GENE EXPRESSION STUDY IN BUDGRAFTED RUBBER PLANTS (*HEVEA BRASILIENSIS* Muell. Arg.) BY mRNA DIFFERENTIAL DISPLAY

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Submitted by

RANI KRISHNA
Reg.NO:21504

MAR ATHANASIOS COLLEGE
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CERTIFICATE

This is to certify that the project work entitled 'Gene expression study in bud grafted rubber plants (*Hevea brasiliensis* Muell.Arg.) by mRNA Differential Display' submitted to Mar Athanasios College for Advanced Studies, Tiruvalla (Mahatma Gandhi University, Kottayam) by Miss.Rani Krishna for the award of degree of Master of Science in Plant Biotechnology, was carried out under my supervision and guidance, in Crop Physiology Division, Rubber Research Institute of India, Rubber Board, Kottayam. It is also certified that this work has not been presented for any other degree or diploma.

Dr. N.Geetha
Scientist S_2
Crop Physiology Division
Supervisor

Joint Director
Crop Physiology Division

Rubber Board P.O, Kottayam – 686 009, Kerala, INDIA
Telephone: 91- 481- 2353311 to 2353320Fax: 91- 481- 2353327
E-mail: rrii@rubberboard.org.inWebsite: www.rubberboard.org.in

CERTIFICATE

This is to certify that the work entitled **"Gene expression study in bud grafted rubber plants (*Hevea brasiliensis* Muell.Arg.)by Differential Display"** submitted to Mar Athanasios college for advanced studies Tiruvalla (Mahatma Gandi University, Kottayam) by Miss.Rani Krishna during the period of the study from may 2008 to july 2008 under our guidance and supervision, for the award of the Degree of Mater of Science in Plant biotechnology. It is also certified that this work has not been presented for any degree or diploma, associateship, fellowship, or similar title to any candidate of any university

Head of the Department

Dr. V. Reghu Rajan

Dept. of Plant Biotechnology
Mar Athanasios College for Advanced
Studies, Tiruvalla(MACFAST)

Supervising Guide

Dr. N.Geetha

Scientist S_2
Crop Physiology Division
RRII, Puthupally, Kottayam

External Examinar

DECLARATION

I, Rani Krishna do hereby declare that the project work titled " **Gene Expression Study in Bud Grafted *Hevea brasiliensis* by Differential display**"submitted to Mahatma Gandhi University,Kottayam for the award of the degree of Master of Science in Plant biotechnology, is a record of original and independent research work by me during may 2008 to july 2008 under the supervision of Dr. N .Geetha scientist S_3 , Plant Physiology Division, Rubber research Institute of India ,Kottayam, and it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship.

Rani

Signature of the candidate

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CONTENTS **PAGE NUMBER**

LIST OF ABBREVIATION

A	Absorbance
APS	Ammonium per sulphate
cDNA	Complimentary DNA
DNA	Deoxyribonucleic acid
DNase	Deoxyribo nuclease
DDPCR	Differential display PCR
DEPC	Diethyl pyro carbonate
dNTP	Deoxy ribonucleotide tri phosphate
°C	Degree Celsius
EDTA	Ethylene Diamine Tetra Acetic ACID
g	Gram
HCHO	Formaldehyde
HCl	Hydrochloric Acid
IAA	Indole acetic acid
LB	Luria bertani
LiCl	Lithium Chloride
M	Molar concentration
ME	Mercapto ethanol
ml	milli litres
MOPS	3-[N-morpholino] propane sufonic acid
µl	micro litre

µg	micro gram
MMLV	Moloney Murine Leukemia Virus
NaCl	Sodium Chloride
nm	Nano metre
ng	nano gram
PCR	polymerase chain reaction
PVP	Poly Vinyl Pyrrolidone
RNA	Ribo nucleic acid
RT PCR	Reverse Transcription PCR
SDS	Sodium dodecyl sulphate
SOC	Super Optimal Catabolite Repression
TBE	Tris borate EDTA
TEMMED	N-N Tetramethyl ethylene diamine

INTRODUCTION

Natural rubber, produced by several plant species, is the most versatile industrial raw material of plant origin. There are about 2000 laticiferous plant species, mostly belongs to the family Euphorbiaceae, Moraceae, Apocyanaceae and Asteraceae are reported to contain rubber in their latex. About 500 species have been tried as a source of natural rubber (Bonner, 1947), however, only a few have been commercially viable. Rubber tree, *Hevea brasiliensis,* which belongs to the family Euphorbiaceae accounts for 99% of the world's natural rubber production (Wycherley, 1992).

Hevea brasiliensis is a quick growing perennial tree with a straight trunk and bark, which is usually grey and fairly smooth. The tree is indigenous to the tropical rain forests of Amazon River basin of South America. The plants need a rainfall of about 80 inches per year and temperature between the range of 75-90° F. Natural habitat of different species of *Hevea* are also found in Brazil, Bolivia, Colombia, Ecuador, French Guiana, Guyana, Peru, Surinam and Venezuela (Wycherley,1992).

Hevea cultivation in India started in 1870 by the British and by the half of 20th century many plantations were successfully established in the country. Today, India is the fourth highest producer of natural rubber in the world having a total of 5.7 lakh ha under rubber cultivation with a production of 605045 t. More than 98% of the rubber production in India is from traditional rubber growing tracts of Kerala and parts of Tamil Nadu and Karnataka.

In rubber tree, the bark on taping yields latex. The unique isoprenoid compound in natural rubber is cis-1,4 polyisoprene,which is present in latex. Rubber constitutes about 90% of the dry weight or about 30-40% of the weight of the latex exuded on tapping (Grill *et al.*,1980). Rubber is the raw material of choice for heavy duty tires and also industrial uses requiring elasticity, flexibility and resilience.

All the earlier plantations in South and South East Asia were raised from unselected seeds .The field potential of these trees being low, the production was poor. Selection work on - *Hevea* to improve the planting materials and the introduction of vegetative propagation by budding (budgrafting) led in course of time, to the establishment of numerous valuable clones.

By budgrafting technique two plants with different genetic make up are joined together to form single plant and function as a new single plant. The upper portion or top of the plant is known as scion and the lower portion is called stock or rootstock. Rootstocks are obtained by germinating open pollinated seeds (heterogeneous in nature) collected from the fields.

Budgrafting consists of replacing a strip of bark of the stock with a strip of bark, containing a dormant bud (bud patch) taken from budwood tree (mother tree/elite tree) and bandaging it. Mother trees are selected after observations on yield and secondary attributes for a couple of years. The popular clones are evolved by this method in India are RRII 105, PB 235, PB 260, PB 311, PB 28/59, RRII 600 and GT 1. In India early hybrid clones developed by Rubber Research Institute of India (RRII) are RRII 100, RRII 200 and RRII 300 series. Among clones of the RRII 100 series, RRII 105 is highly successful and popular clone.

Stock-scion interaction is a phenomenon observed among budgrafted plants. There is an appreciable amount of variability exists among budgrafted plants due to stock-scion interaction. When the two partners of the bud grafted plants have different genetic constitution there are chances of incompatibility symptoms which affect the metabolic activities of these plants (Andrews and Marquez,1993). Rootstock-scion interaction are generally complex, and many tree characteristics such as tree size, temperature tolerance, fruiting and disease resistance may be affected by the presence of a graft union and stock-scion interaction (Hartmann and Kester,1976).

Isozymes which can be considered as markers of gene expression were also reported to be influenced by rootstock-scion interaction. Effect of rootstock-scion interaction on isozyme

polymorphism was reported in *Hevea* (Krishnakumar *et al.*, 1992) and mango (Degani *et al.*, 1990). In citrus, bands for peroxidase differed in their position depending upon the rootstock-scion genomic diversity and degree of compatibility (Protopapadakis,1988).

Despite these interesting observations, the molecular basis of grafting induced effects remains obscure. It was reported in grafted tomatoes that the specific species of mRNA can migrate from the rootstock to scion (Kim *et al.*, 2001) whereas in grafted potatoes they migrate from the scion to stock (Peres *et al.*, 2005). These reports indicate there is a possibility of influence of stock on scion and *vice versa*. Jensen *et al.*, (2003) described the effects of rootstocks on gene expression patterns in apple tree scions. They performed a comprehensive comparison of gene expression patterns in scions of the apple cv. Gala grafted to either M7EMLA or M9T337 by cDNA-AFLP technique. Scions grafted to the M9T337 rootstock showed elevated expression of a number of genes related to photosynthesis, transcription/translation, and cell division while scions grafted to the M7EMLA rootstock showed increased expression of some stress related genes. Recently Zhang *et al.*, (2008) studied a possible effect of tomato rootstocks on gene expression of egg plant scions. It was found that the tomato rootstock could up or down regulate gene expression of egg plant scions. The effected genes are related to diverse functions, including general metabolism, signal transduction, stress response, cell cycle/division, and transcription/translation.

Hevea brasiliensis is propagated commercially through budgrafting using genetically heterogeneous seedling rootstocks. Though budgrafting is considered as the most effective method of vegetative propagation of the true-to type elite clones in *Hevea*, these heterogeneous rootstocks impart intraclonal variations. Root stock influence on growth and yield in *Hevea* has been reported earlier (Templeton,1960; Buttery ,1961; Ng *et al.*, 1981; Cardinal *et al.*, 2007).. Ahmed (1999) demonstrated the effect of rootstock on growth and water use efficiency of different rubber tree clones. TPD is considered as a physiological disorder resulting in drying up of the tapping panel in *Hevea* and one of the suspected reasons is the influence of rootstock. Wider genetic difference between the rootstock and scion may lead to TPD incidence in *Hevea* (Sobhana *et al.*, 2005).

