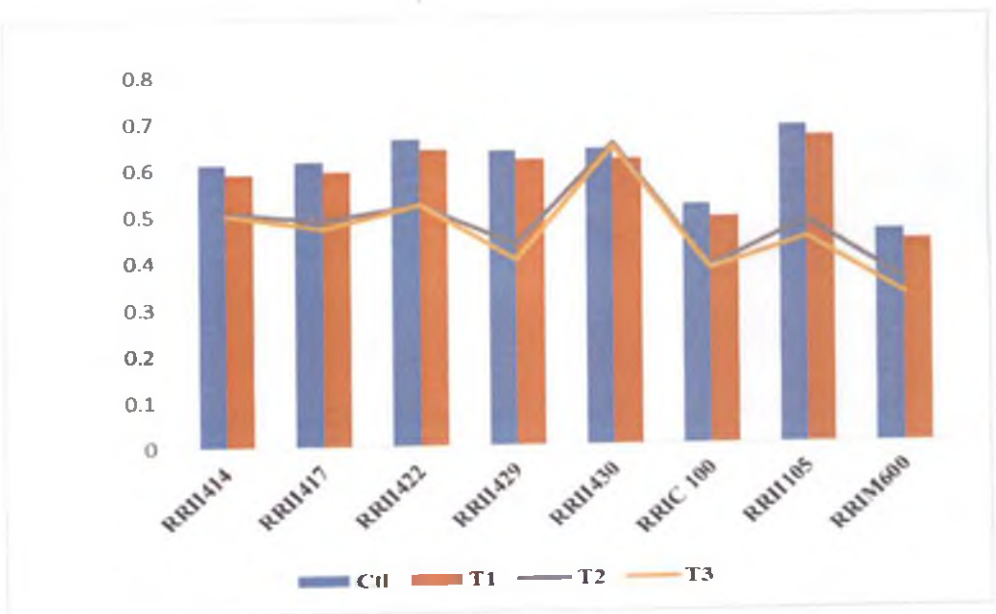

Fig.5. Heat stress assay (TTC reduction assay) in *Hevea* genotypes, Ctl- control; T1- 35°C; T2- 35°C →50°C; T3-50°C. Significant at $t \leq 0.05$

A clear difference was noticed in cellular viability/activity expressed as percentage of the control by the heat stress assay. Under sub lethal temperature treatment all clones have almost same level of cellular activity or viability (Fig.6). The cellular activity was found high in clone RRII 430 followed by RRII 414, RRII 422, RRII 417 and RRIM 600 after acclimatization treatment (35°C, 2h→50°C, 1h). When the leaf discs challenged by lethal temperature (50°C) for 1hr, same trend in clonal variation was noticed as in the case of acclimation treatment. It was found that under high temperature the rate -of activity was found high in clone RRII 430 followed by RRII 414, RRII 422,RRII417andRRIIM600.