There is no study in *Hevea* in the stock scion interaction phenomenon to confirm whether root stock has any influence on scion. So in the present investigation we analyzed the mRNA profiles in the latex collected from scion portions of mature trees and in the scion leaves collected from growth chamber acclimatized plants of *Hevea brasiliensis.* And also we report the cloning and characterization of some scion specific cDNAs from growth chamber acclimatized plants with the following objectives:

1. Isolation of total RNA from latex cells and leaves of *Hevea* clone RRII 105.
2. cDNA synthesis by RT-PCR and PCR amplification.
3. Cloning and characterization of some scion specific cDNAs.

REVIEW OF LITERATURE

Grafting is a popular method of vegetative propagation in several plantation and horticultural species. Due to the asexual nature of this technique, the traits of the scion are perpetuated without any change. The Chinese had been practicing this technique since 1000BC and the Greeks too were familiar with this horticultural art since the times of Aristotle (Rom, 1987).

Various anatomical, physiological and genetic factors determine the success of the graft union and its eventual establishment into a healthy plant. If the stock and scion are genetically different as in the case of two different species or genera, their biochemistry and physiology may be too incompatible to establish a successful bud union between them. Grafting compatibility is genetically controlled and multiple genes are involved (Copes, 1970 and 1978). The chance of grafting success and hence establishment of a healthy grafted plant increases as the genetic, physiological and anatomical differences between the stock and scion are minimal.

One of the most dramatic rootstock effects are those exerted on plant scion quality . For example, Ough *et al.*,(1968) found that the choice of grape rootstocks for a wine variety could significantly alter the juice composition and thus affect the fermentation rate to an economically important degree. Riquelme *et al.,* (2004) found that the rootstock was an important agronomic factor affect the total flavonoid content of lemon juice. Verzera *et al.*, (2003) reported that rootstock could influence the quality of bergamot essential oils based on extensive measurements during the years 1997-1998, 1998-1999 and 1999-2000.

Hirst and Ferree (1995) studied the effect of rootstock and cultivar on the growth and precocity of young apple trees. It was observed that rootstocks exerted more influence than cultivar on total growth. In cherry also rootstock influence on tree size and yield was reported (Facteau *et al.*, 1996). The effect of combinations of three scions and three rootstocks on yield and quality of pistachio nuts was reported by Panahi *et al.*, (1996) and they found that rootstocks had large and significant effect on all the characters. Anatomical and biochemical studies conducted by Shklarman *et al.,* (1992) in compatible and incompatible combinations of citrus species showed symptoms of poor graft union, callose development and irregularities in water translocations.

Scion cultivars of Vitis on different rootstocks reported to yield differently with varying levels of fruit quality (Howell, 1987). Similarly rootstock effect on cold hardiness of scion cultivars of Vitis was also observed. Increased yield varieties like *Vitis labrusca* grapes were obtained when grafted on vigorous rootstocks.

Another remarkable rootstock effect on scions induces phenotypic changes. For example, the recessive petunia mutant *dad*1-1 allele displays a phenotype characterized by highly branched growth, late flowering, adventitious root formation, shortened internodes and mild leaf chlorosis traits, which are associated with cytokinin and auxin overexpression as well as gibberellin limitation (Napoli, 1996).

Rootstocks also have the abilities to improve the scion disease resistance. For example, the apple tree M7EMLA rootstock has resistance to *Erwinia anylovora*, the causal agent of Fire Blight disease. The scion of the apple cv. Gala grafted to the M7EMLA rootstock can reduce its susceptibility to *Erwinia anylovora* (Jensen *et al.*, 2003). Similarly, the *Lagenaria* accession Sus is resistant to the carmine spider mites, whereas *Cucurbita* accession Brava has no such resistance. Grafting the susceptible Brava onto the Sus rootstock increased the resistance of the scion to the same level of as that of ungrafted Sus (Edelstein *et al.*, 2000). The mango cultivar, Alphonsa grafted onto eight different rootstocks were compared and found that tree vigour, height and yield were influenced by the rootstock (Reddy *et al.*, 1989). Rootstock also have a role in influencing the sensitivity of the scion cultivars to pests (Ferree and Carlson,1987).

It has been suggested that the physiology of rootstock-scion relationship is mediated through the movement of endogenous growth factors and other biochemical components between rootstock and scion. Exchange of such factors between stock and scion particularly can have profound effects. E.g. juvenile characters in ivy (*Hedera helix)* could be induced in the adult form by grafting the scion onto juvenile plants and hormones play a crucial role in this (Hartman and Kester, 1976).

There has been a recent report on long distance movement of mRNA from the stock to the scion in tomato grafts (Kim *et al.,* 2001). The translocated mRNA caused morphological changes in the scion leaves similar to the morphology of the stock leaves suggesting that the translocated mRNA was functional.

During the brief course of the history of domestication of natural rubber, which stretches to more than one century, there was a tenfold increase in its productivity (Sethuraj, 1996). One of the important attributes for this spectacular increase in yield has been the development of high yielding elite clones either through ortet selection or hand pollination and their subsequent multiplication through bud grafting. This procedure ensured that the genetic constitution and hence the yield potential of the selected high yielding clones remained the same during the course of their multiplication into millions of bud grafted plants.

Although the genetic constitution of the scion could be maintained constant in a large *Hevea* population through bud grafting it may be noted that the root stocks are always grown from heterogeneous rubber seeds that have different genetic constitutions. Hence, rootstock is a potential source of functional variability among the individual trees of the same clone despite their genetic homogeneity (Djikman,1951). Such variations exist not only in rubber, but in almost all budgrafted species.

Rootstock has a profound effect on the vigour of the scion and the size and shape of its canopy in various species. In spite of such large tree-to-tree variations in latex yield in a

particular clone of *Hevea*, the mean yield is always higher in a high yielding than in a low yielding clone. It has been suggested that girthing in scion is more influenced by the rootstocks than its yield is (Templeton, 1960, Seneviratne, 1996). Rootstock influenced girth, height and leaf area of the scion in *Hevea brasiliensis* (Sobhana, 1998).

Shobhana (1988) have shown a clear influence of rootstock on the photosynthetic rate of the scion leaves. The greater the photosynthetic rate of the rootstock seedlings (before budgrafting), the greater the photosynthetic rate of the scion leaves (after budding).

The rooting behaviour of the rootstocks has obvious effects on the water relations of the scion leaves. It was found that a scion grafted to monoclonal seedlings of GT1 and RRIM 623 was better adapted to drought than the monoclonal seedlings of RRIM 600 used as rootstocks (Ahmad, 1999). This was due to better root characteristics in the monoclonal seedlings of GT1 and RRIM 623 than RRIM 600.

Root stocks have been known to influence the cation exchange capacity (CEC) of the roots which influences the mineral uptake. Similarly the NPK contents in the scion leaf also were determined by the scion itself and rootstock had no effect in it . There has been a positive correlation between Magnesium and Manganese contents in the leaves of the root stock and scion in some clones but not in others (Sobhana, 1998).

Several reports are available on the effects of the root stock on the biochemical composition of the scion in various species (Brown *et al.*, 1985) and in *Hevea* also it has been found that root stocks have profound influence on the biochemical composition of the leaves such as enzymes, reducing sugar, phenol and amino acids (Sobhana, 1998).

Several enzymes such as aspartate aminotranferase, leucine aminopeptidase, acid phosphatase, alkaline phosphatase, and phosphogluco isomerase showed polymorphism in the scion tissues collected from rubber trees grafted to different *Hevea* rootstocks (Krishnakumar *et*

al., 1992). Rootstocks have been found to influence isozyme patterns of peroxidase, esterase and catalase in the bud graft plants of five *Hevea* clones (Sobhana *et al.*, 2000).

Wider genetic differences between rootstocks and scion could lead to incompatibility symptoms in budgrafted plants (Hartman and Kester, 1976). It is possible that when the genetic distance between the rootstock and the scion is higher the chances of incompatibility will be higher (Hartman and Kester, 1976). In other words, a "genetic conflict" may be existing between the genetically divergent rootstock and scion. The large tree to tree variations noticed in the growth and yield of budgrafted *Hevea brasiliensis* are likely due to the effect of the genetically heterogeneous rootstocks (Dijkman, 1951).

The 'genetic conflicts' resulting from the genetic differences between the rootstock and the scion may lead to variations in the metabolic activities of the budgrafted plants as evident from the isozyme polymorphism in the scion leaves and RAPD analysis of the rootstock and the scion in *Hevea brasiliensis* (Sobhana *et al.*, 2000). RAPD analysis of the rootstock and scion in *Hevea brasiliensis* revealed that as expected the DNA profiles of the scion tissues of each cultivar were identical and the genetic distance between them was zero indicating the genetic homogeneity of the scion. But the DNA profiles of the rootstocks were different indicating their genetic heterogeneity because the rootstocks in *Hevea* were raised from polyclonal / assorted seeds.

Tapping panel dryness syndrome in *Hevea* is a physiological disorder resulting in the complete shut down of the latex production and preliminary studies in such trees indicated that the genetic distance between the rootstocks and the scion was higher than the normal healthy trees. The results lead to the assumption that when the genetic distance between the rootstock and the scion is large there is a possibility of "genetic conflict" resulting in metabolic disorders in the budgrafted plants (Sobhana *et a.l*, 1999).

MATERIALS AND METHODS

Plant materials

Normal budgrafted mature trees of *Hevea brasiliensis,* clone RRII 105 (19 years old) from the field of Rubber Research Institute of India, Kottayam and growth chamber (Conviron-E 15) acclimatized plants at their two whorled stage were used as the experimental materials in this study. For total RNA isolation, mature trees of *Hevea brasiliensis* were tapped in the scion portion and latex was collected (Fig.1) and scion leaves were collected from the growth chamber acclimatized plants.

Total RNA Isolation from Latex

Total RNA was isolated from the latex cells according to the following procedures:

I. Rubber trees were tapped and the fresh latex was collected in 2 ml eppendorf tube containing RNA extraction buffer. While collecting, the latex was continuosly mixed with an equal volume of RNA extraction buffer and kept at room temperature while transportation from field to lab.

RNA extraction buffer

0.2 M NaCl

0.1M Tris HCl, pH 7.0

0.01M EDTA

1.5% SDS

0.02 % PVP and

2% β-ME

II. After thorough mixing or vortexing, the mixture was centrifuged at 14000 rpm for 15 minutes at room temperature to separate the phases.
III. Equal volume of phenol was added to the upper aqueous phase and spun at 12000 rpm for 10 minutes at room temperature.
IV. Next the collected upper aqueous phase was extracted with equal volumeof chloroform : phenol : IAA and centrifuged at 12000 rpm for 10 minutes at room temperature.
V. The total RNA of aqueous phase was precipitated by the addition of 1/3 volume of 8M LiCl.
VI. The mixture was incubated at 4°C for overnight and the RNA was pelleted by centrifugation at 15000 rpm for 15 minutes at 4°C.
VII. RNA pellet was washed with 70% ethanol and dried and dissolved in sterile DEPC water and stored at -80 °C.

Total RNA isolation from leaves

Total RNA was isolated from leaves according to the manufactures' protocol for the RNAaqueous kit, Ambion ,USA.

I. Briefly, 0.1gm leaf tissue was ground in liquid nitrogen to a fine powder with a mortar& pestle . The tissue was transferred to a 2ml eppendorf tube to which 1.2 ml lysis /binding solution and 0.1ml of plant RNA isolation aid were added and mixed thoroughly by vortexing. The mixture was centrifuged at 12000 rpm for 5 minutes at room temperature. The clear aqueous phase was taken in a fresh eppendorf tube to which equal volume of 64% ethanol was added. Ethanol solution was used to make the RNA competent for binding to the glass filter in the RNA aqueous filter cartridge.
II. The mixture was applied to a filter cartridge assembled in a collection tube and centrifuged at 12000 rpm for 1 minute. Flow through was discarded and the collection tube was reused for the washing steps.
III. The filter cartridge was washed three times to remove contaminants, and the RNA was eluted in a very low ionic strength solution.
IV. The elution solution containing total RNA was precipitated by the addition of LiCl.

V. The mixture was incubated at 4°C for overnight and the RNA was pelleted by centrifugation at 15000 rpm for 15 minutes at 4°C.

VI. RNA pellet was washed with 70% ethanol and dried and dissolved in sterile DEPC water and stored at -80°C.

Quantification of Total RNA

The total RNA was dissolved in DEPC treated water and concentration and quality were determined using the NanoDrop® ND-1000 Spectrophotometer (USA) according to the manufactures' instructions. It measured 1 µl samples with high accuracy and reproducibility.

RNA can be quantified using absorption of light of 260 and 280 nm (A260/280). A ratio of ~2.0 was generally accepted as "pure" for RNA. If the ratio was appreciably lower than this value, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm.

Sample concentration in ng/µl was based on absorbance at 260nm. For nucleic acid quantification, the Beer –Lambert equation was modified to use an extinction coefficient with units of ng-cm/ml.

$$C=(A^{x}e)/b$$

Where :

C is the nucleic acid concentration in ng/ µl

A is the absorbance at 260nm

e is the wavelength dependent extinction coefficient in ng-cm/ µl (for RNA 40ng-cm/ml)

b is the path length in cm (for RNA data are normalized to 1.0 cm path)

Removal of genomic DNA contamination

Genomic DNA contamination was removed from total RNA by a treatment of 1 µg RNA with 1U RNase free DNase (Sigma).

The DNase treatment procedure is given below.

I. In a 1.5 ml micro centrifuge tube, 40 µg total RNA, 40 µl of DNase I reaction buffer, 40 µl RNase free DNase were taken and final volume was brought to 100µl using DEPC treated water and mixed gently.
II. The mixture was kept at room temperature for 15 minutes.
III. After 15 minutes 40 µl stop solution was added and kept at 70°C for 10 minutes.
IV. After the incubation the mixture was cooled immediately using the watery ice.
V. The mixture was spun briefly and RNA concentration was measured once again and integrity of the total RNA was checked on a denaturing gel.

Electrophoresis of RNA

Preparation of 10X MOPS buffer:

Concentration	Component
0.4 M	MOPS , pH 7.0
0.1 M	Sodium acetate
0.01M	EDTA

1. For 1.2 % (W/V) gel (100 ml),1.2 g of agarose was weighed into a 250 ml conical flask containing 72 ml of DEPC treated water and boiled till the agarose was completely melted.
2. The agarose solution was cooled to 60°C and 10 ml of 10X MOPS buffer and 18 ml 37% formaldehyde (12.3M) were added to it.

3. After mixing, the solution was poured into a gel casting tray and allowed to solidify at room temperature.

4. The isolated total RNA samples were prepared by mixing the following components in a 1.5 ml eppendorf tube as follows:

   1μl RNA containing 2.5μg RNA
   4μl DEPC water
   5μl Ambion RNA loading dye

5. The above components were mixed and kept at 65°C for 5 minutes in the thermo mixer (Eppendorf).
6. The heat denatured RNA samples were spun briefly and then loaded carefully into the solidified gel.
7. Electrophoresis was carried out for 1.5 h at 60V using 1X MOPS buffer.

Detection of Differentially expressed genes by DDRT-PCR

Analysis of gene expression is a central aim in most studies in molecular and cell biology. Expression of specific genes can now be visualized by a powerful technique mRNADD (mRNA Differential Display) or DDRT-PCR (Differential Display Reverse Transcription-Polymerase Chain Reaction), that allows a rapid screening of RNA populations from any biological experimental system (Liang and Pardee, 1992; Bauer *et al*., 1993; Liang *et al* .,1993). In plants, mRNA differential display method has been successful in identifying a number of differentially transcribed genes of populations in different physiological states (Wilkinson *et al* .,1995), at different developmental stages (Opsahl- Ferstad *et al*., 1997 ; Heck *et al* .,1995) and induced /non-induced by an abiotic/biotic agent (Benito *et al*., 1996; van der Knapp and Kende ,1995). Sharma and Kumar (2005) identified 3 drought responsive expressed sequence tags in tea by this method. It was also applied to identify genes related to touch regulated gene from *Arabidopsis* mutant plants Chotikacharoensuk *et al*., 2006) and to identify genes in ginger (Kavitha and George Thomas, 2008) whose expression was altered in response to *Pythium apahidermatum*.

The strategy of this method consists of three basic (**Fig.2**) and two additional steps:

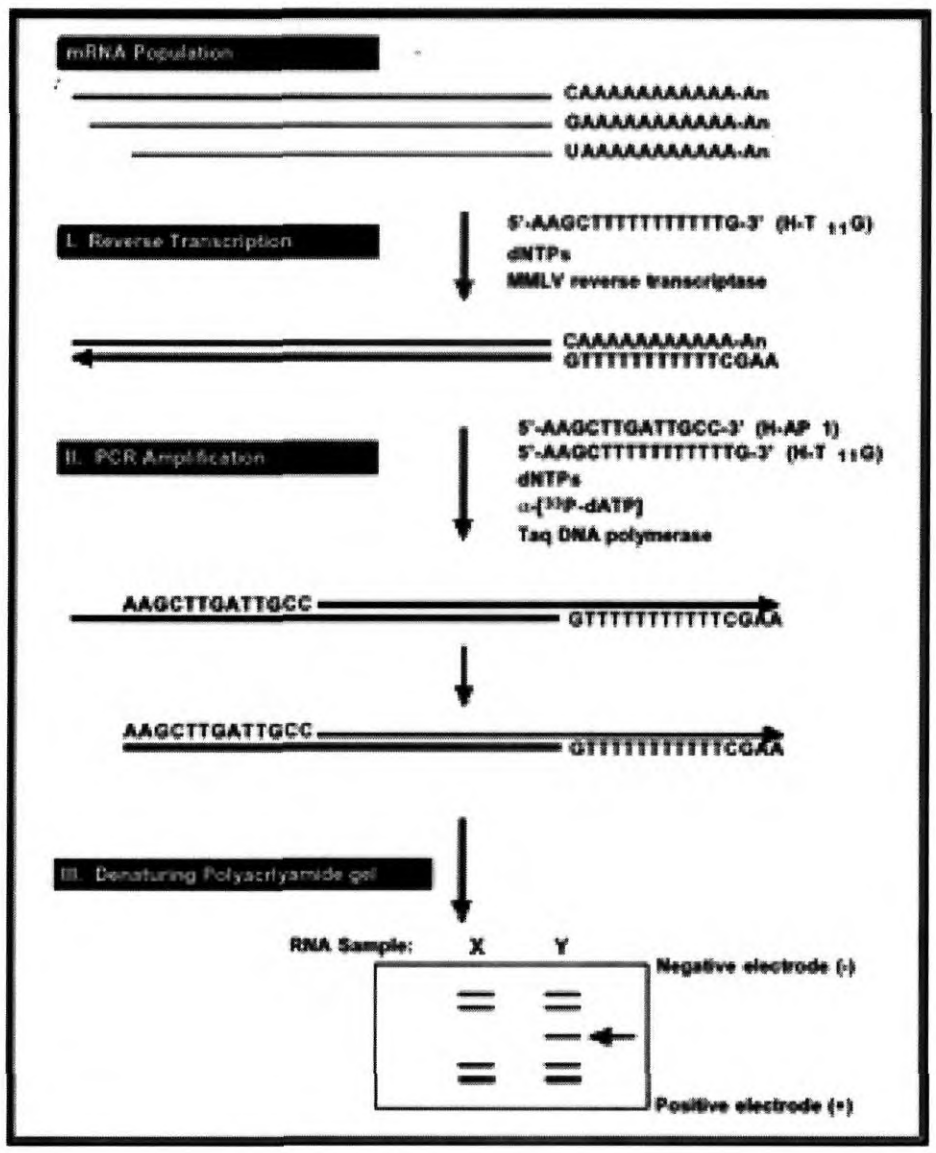


Fig. 2 Schematic representation of 3 basic steps of mRNA differential display method

1. Reverse transcription in fractions using a set of anchored primers,
2. Amplification of cDNA species from each fraction using a set of arbitrary primers and anchored primers,
3. Electrophoretic separation of the resulting fragments,

4. Reamplification of fragments that are different in two situations, cloning and sequencing, and
5. Confirmation of differential expression by an independent RNA analysis technique (Northern bloting, RNase protection, and/or nuclear run-on).