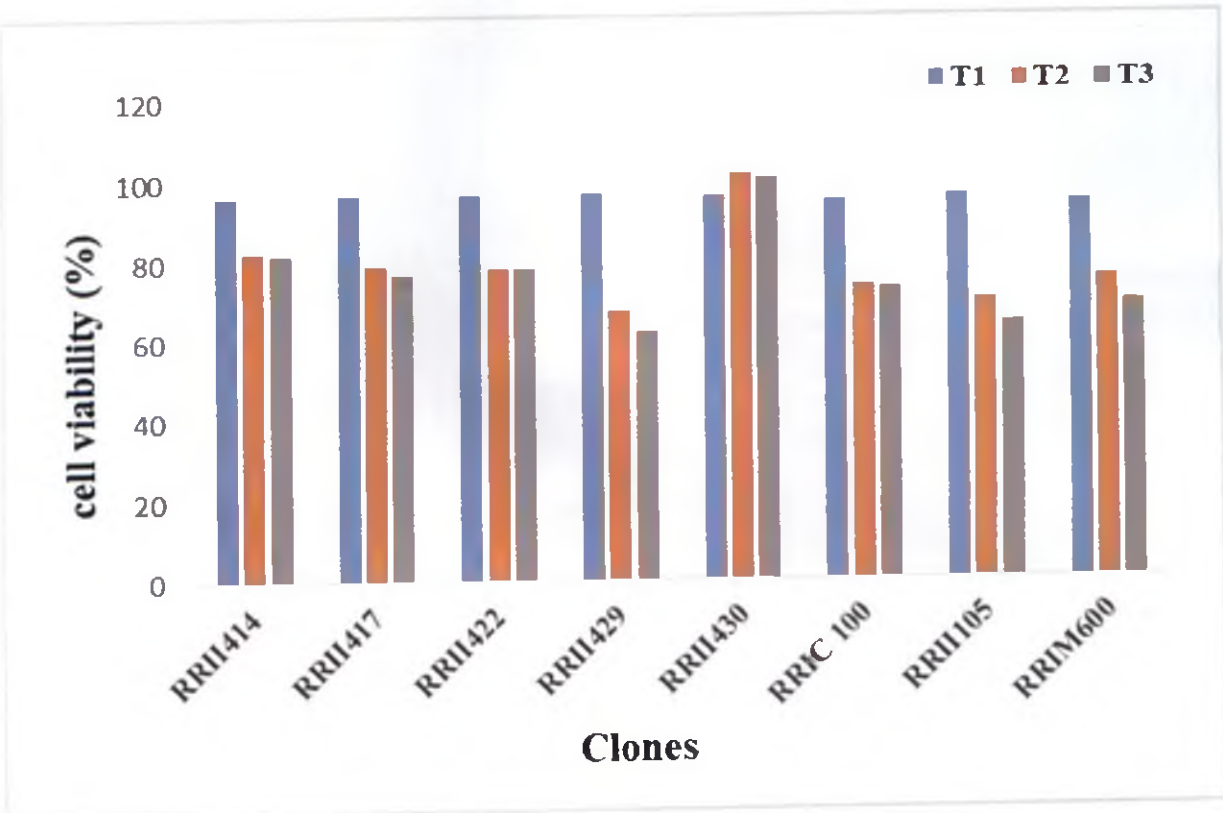

Fig. 6. Cell viability expressed as percentage to control in *Hevea* genotypes under different temperature treatments. T1- 35°C; T2-35°C→50°C; T3-50°C. Significant at $t \leq 0.05$

The acquired thermotolerance (ATT) ranged with a high value for clone RRII 430 to a low for the clone RRII 429. Clones such as RRII 414, 417, RRII 422 and RRIM 600 were on par with each other with respect to ATT. The decrease in cell viability in response to high temperature may be attributed to the uncoupling of electron transport chain through disruption of mitochondrial inner membrane or inactivation of enzymes of respiratory pathway (Porter *et al.*, 1994). Clone RRII 430 have high mitochondrial activity followed by RRII 414, RRII 417, RRII 422 and RRIM 600. (Fig.7). The results indicate that acclimatized tissues were capable of acquiring acquired thermotolernace. Most research reported were found to be done with seedlings or whole plant temperature treatment. In the present study, heat stress assay was used to determine the differences in varieties using leaf samples from the nursery. Hence establish at whole plant level a study under the controlled condition is very much needed.