First strand cDNA synthesis by RT-PCR

1. First strand cDNA synthesis was done essentially using RNAimage kit (Gen Hunter Corporation, USA) according to the manufactures' instructions.
2. Total RNA isolated from latex as well as from leaves was used as the template for the reverse transcription reaction.
3. The first strand synthesis reaction was set up in a 1.5 ml eppendorf tube on ice. The reaction mixture was prepared as follows:

Components	**Volume / reaction(µl)**
DEPC H_2O	9.4
5X RT buffer	4.0
DNTP (250µM)	1.6
Total RNA (DNA –free)	2.0(0.1µg/µl, freshly diluted)
Oligo dT primer	2.0
TOTAL	**19.0**

RT–PCR mix was kept in the PCR machine and it was programmed as follows:

Initial denaturation at 65°C for 5minute. ->37°C for 60 minute. ->75°C for 5 minutes. ->4°C. After the tubes have been at 37°C for 10 minutes, PCR reaction was paused and 1µl MMLV reverse transcriptase enzyme was added to each tube, and quickly mixed well by finger tipping before continuing incubation. The products of reverse transcriptions were stored at 4°C for recent use or at -20°C for later use.

PCR amplification or DDRT-PCR

The first strand cDNA synthesis reaction mixture was used for the amplification of cDNAs. PCR was performed with 8 arbitrary primers (Table 1.)

Table.1 Arbitrary primers used for mRNA differential display.

Nos.	Primer	Sequence
1.	H-AP1	5'-AAGCTTGATTGCC-3'
2.	H-AP2	5'-AAGCTTCGACTGT-3'
3.	H-AP3	5'-AAGCTTTGGTCAG-3'
4.	H-AP4	5'-AAGCTTCTCAACG-3'
5.	H-AP5	5'-AAGCTTAGTAGGC-3'
6.	H-AP6	5'-AAGCTTGCACCAT-3'
7.	H-AP7	5'-AAGCTTAACGAGG-3'
8.	H-AP8	5'-AAGCTTTTACCGC-3'

DDRT-PCR mix was prepared as follows:

Component	**Volume/ reaction (µl)**
Distilled H_2O	10.0
10X PCR buffer	2.0
dNTP(25µM)	1.6
Arbitrary primer	2.0
Anchored primer	2.0
RT mix	2.0
Taq DNA polymerase (Sigma)	0.2
Total	**20.0**

The amplification was carried out in thermocycler (Techne, USA).

The PCR condition were as follows: 94°C for 30s; 40°C for 2 min; 72°C for 30s for 40 cycles and finally 72°Cfor 5 min.

Preparation of 6% denaturing polyacrylamide electrophoresis

The amplified cDNAs were size fractionated by 6% denaturing polyacrylamide electrophoresis using Bio-Rad Mini protein Electrophoresis Cell.

A 6% denaturing polyacrylamide gel in TBE buffer was prepared as follows:

Components	**volume**
Acrylamide (40%)	938 µ
10X TBE buffer	625 µl
sterile H_2O	2.4 ml
Urea	3.13g
TEMMED	2.5 µl
APS(10%)	37.5 µl

I.The gel was allowed to polymerize for atleast 1 hr.

II.The gel was prerun in 1X TBE buffer for 30 minutes.

III.5 µl loading dye was added to each sample, mixed well, heated at 80°C for 2 minutes prior to loading. The gel was run at constant volt (150 volt) until xylene dye touch the bottom of the gel.

Silver staining

Silver staining was accomplished by following the method of Benbouza *et al.*(2006). It comprised the following steps:

I. After electrophoresis, gel was fixed in 50 ml cold (10-12°C) fixing solution (10% absolute ethanol, 0.5% acetic acid) for 5 minutes.

II. The fixed gel was soaked for 6-7 minutes at room temperature in a 50 ml solution containing 1.5% Silver nitrate ($AgNO_3$), 75 µl of 37% HCHO.

III .Gel was rinsed quickly (10-15 sec.) once with 200 ml distilled H_2O.

IV. It was then developed by soaking it at room temperature in a 50 ml developing solution (1.5% NaOH, 100 µl 37% HCHO) until the bands appear with a sufficient intensity (3-5min.).

V. When the desired intensity was achieved development was stopped by impregnating the gel in a 50 ml stop solution (10% absolute ethanol, 0.5% acetic acid) for 2 min.

VI. Gels were washed several times thoroughly with distilled H_2O and DNA bands were viewed and photographed using Genius Bio Imaging System, Syngene, Cambridge, UK.

Recovery, Reamplification and cloning of cDNA fragments

Recovery of cDNA fragments

I. The gel band of differentially expressed cDNA fragments was cut down with a sterile knife and added to an eppendorf tube to boil for 15 minutes after the addition of 100 µl sterile distilled H_2O.

II. The tubes were centrifuged at 12000 rpm for 5 minutes at room temperature and the supernatant were transferred to a new eppendorf tubes.

III. 10 µl of 3M sodium acetate (pH 5.2),5 µl of glycogen (10mg/ml) and 450 µl of 100% ethanol were added to the supernatant and kept in -80°C for 30 minutes.

IV. The tubes were spun at 12000 rpm for 10 minutes at 4°C to pellet DNA.

V. The supernatant was removed, the pellets were rinsed with 85% ice cold ethanol.

VI. Finally the pellet were allowed to air dry and dissolved in 10 µl distilled H_2O

Reamplification of cDNA fragments

Reamplificatin was done using 4 µl (out of 10 µl) eluted DNA in a 40 µl reaction volume using the same primers and PCR conditions except the dNTP concentrations as mentioned below.

Reamplification reaction mixture

Components	40 .0 µl
Distilled H_2O	20.4
10X PCR buffer	4.0
DNTP (250 µM)	3.2
Oligo dT primer	4.0 µl
Arbitary primer	4.0 µl
cDNA (eluted DNA)	4.0 µl
Taq DNA polymerase	4.0 µl

Transformation protocol

4 µl of TOPO cloning reaction from the preformed TOPO cloning reaction mixture was added into a vial of one shot chemically competent E.*coli* (DH5α) and mixed gently. The mixture was incubated on ice for 30 minutes and the cells were heat shocked for 30 seconds at 42°C without shaking. The tubes were immediately transferred to ice. About 250 µl of SOC medium was added. The tube was capped tightly and was kept horizontally in an incubator shaker (200 rpm) at 37°C for 1hour.

Plating of transformed cells

Transformed cells were plated onto LB medium containing antibiotic ampicillin (50mg/l).

Preparation of antibiotic selection medium.

LB medium (Sambrook *et al.*, 1989) was prepared as follows:

Components	**Quantity**
Distilled H_2O	1 litre
Bacto-tryptone	10 g
Bacto-yeast extract	5g
NaCl	10g
Bacto-agar	15g

Conical flasks were plugged and autoclaved for 15 minutes at 15lbs. Ampicillin (50mg/l) was added into the medium, after cooling to 50°C (mixed gently to avoid air bubble formation) and the medium was poured into plates. Transformed cells (75µl) were plated onto prewarmed antibiotic selection plate under laminar air flow. Plates were incubated at 37°C for over night. Colony PCR was performed to identify the positive colonies (transformed colonies).

20µl of the PCR samples were run on a 1.5% agarose gel to check the reamplification of cDNA probe in the first round of PCR. The remaining 20 µl PCR samples were stored at -20°C for cloning.

Cloning of cDNA fragments

The reamplified cDNA probe was then cloned into TOPO-TA cloning vector (Invitrogen,USA) according to the manufactures instructions. The principle of TOPO-TA cloning is that, Taq polymerase has a nontemplate dependent terminal transferase activity, which adds single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector in the kit had single, overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

To promote the TOPO cloning the PCR products, the reagents provided in the kit were taken as per the manufactures' instruction and reaction mixture was prepared as follows:

Reaction components for TOPO cloning:

Components	**volume**
Fresh PCR products	4 µl
Salt solution	1 µl
TOPO TA vector	1 µl
Total	**6 µl**

The reaction components was gently mixed and incubated for 5 minutes at room temperature (22°C). After the incubation the solution was placed on ice and transformation was proceeded.

Colony PCR profile

Step 1	Initial denaturation	94°C	4 minutes
Step 2	Denaturation Annealing extension	94°C 50°C 72°C	1 minute 1.30minutes 2 minutes
Step 3	Repeat step 2	39 cycles	
Step 4	Final elongation	72°C	7 minutes.

The selection of transformed clones was performed by agarose gel electrophoresis using colony PCR products.

LB plate containing 5 different /independent transformed clones were selected for sequence analysis. The complete sequence of each cloned fragment was obtained using Automated DNA sequencer M/s. Banglore genei, Banglore, India. The DNA sequence was analysed for homology in the GenBank database using the BLAST N programme (Altschul *et al.*, 1990).

Fig.1 Collection of latex by tapping the scion portion of mature tree of *Hevea brasiliensi*

RESULTS AND DISCUSSION

RESULTS

The present study describes gene expression profile of latex collected from two different scions of the two mature trees of *H. brasiliensis* , clone RRII 105. It also explains the isolation, cloning and sequence characterization of the cDNAs specific to scion leaves of two growth chamber acclimatized plants. The mature trees were free from disease as well as TPD syndrome. The leaves collected from growth chamber acclimatized plants were free from disease and also of same physiological maturity.

Total RNA isolation

It is important that the total RNA used for differential display must be absolutely free from DNA contamination. Total RNAs were prepared from latex as well as from leaves, treated with DNase I, checked for the integrity by formaldehyde agarose gel electrophoresis and used for RT-PCR. The quality of the RNA after DNase I treatment was assessed by the appearance of large and small ribosomal RNAs, which appeared as sharp intense bands (Fig.3).

cDNA synthesis by Reverse Transcription polymerase Chain reaction

Total RNA isolated from latex as well as from leaves was used as template for the reverse transcription reaction. The first strand cDNA synthesis reaction was performed in 20µl reaction volume using oligo dT primer (H-T11 G) in the presence of MMLV reverse transcriptase at 37°C for 1 hour.

Differential Display Reverse Transcription Polymerase Chain Reaction

After the reverse transcription reaction the first strand cDNA was used as template for PCR amplification using 8 different arbitrary primers (HAP_1- HAP_8) in combination with oligo dT primer (H-T11 G) for gene expression profile study of 2

samples (**sample 1**- two different scion latex; **sample 2**- scion leaves of two growth chamber acclimatized plants). PCR was performed in a DNA thermal cycler as follows: 94°C for 30s; 40°C for 2 min; 72°C for 30s for 40 cycles and finally 72°C for 5 min.

Visualization of amplified cDNA fragments

Differential display experiment was repeated two times for the confirmation of the results. The amplified cDNA fragments were separated on a 6% denaturing polyacrylamide gel containing 7M urea and visualized by silver staining according to the method of Benbouza *et al.*, (2006).

From the combination of one anchored oligo dT primer (H-T11 G) and 8 arbitrary primers numerous amplified cDNAs were observed for all the 3 samples. On the gel with each primer combination only the differentially expressed cDNA bands were recorded for two samples.

Identification of differentially expressed cDNAs

Out of 8 arbitrary primers 3 primers produced differentially expressed mRNA bands for samples 1. With the primer combination of one anchored primer H-T11G and arbitrary primer HAP_2, a total of 4 differentially expressed cDNA bands were detected, Whereas 3 differentially expressed genes were noticed with the combination of anchored primer H-T11G and arbitrary primer HAP_3. However, only one differentially expressed cDNA appeared when the combination of H-T11G and HAP_5 was used (Fig.4).

Among 8 arbitrary primers used, only 4 primers produced differentially expressed cDNAs for sample 2. Three differentially expressed genes were identified by H-T11G+HAP_5 whereas only one band was noticed with H-T11G+HAP_3 combination. The other 2 primer combinations (H-T11G+HAP_4 and H-T11G+HAP_7) produced 2 differentially expressed genes each (Fig.5).

Reamplification and cloning of differentially expressed genes/cDNAs

A total of 8 differentially expressed cDNAs were selected and purified from the gel. Purified cDNAs were reamplified using the same primer combinations. Reamplified products were purified and cloned into T-vector. The presence of the cDNA inserts in the

plasmid was reconfirmed by colony PCR (Fig.6). After PCR confirmation positive clones (plasmid with insert) were selected for DNA sequencing analysis.

Nucleotide sequence characterization

A total of 5 cDNA clones were sequenced and DNA sequence data were edited to remove vector sequences. Edited sequences were compared with GenBank database using Blast N programme (Altschul *et al.*, 1990). Among the 5 clones analysed, 4 clones did not share any homology or similarity with the reported sequences in the databse whereas DD4 clone showed homology (77%) with GA20 oxidase gene of *Phaseolus vulgaris* (U70530) and of hybrid popular (80%) (AJ001326) (Table 2).

Table 2. DD PCR products and homology search results.

DD-PCR clone designation	Clone size in base pairs	Homology search results	E-value
DD1	107	No homology identified	—
DD2	110	No homology identified	—
DD3	252	No homology identified	—
DD4	205	showed homology (77%) with GA20 oxidase gene of *Phaseolus vulgaris* (U70530)	0.045
DD5	193	No homology identified	—

DISCUSSION

Identification of differentially expressed genes

To analyze and identify genes that were differentially expressed in the latex of *Hevea* scions as well as in the leaves, we applied mRNA differential display technique. mRNA differential display is a powerful technique for isolating cDNAs specifically expressed in particular type of cells or induced in cells by various stress factors. Even

genes that express at low levels, such as transcriptional factors, can be cloned after isolation them using this technique.

The grafting technique has been extensively used for fruit trees and other plants to improve fruit qualities. Several recent studies provided convincing evidence that specific traits of the scions can be dramatically altered as a result of grafting to suitable stocks (Salm *et al.*, 1998; Pantalone *et al.*, 1999; Napoli, 1996 and Beveridge *et al.*, 1996). Most recently Zhang *et al.* (2008) studied the possible effect of grafting on tomato+eggplant grafts in which they showed the expression of a number of genes related to diverse functions, including general metabolism, signal transduction, stress-response,cell cycle/division, and transcription/translation. In their study, they used eggplant as a scion and tomato as a rootstock to study the effects of rootstocks on scions from a molecular perspective. They also studied the gene expression pattern in the leaves of ungrafted tomatoes, ungrafted eggplants and self grafted controls. It is reported that the alterations in gene expression resulted from the effects of tomato rootstock because they were only observed in eggplant scions grafted onto tomato rootstock and absolutely absent from the ungrafted and self grafted controls.

Previously a similar type of study on apple was carried out by Jensen *et al.*, (2003), who demonstrated that rootstock influences on gene expression patterns in apple tree scions. In this study they performed a comprehensive comparison of gene expression patterns in scions of the apple cv. Gala grafted to either M7EMLA or M9T337 by using the cDNA-AFLP technique. Scions grafted to the M9T337 rootstock showed elevated expression of a number of genes related to photosynthesis, transcription/translation, and cell division, while scions grafted to the M7EMLA rootstock showed increased expression of some stress related genes.

In *Hevea* grafting technique has been practiced for more than a century to obtain uniform plants. Even though we are getting uniform plants we could not avoid tree to tree variation due to unavailability of true to type plants. These tree to tree variations are often reflected at phenotypic level (growth and productivity and root system),

physiological level (photosynthesis, biotic and abiotic stresses and mineral nutrition) and biochemical level (biochemical parameters and isozymes) changes (reviewed by Shobhana *et al.*, 2007).

The present investigation was under taken to find out the changes at gene expression level between trees and between growth chamber acclimatized plants using total RNA of latex and total RNA of leaves, respectively.

We have observed the variation at gene expression level between two scion latex and between leaves of two growth chamber acclimatized plants.

There are chances for environmental influences like light, temperature, humidity etc. on mature field grown rubber trees in addition to stock scion interaction. In this condition we could not say very confidently that the variation in gene profile pattern between stock and scion, between scion and scion and between stock and stock is due to only grafting or stock scion interaction. In order to avoid or minimize the environmental influences we have kept some polybag plants inside the controlled environmental chamber for 2-3 weeks for acclimatization. Since the growth chamber acclimatized plants are of two whorled stage it is not possible to collect latex either from stock portion or from scion portion for total RNA isolation. So we have collected leaves of same developmental stage from two growth chamber acclimatized plants for DDRT-PCR experiment. Using total RNA from leaves DD-PCR was carried out with 8 primer combinations. About 8 prominent differentially expressed genes were noticed from one of the growth chamber acclimatized plants. After reamplification, 5 genes were cloned and sequence characterized. Among them, 4 clones did not show any homology to the known sequences in the databse, while one clone (DD4) showed 77% and 80% homology with GA20 oxidase gene of *Phaseolus vulgaris* and hybrid popular, respectively.

Gibberellins (GAs), a group of tetracyclic diterpenes, some of which are biologically active, that act as hormones in higher plants by controlling diverse growth

and developmental processes such as shoot elongation, expansion and shape of leaves, flowering, seed germination and fruit development through promoting cell division and elongation (Olszewski *et al.*, 2002; Sponsel and Hedden, 2004). GA20 oxidase (GA20 ox) is one of the catalytic enzymes of biosynthetic pathway of GAs. Biochemical and molecular evidences showed that GA20 oxidase transcript levels are regulated by bioactive GAs through a negative feedback mechanism (Hedden and Kamiya, 1997). The isolation and characterization of this gene was reported from various sources like *Arabidopsis*- (Huang *et al.*, 1998 and Coles *et al.*, 1999), Hybrid aspen (Eriksson *et al.*, 2000), apple (Bulley *et al.*, 2005) and Citrus (Vidal *et al.*, 2003 and Fagoage *et al.*, 2007).