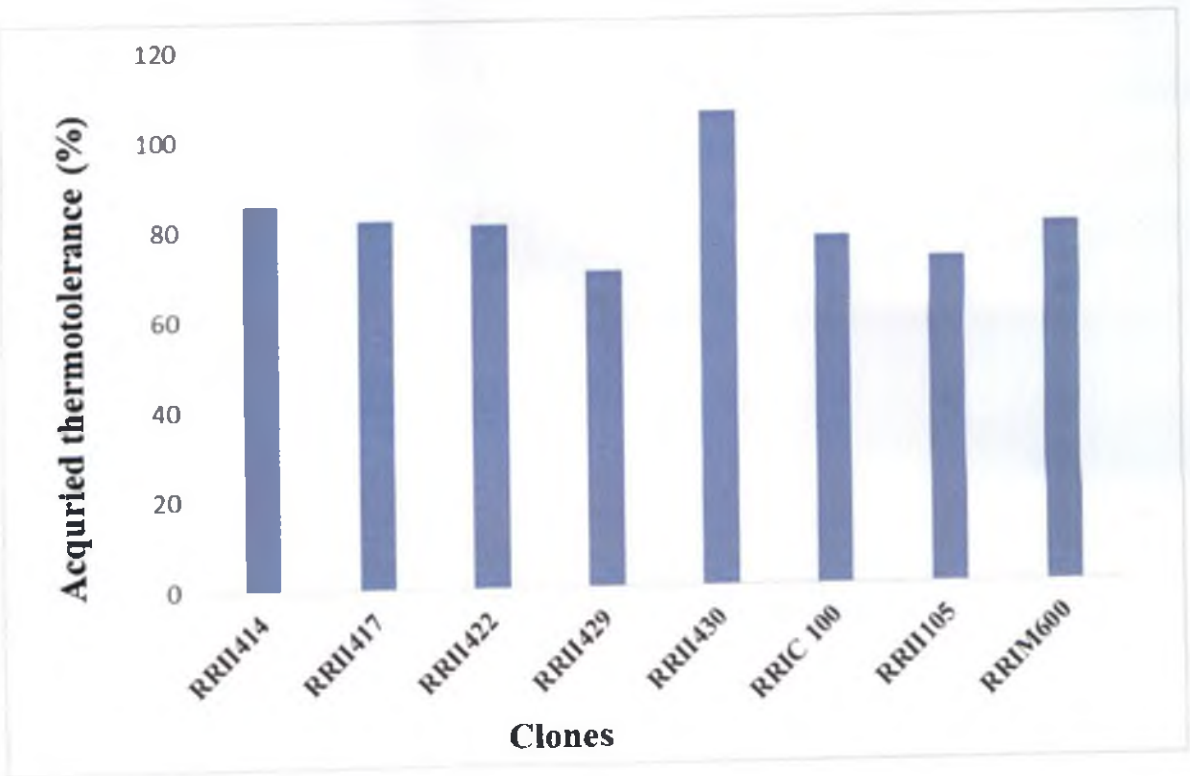

Fig. 7.Acquired thermotolerance (ATT%) estimated by heat stress assay in *Hevea* genotypes.

Chlorophyll Bioassay

The chlorophyll bioassay indicates a loss in the level photosynthetic pigment content. Temperature sensitivity of chlorophyll accumulation is considered as an important indicator of acquired thermo tolerance (Burke *et al.*, 2000; O'Mahony *et al.*, 2000; Camejo *et al.*, 2005). When temperature treatment was imposed directly, loss of pigment level was noticed compared to the control (Fig.8). When the leaf disc were acclimatized for 2hrs at 35°C and the challenged to lethal temperature 50°C the level was slightly improved in some of the clones than direct exposure to lethal temperature (Fig.8).

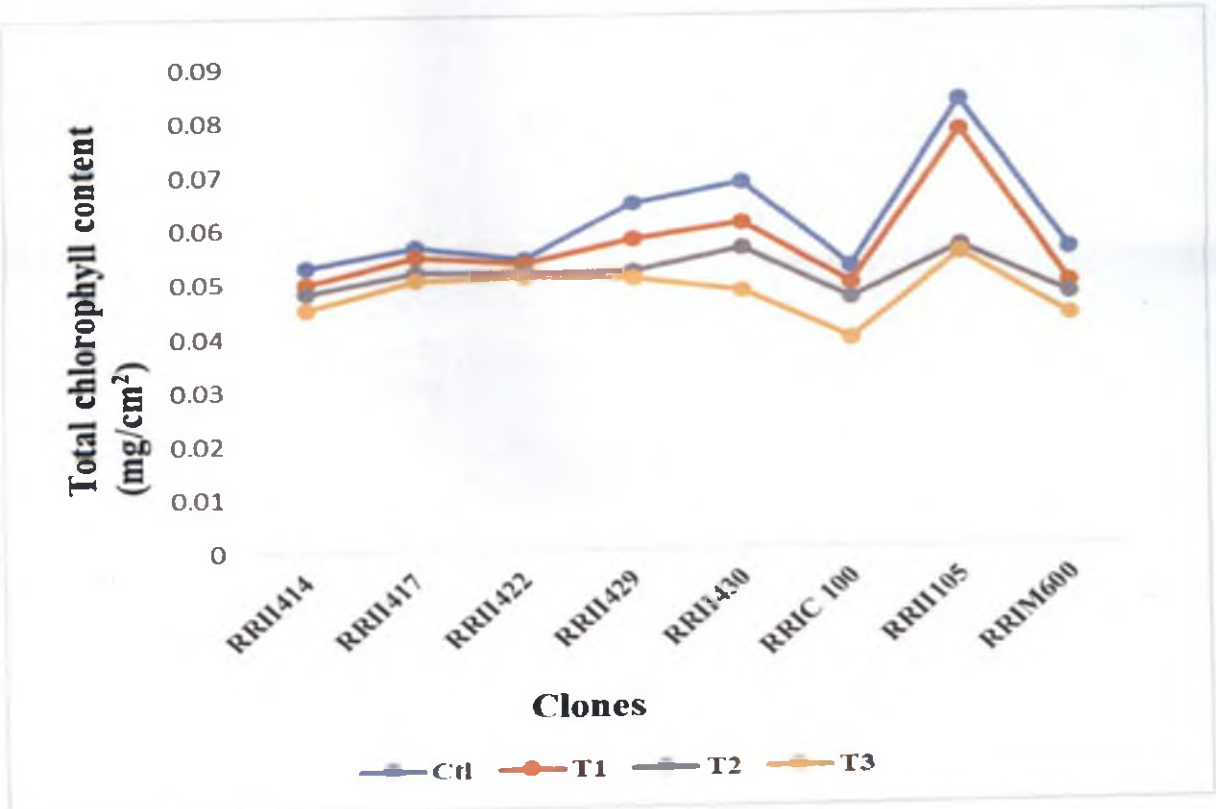

Fig. 8. Total chlorophyll composition in *Hevea* genotypes under different temperature treatments. Ctl- control; T1- 35°C; T2-35°C 2hrs→50°C 1hr; T3-50°C. Significant at $t \leq 0.05$

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Plants and other organisms have both an inherent ability to survive exposure to temperature and maintain optimal growth (basal thermotoerance, BTT) and an ability to acquire tolerance to lethal temperature (ATT). Hence total chlorophyll content in 35°C/Control considered as BTT and 35°C→50°C as ATT. In this context it was observed a high BTT in clones, RRII422 (1.26% decrease), RRII 417 (2.88% decrease) and RRII 414 (5.77% decrease) (Fig. 9). ATT also showed a same trend that % decrease in RRII422 (4.57%), 7.87% in RRII 417 and 9.18% in RRII 414 (Fig. 9). This may be due to the initial low level of chlorophyll content in these clones and vice versa as it was evident in RRII 105 (Fig.8).