Results of the two plants grown in controlled environmental conditions showed distinct differences in gene expression pattern by DD-RTPCR. It is quite evident that tree to tree variation exist between the plants. It is possible that GA20 oxidase gene may influence the growth of one plant compared with the other used in this experiment. The exact role of GA20 oxidase gene on stock scion interaction is not known. Further study is required to verify the expression pattern of this gene with more number of growth chamber acclimatized plants.

In conclusion, the present study revealed that tree to tree variation exists between plants under field grown conditions as well as under controlled environmental conditions. In addition, one of the cell growth regulator genes was identified by DD-RTPCR.

Fig. 3 Total RNA isolated from latex and leaf on a denaturing 1.2% agarose formaldehyde gel

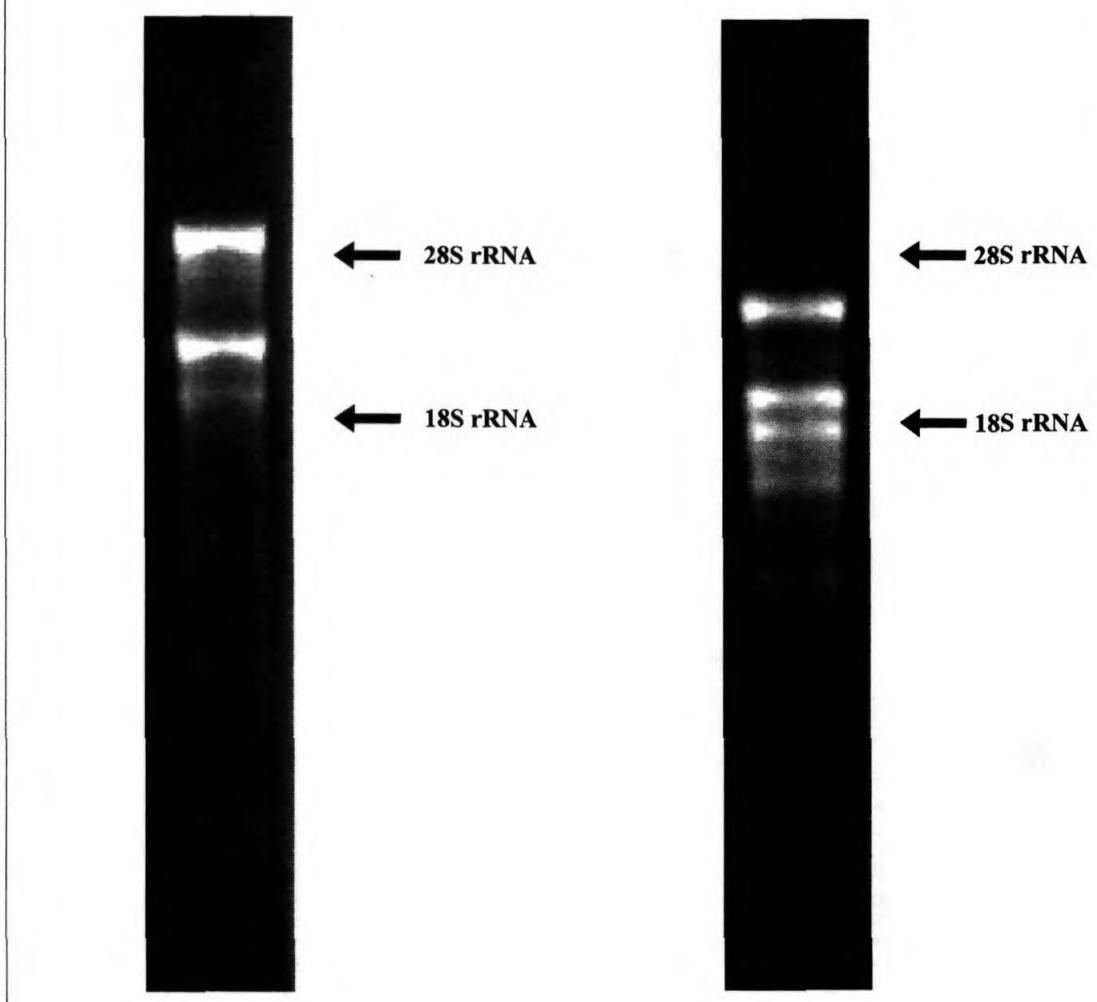
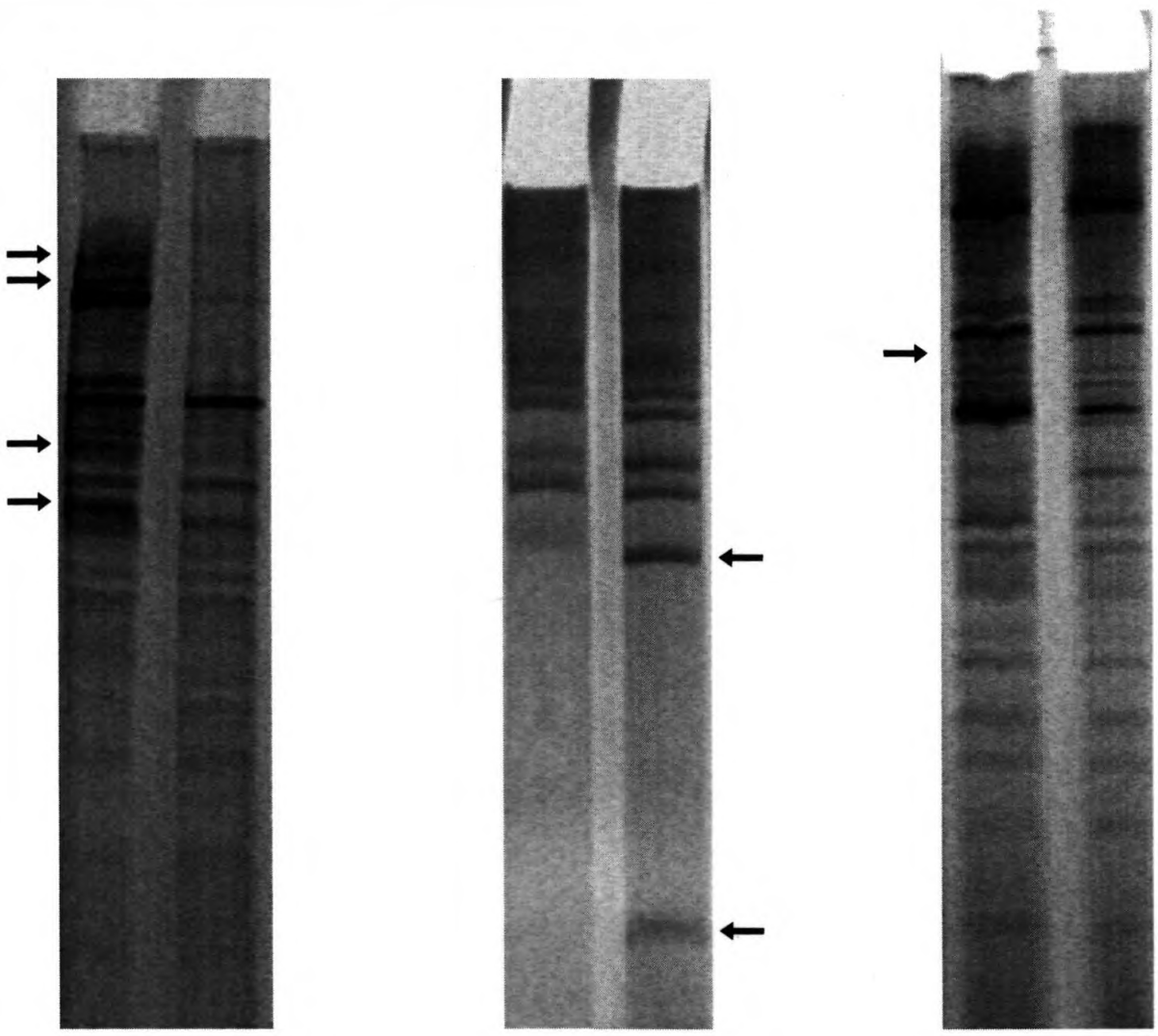


Fig. 4. DD RT-PCR using two different scion latex of mature tree of *H.brasiliensis*