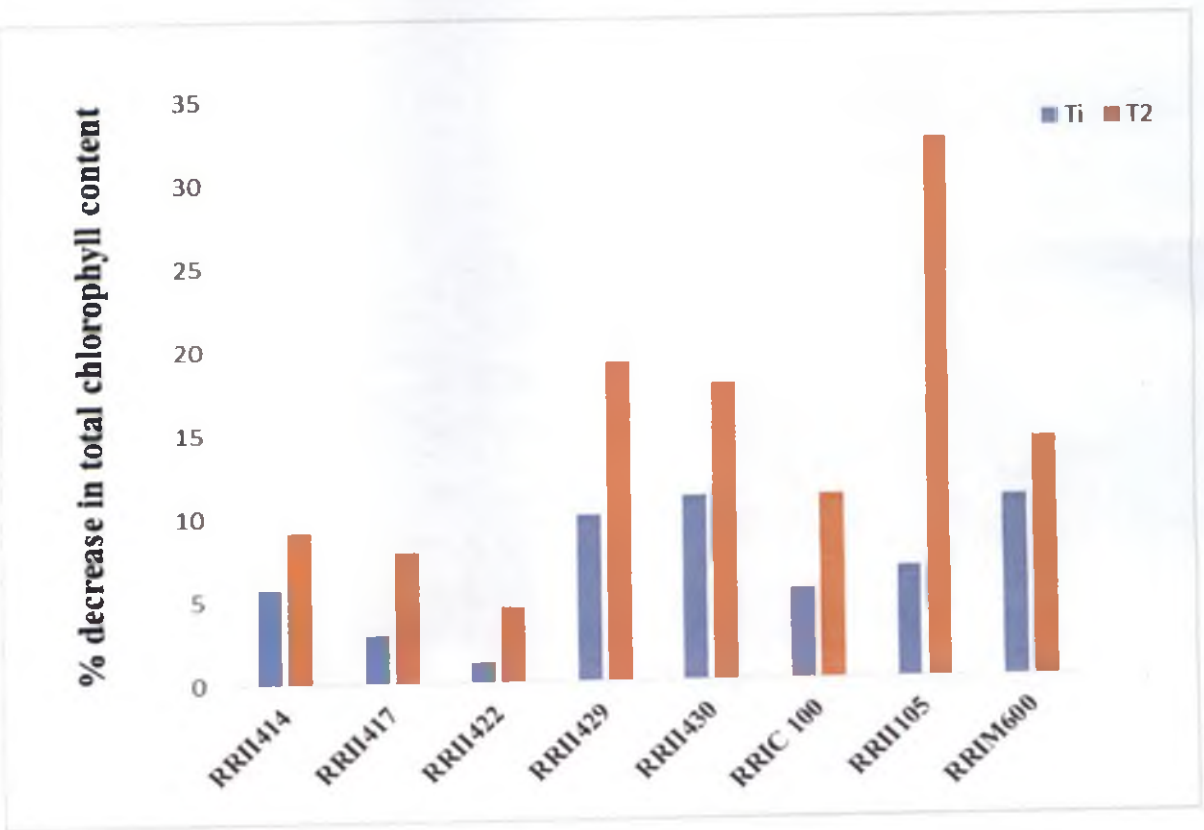

Fig.9.Percentage decrease in total chlorophyll content in *Hevea* genotypes under temperature treatments.T1- 35°C; T2-35°C→50°C. Significant at $t \leq 0.05$

CMS assay

Membrane damage is one of the important trait in abiotic and biotic stress studies. Genotypic variation were observed in cell membrane stability (Fig. 10). In the present study, direct exposure to high temperature both 35°C and 50°C showed membrane injury to a larger extent than 35°C→50°C or acclimatized condition. Relative conductivity is an important trait denoting the degree of damage or injury as cell membrane is sensitive to high temperature (Zarrin *et al.*, 2011). The BTT for membrane damage was almost same in clones RRII 430, RRII 422 and RRIM 600 (Fig.10). Electrolyte leakage was high in RRII 414. At lethal temperature clones such as RRII 417 and RRIM 600 showed less membrane damage or electrolyte leakage. Variation in cell membrane stability under temperature treatment reflects the differences in heat tolerance of the genotypes evaluated using this assay. It was noticed that in acclimated condition (35°C→50°C) the membrane damage or electrolyte leakage is lesser than lethal temperature. Hence it is clear that by giving an induction for a short period can able to reduce the membrane damage in all the genotypes.

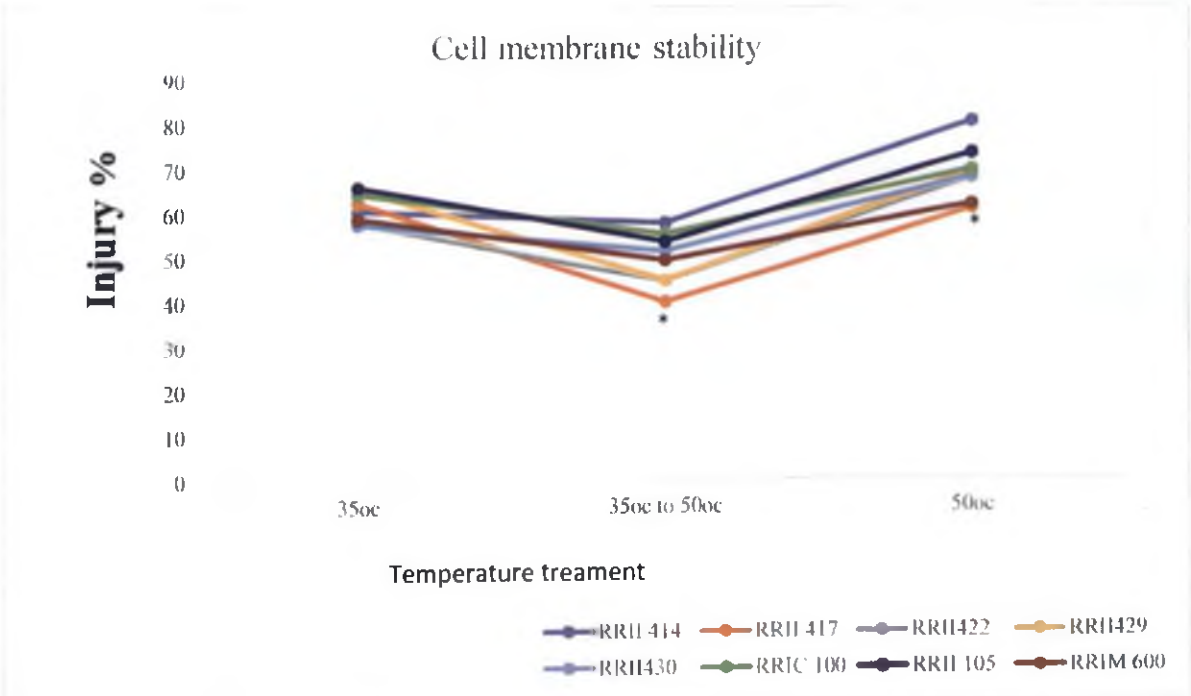

Fig.10 Injury %/Cell Membrane Stability in *Hevea* genotypes under temperature treatments.T1- 35°C; T2-35°C→50°C; T3- 50°C.,Significant at $t \leq 0.05$

Rapid physiological assay enables to differentiate genotypic or varietal difference to heat tolerance. It appears that a rapid screening is possible by establishing ATT through following the temperature induction response and other related method such as relative conductivity, tissue viability, *etc*. Sufficient replications is very much essential for this type of experiments. Results of the study revealed that changes in responses of genotypes to induction was prominent. It is expected that with a whole plant level treatment may get clearer picture of the induction responses. However for a rapid screening of a large number of diverse genotypes this methodology can be employed to determine the relative heat tolerance. From this initial screening we can able to short list the large number of genotypes to relatively thermotolerant ones. These can be utilized for other multi-level approaches to assess better performing varieties under heat stress. Thus the induction response approach will maximize the efficacy of screening program for heat stress and can be implemented as part of breeding programme for the detection of heat tolerant genotypes.

Summary and conclusions

Rapid physiological assay enables to differentiate genotypic or varietal difference to heat tolerance. It appears that a rapid screening is possible by establishing ATT through following the temperature induction response and other related method such as relative conductivity, tissue viability, etc. Sufficient replications is very much essential for this type of experiments. Results of the study revealed that changes in responses of genotypes to induction was prominent. It is expected that with a whole plant level treatment may get clearer picture of the induction responses. However for a rapid screening of a large number of diverse genotypes this methodology can be employed to determine the relative heat tolerance. From this initial screening we can able to short list the large number of genotypes to relatively thermotolerant ones. These can be utilized for other multi-level approaches to assess better performing varieties under heat stress. Thus the induction response approach will maximize the efficacy of screening program for heat stress and can be implemented as part of breeding programme for the detection of heat tolerant genotypes. And it is an efficient tool to solve the increasing threat of climatological extremes including very high temperature that demands for the generation of those varieties, with higher average temperature and larger temperature fluctuation tolerance, besides sustainable yield production, by evaluation of varieties that have ability to acquire thermotolerance, to lethal temperature, paving much importance, in their selection and propagation, besides other technique for the crop improvement to heat stress tolerance, in an efficient manner.

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