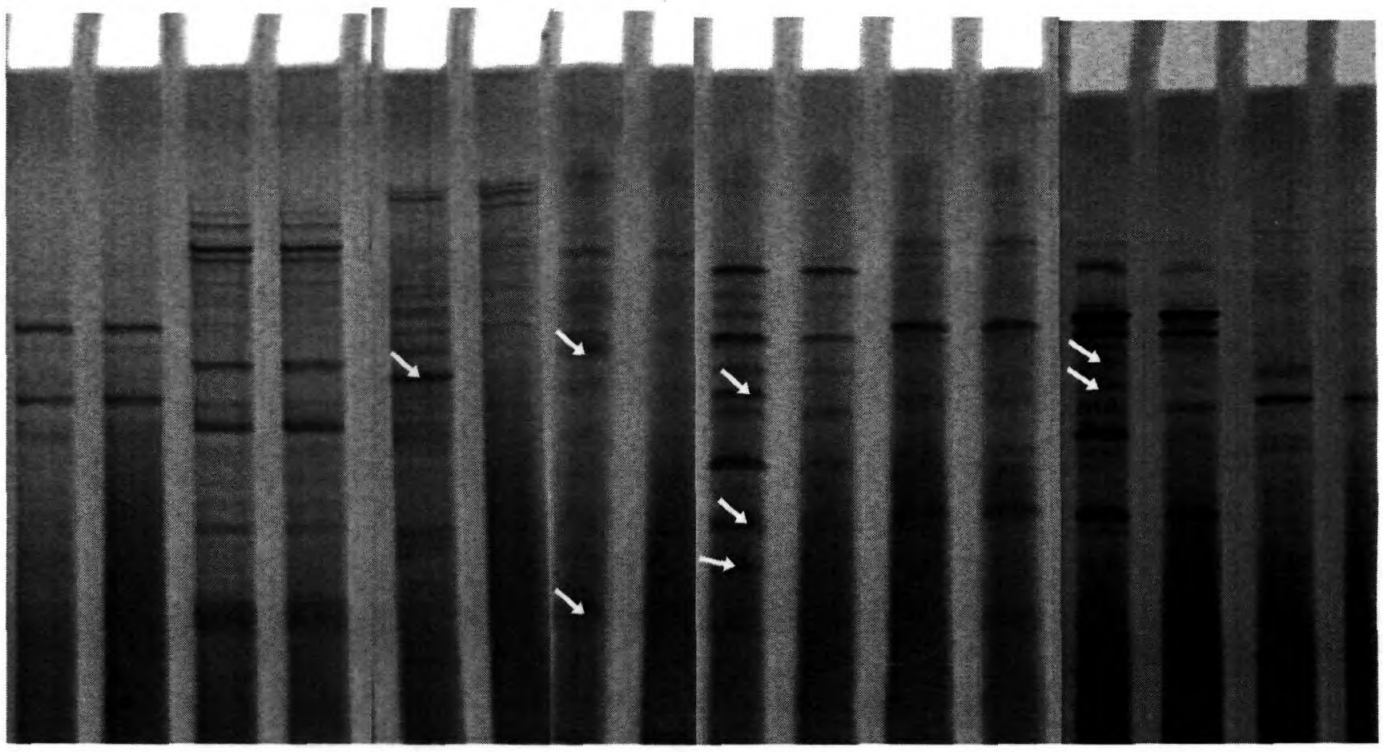
scion latex A scion latex B
oligo dT primer H-T11 G + HAP_2

scion latex A scion latex B
oligo dT primer H-T11 G + HAP_3

scion latex A scion latex B
oligo dT primer H-T11G + HAP_5

Differentially expressed band

Fig. 5 DD RT-PCR using leaf total RNA of two growth chamber acclimatized plants of *H.brasiliensis* in the combination of oligo dT primer H-T11 G and 8 arbitrary primers

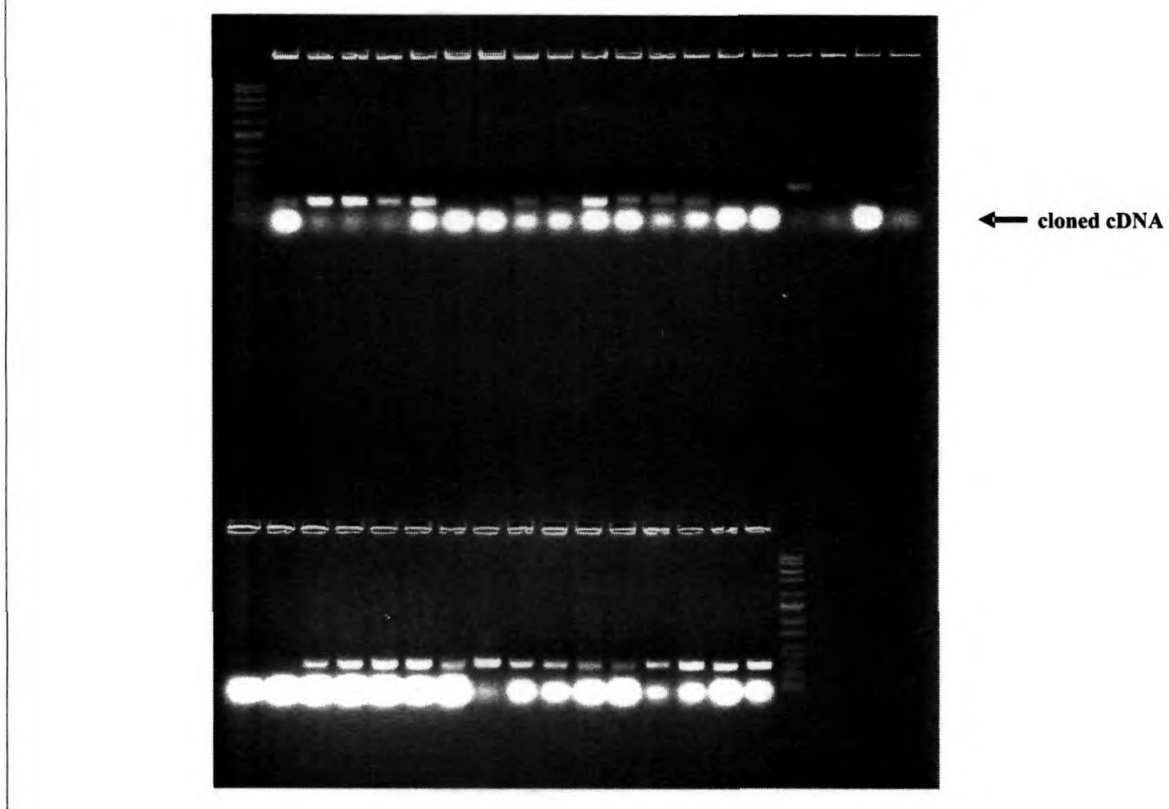


G1 – Growth chamber acclimatized plant 1

G2 – Growth chamber acclimatized plant 2

Differentially expressed band

Fig.6 Colony PCR



SUMMARY AND CONCLUSION

The present study was done to find out the changes at gene expression level between field grown trees and between growth chamber acclimatized plants .

Normal budgrafted mature trees of *Hevea brasiliensis* clone RRII 105 and growth chamber acclimatized plants at their two whorled stage were used as the experimental materials in this study. We have applied mRNA differential display technique to identify genes that were differentially expressed in the latex of *Hevea* scions as well as in the leaves.

Total RNA isolated from latex as well as from leaves was used as template for the reverse transcription reaction. After the reverse transcription reaction the first strand cDNA was used as template for PCR amplification using 8 different arbitrary primers(HAP_1-HAP_8) in combination with oligo dT primer(H-T11G)for gene expression profile study of these two samples.

From the combination of one oligo dT primer (H-T11G) and 8 arbitrary primers(HAP_1-HAP_8) numerous amplified cDNAs were observed for the two samples. From sample 2, a total of 8 differntially expressd cDNAs were selected , purified and reamplified and cloned. A total of 5 cDNA clones were sequenced . Among 5 clones analysed ,4 clones did not share any homology or similarity with the reported sequence in the database whereas DD4 clone showed homology (77%)with GA20 oxidase gene of *Phaseolus vulgaris*(U70530) and of hybrid popular(80%) (AJ001326).

The present study revealed that tree to tree variation exists between plants under field grown condition as well as under controlled environment conditions.

REFERENCES

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

2. Altschul,Stephan F., Warren Gish,Webb Miller ,Eugene W. Myers and David J.Lipman (1996). Basic local alignment search tool.*J.Mol.*Biol.215:403-10.

3. Andrews,P..K. and Marquez,C.S. (1993). Graft incompatibility.*Horticulitural Reviews*, 15: 183-232.

4. Bauer,D., Muller, J.Relch., v.Ahrenkiel,P.Warthoe, and M.Strauss.(1993). Identificaton of differentially expressed mRNA species by an improved display technique(DDRT-PCR).*Nucelic* Acids Res.21:4272-4280.

5. Benito., E. P., T. Prins, and J. A. van Kan. (1996). Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction. *Plant Mol. Biol.* 32:947-957

6. Bonner ,J Galston,A. (1947) The Physiology and Biochemistry on Rubber formation in plants. *The Bot .Rev.*Dec .13: 543-596

7. Beveridge, C.A., Ross,J.J., and Murfet,I.C., (1996) Branching in Pea(Action of Genes *rms3* and *rms4*), *Plant Physiol.*, ,vol.110,pp.859-865

8. Brown,C.S., Young,E. and Pharr,D.M. (1985). Rootstock and scion effects on carbon partitioning in apple leaves. *Journal of American Society for Horticultural Science,* 110(5): 701-705.

9. Buttery, B.R. (1961). Investigations into the releationship between stock and scion in budded trees of *Hevea Brasiliensis . Journal of Rubber Research Institute of Malaysia*, 17 :46-76

10. Chotikacharoensuk T., Arteca RN., Arteca JM (2006) Use of differential display for the identification of touch-induced genes from an ethylene-insensitive *Arabidopsis* mutant and partial characterization of these genes. *J Plant Physiol* 163:1305–1320

11. Coles., J.P.,Phillips, A.L., Croker, S.J., Garcia-Lepe, R.,Lewis, M.J., and Hedden, P., (1999) Modification of gibberellin production and plant development in Arabidopsis by sense and antisense expression of gibberellin 20-oxidase genes. *The Plant Journal*, vol.17 pp.547-556.

12. Copes, D.L. (1970). Initiation and development of graft incompatibility symptoms in Douglas-fir. *Silvae Genetics*, **19**: 77-82.

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

14. Dijkman, M.J. (1951). *Hevea: Thirty Years of Research in the Far East.*University of Miami Press, Coral Gabler, Florida.

15. Facteau,T.J., Chesnut N.E. and Rowe, K.E(1996). Tree,fruit size and yield of 'Bing' sweet cherry as influenced by rootstock,replant area and training system .*Scientia Horticultural*,67:13-26.

16. Ferree, D.C. and Carlson,R.F. (1987). Apple rootstocks. In:Rootstocks for fruit crops.p.107. Roy C.Rom and Robert F.Carlson (eds). *A Wiley Interscience Publication.* John Wiley and Sons,Newyork

17. Grilli,E.R.,Agostini,B.B.,'t-Hooft –Welvaars,M.J. (1980). *The world rubber economy world bank*/John Hopskins press, Baltimore, pp .120-130.

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

19. Hartmann, H., Kester, D.E. and Davies,F.T. (1990). Theoretical aspects of grafting and budding. In: *Plant propagation*.. 5th ed. Prentice-Hall, Englewood Cliffs, New Jersey.

20. Heck GR., Perry SE., Nichols K.W. and Fernández DE (1995) AGL15, a MADS domain protein expressed in developing embryos. *Plant Cell* 7: 1271-1282.

21. Hedden, P., and Kamiya, Y., Gibberellin biosynthesis: enzymes, genes and their regulation. *Annual Review of Plant Physiology*.,1997, vol.48 pp.431-460

22. Howell, G.S (1987). Vitis rootstocks. In: Rootstocks for fruit crops. Roy C .Rom and Robert F.Carlson(eds). *A Wiley- Interscience Publication*, JohnWiley and sons,New York.

23. Hirst, P.M and Ferree, D.C (1995). Effect of rootstock and cultivar on the growth and precocity of young apple tree. *Fruit varieties Journal*, 49 (1): 34-41.

24. Huang, S.S., Raman, A.S., Ream, J.E., Fugiwara, H., Cerny, R.E., and Brown S.M., Over expression of 20-oxidase confers a gibberellin-over production phenol type in Arabidopsis., *Plant Physiology*, 1998,vol.118 pp.773-781

25. Jenson,P.J., Rytter,J., Detwiler,E.A., Travis,J.W. and McNellis,T.W. (2003). Rootstock effects on gene expression patterns in apple tree scions. *Plant Molecular biology,*493:493-511.

26. Kavitha P.G and Thomas G (2008). Defence transcriptome profiling of *Zingiber zerumbet* (L.) Smith by mRNA differential display;*J.Biosci.*33 .81-90

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

28. Liang,P. and A.B.Pardee.1992., Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction.*Science* 257: 967-971.

29. Liang,P.,L.Averboukh and A.K Pardee (1993) .Distribution and cloning of Eukaryotic mRNAs by means of differential display; Refinements and Opyimisation.*Nucleic Acids Res.*18:6531-653

30. Napoli, C., Highly Branched Phenotype of the Petunia *dadI-1* Mutant Is Reversed by Grafting, *Plant Physiol.*, 1996 vol.111,pp.27-37.

31. Ng ,A.P., Ho, C.V., Sultan ,M.O., Ooi ,C.B ., Lew.H .L and Yoon ,P.K .(1981) .Influence of six root stocks on growth and yield of six scion classes of *Hevea brasiliensis. Proceedings of the RRIM Planter'Conference* ,Kualalumpur,Malaysia,pp.134-151.

32. Olszewski, N., Sun, T.P., and Gubler, F (2002) Gibberellin signaling: biosynthesis, catabolism,and response pathways., *The Plant Cell* vol.14 pp.S61-S80.

33. Opsahl-Ferstad,,H.G., Le Denuff, E., Dumas,C., and Rogowsky,P.M (1997) ZmEsr, a novel endosperm specific gene expressed in a restricted region around the Maize embro.*Plant J.*12,235-246.

34. Ough, C.S., Lider, L.A., and Cook, J.A., (1968) Rootstock-Scion Interactions Concerning Wine Making. I. Juice Composition Changes and Effects on Fermentation Rate with St. George and 99-R Rootstocks at Two Nitrogen Fertilizer Levels, *Am. J. Enol. Vitic.*, , vol. 19, pp. 213–227.

35. Panahi, B., Sheibani, A. and Peat, W.E. (1996). The effect of combinations of three scions and three rootstocks on yield and quality of Iranian Pistachio nuts. *HortScience*, 31(4): 634. (Abstract)

36. Pantalone, V.R., Rebetzke,G.J., Burton, J.W., Carter, T.E., and Israel, D.W., Soybean PI 416937 Root System Contributes to Biomass Accumulation in Reciprocal Grafts, *Agron. J.*,1999,vol.91,pp.840-844.

37. Peres,L.E.P.,Carvalho,R.F.,Zsogon,A.,Bermudez-Zambrano,O.D.,Rebles W.G.R.and Tavares,S.(2005). Grafting of tomato mutants onto potato rootstocks: An approach to study leaf derived signaling on tuberization. *Plant science*,169:680-688.

38. Protopapadakis,E.E. (1988)Effect of rootstocks on isoenzymic composition of *citrus*. *In: Sixth Internaional Citrus Congress*. Middle-East, Tel Aviv Israel. Volume 1 (Rehovot,Israel, Balaban Publishers) pp. 609-614.

39. Reddy,Y.T.N., Kohli, R.R. Singh,G. and Bhargava, B.S. (1989).Effect of rootstocks on growth,yield and leaf nutrient composition of mango (Mangifera indica L.). Fruits (Paris, 44 (7-8):409-413

40. Riquelme, M.T., Porras, R.I., and Ferreres, F., (2004) Effect of the Rootstock and Interstock Grafted in Lemon Tree (*Citrus limon* (L.) Burm.) on the Flavonoid Content of Lemon Juice, *J. Agric. Food Chem.*, , vol. 52, pp. 324–331

41. Rom, C.R.(1987). In :Rootstocks for fruit crops.Eds.Rom, C.R and Carlson ,R.F.John Wiley &Sons,Inc.

42. Salm, T., Bouwer, R., Dijk, A., Keizer, L., Cate, C.H., Plas, L., and Dons, J., (1998)Stimulation of Scion Bud Release by *rol* Gene Transformed Rootstocks of *Rosa hybrida* L., *J. Exp. Bot.*, 1998, vol. 49, pp. 847–852

43. Sambrook,J., Fritsch,E.F. and Maniatis ,T (1989), Molecular cloning: A labortary Manual.Cold Spring Harbour,New York: Cold Spring Harbour Labortary Press.

44. Senevirratne, P.,Nugawela, A.,Samarokoon, S.M.A and Ramavikrama,D. (1996). The growth of stock plantsand its effects on the scion growth of young buddings of *Hevea brasiliensis* (Muell Arg). *Journal of plantation crops,* 24(2):119-125.

45. Sethuraj, M.R. (1996). Emerging trends in Productivity Improvement in Rubber (*Hevea brasiliensis*). Proceedings of Second International Crop Science Congress, 1996. Eds. V.L. Chopra, R.B. Singh and Anupam Varma.

46. Sharma, S.K. and Singh, R. (1989). Photosynthetic characteristics and productivity in citrus. II. Effect of rootstocks. *Indian Journal of Horticulture*, *46*(4): 422-425.

47. Sobhana, P., Jacob, J and Sethuraj, M.R., (2007) Root stock Scion Relationship in Budgrafted plants with special reference to to Hevea Brasiliensis., *Nat. Rubb. Res.*,vol.20(1&2)pp.94-107

48. Sobhana,P.Thomas,M .and Jacob,J. (2005). Genetic distance between the root stock and scion of healthy and TPD affected trees of *Hevea brasiliensis*. *Natural Rubber Research*. 18:101-104.

49. Sobhana,P., Molly Thomas., Krishnakumar,R. and James Jacob. (2000). Can there be possible genetic conflicts between genetically divergent rootstocks and scion in budgrafted plants? National symposium on Recent Trends in Plant Science Research, University of Kerala ,Thiruvanathapuram.

50. Sobhana,P (1998). Physiology of rooting and stock-scion interaction in Hevea.Ph.D thesis,Kerala university,kerala,India.

51. Shklarman, Y., Safran, H. and Sagee, O. (1992). Microscopic study of graft union characteristics and water translocation in young citrus buddings. In: *Proceedings of International Society of Citriculture: Volume 1. Taxonomy, breeding and varieties, rootstocks and propagation, plant physiology and ecology: 7th International Citrus Congress*, Acireale, Italy, 8-13, 1992

52. Templeton, J.K. (1960). Some aspects of the growth of *Hevea* buddings. *Proceedings of Natural Rubber Research Conference*., 297-311.

53. Verzera, A., Trozzi, A., Gazea, F., Cicciarello, G., and Cotroneo, A., (2003) Effects of Rootstock on the Composition of Bergamot (*Citrus bergamia* Risso et Poiteau) Essential Oil, *J. Agric. Food Chem.*, , vol. 51, pp. 206–210

54. Van der knapp E.Kende H. (1995) Identification of a Gibberllin induced gene in deep water rice using Differntial display of mRNA.Plant Mol. Biol 28: 589-592.

55. Vidal, A.M., Ben-Cheikh, W., Talon, M., and Garcia-Martinez, J.L., (2003) Regulation of gibberellin 20-oxidase gene expression and gibberellin content in citrus by temperature and citrus exocortis viroid., *Planta*, vol.217,pp.442-448.

56. Wycherly,P.R. (1992). The genus Hevea:botanical aspects in biology, cultivation and technology(Eds M.H Sethuraj and N.H Mathew) Elsevier,Amsterdam,pp.50-66

57. Wilkinson, J.Q., Lanahan, M.B., Conner, T.W., and Klee, H.J. (1995). Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display. *Plant Mol. Biol.* 27:, 1097–1108

58. Zhang, Z.J., Wang, Y.M., Long, L.K., Lin, Y., Pang, J.-s., and Liu, B., Tomato (2008). Rootstock effects on gene expression patterns in eggplant scions, *Russian J. Plant Physiol.*, vol.55(1) pp.93-100.