Biotechnological approaches to impart tolerance to Corynespora leaf fall disease in *Hevea brasiliensis*

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By

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Under the supervision and guidance of

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Dedicated to my family……

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ABSTRACT

Hevea brasiliensis is a perennial tree crop that is widely cultivated as the sole commercial source of Natural Rubber (NR) in Asian countries. Disease incidences increased with domestication, as it did with any other agricultural crop, and more than a hundred pathogens have been identified as capable of attacking the rubber tree. The fungal pathogen *Corynespora cassiicola* causes Corynespora Leaf Fall Disease (CLFD). It is one of the challenging diseases that has spread with the introduction of new breeds. The toxin cassiicolin secreted by the fungus, is thought to be the main determinant of Corynespora pathogenicity. These fungal diseases not only reduce crop yield, but they also have a significant impact on production quality and consistency year after year, weakening the efforts to promote sustainable agriculture. The health and environmental hazards posed by various chemical fungicides used to control these diseases are very serious. Although traditional breeding schemes are being used to develop tolerant *Hevea* varieties, these efforts are often time consuming and labor intensive, and they frequently fail to identify the distinct varieties. With the advancement of biotechnology and gene manipulation techniques, quick and cost effective screening methods have emerged to improve the efficiency and success rate of resistance breeding programs. One such promising strategy is to use a variable selection scheme in which pathotoxin or culture filtrate can be used as the selection agent for cultured cells, resulting in the selection of individuals with suitable levels of resistance to the selection agent.

The goal of our study was to develop CLFD resistant *Hevea* varieties through an *in vitro* selection technique with *Corynespora cassicola* crude culture filtrate as the selection agent. As an initial step towards this goal, various *in vitro* screening experiments were conducted on some selected *Hevea* clones to determine whether the results of these experiments agree with field observations regarding sensitivity to CLFD. Accordingly, a total of eight *Hevea* clones belonging to two groups, susceptible and tolerant to CLFD, with four clones in each group were selected and leaves of these clones were subjected to different Corynespora leaf fall disease sensitivity experiments such as leaf wilt bioassay, vacuum infiltration experiments and electrolyte leakage tests. Results of these studies revealed that sensitivity of the two

groups of clones towards phytotoxic CCF was well in accordance with the already recorded field observations.

Plants have also developed various defense responses to overcome stressful situations caused by the fungal diseases. Various biochemical parameters related to these defense systems need to be analyzed in order to identify the key factors involved in the development of disease resistant plants. Accordingly, in all selected *Hevea* clones, the results of the analysis of various biochemical parameters, such as the activity of chitinase, catalase and peroxidase, as well as the accumulation of ROS were found to be comparable to the CLFD sensitivity of the corresponding *Hevea* clones. Also it was found that chitinase enzyme played an important role in the defense mechanism of all selected clones. Hence *chitinase* gene was chosen as a suitable candidate for genetic transformation in *Hevea* to confer CLFD tolerance because of its potential in conferring tolerance against infection. As part of this, we were able to successfully construct a *chitinase* gene expression cassette in the binary vector pCAMBIA 1301, which can be used in future plant transformation experiments to create transgenic *Hevea* plants overexpressing the *chitinase* gene.

The possibility of developing CLFD tolerant plants of *Hevea brasiliensis* through *in vitro* selection technique by exposing or challenging callus cultures has been explored in detail. As a result of extensive studies in this direction, Corynespora tolerant plants of a susceptible *Hevea* clone RRII 105 could be developed through *in vitro* challenging of embryogenic calli against the pathogenic culture filtrate of Corynespora, followed by selection and further culture development. On laboratory level screening with CCF, most of these plants showed CLFD tolerance as evidenced from the results of leaf wilt bioassay, vacuum infiltration of leaf segments and electrolyte leakage whereas the parental clone RRII 105 is highly susceptible. Biochemical assay of various parameters carried out on these tolerant plants after subjecting to CCF treatment showed an enhanced activity of chitinase enzyme, absence of ROS production and very low levels of catalase and peroxidase acitivity which are signs of better tolerance of these plants towards CLFD. This also demonstrates that the CLFD tolerance trait of *Hevea* callus cultures achieved at the cellular level *via* CCF selection is observable at the plant level and is not lost during the various stages of plant development. These plants are well established under the

field conditions and their disease tolerance in the field need to be yet confirmed. For the same purpose these tolerant plants have been multiplied by bud grafting and are at different stages of development.

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ABBREVIATIONS

2, 4-D	:	2, 4- Dichlorophenoxy acetic acid
ABA	:	Abscisic acid
ANOVA	:	Analysis of Variance
CAT	:	Catalase
POD	:	Peroxidase
ROS	:	Reactive oxygen species
SOD	:	Superoxide dismutase
H_2O_2	:	Hydrogen peroxide
BA	:	Benzyl adenine
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
C.cassiicola	:	*Corynespora cassiicola*
CaMV	:	Cauliflower Mosaic Virus
CCF	:	Crude culture filtrate
cDNA	:	Complimentary DNA
CLFD	:	Corynespora leaf fall disease
DNA	:	Deoxyribo nucleic acid
DNS	:	Dinitro salicylic acid
EDTA	:	Ethylene diamine tetra acetic acid
EL	:	Electrolyte leakage
GA_3	:	Gibberellic acid
GT	:	Godng Tapen
H.brasiliensis	:	*Hevea brasiliensis*
HCl	:	Hydrochloric acid
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butyric acid
KIN	:	Kinetin

LB	:	Luria Bertani
MCS	:	Multiple cloning site
mRNA	:	Messenger RNA
MS	:	Murashige and Skoog
NBT	:	Nitroblue tetrazolium
NCBI	:	National Center for Biotechnology Information
NR	:	Natural rubber
OD	:	Optical density
PB	:	Prang Besar
PCR	:	Polymerase Chain Reaction
PR	:	Pathogenesis related
rDNA	:	Recombinant DNA
RRIC	:	Rubber Research Institute of Ceylon
RRII	:	Rubber Research Institute of India
RRIM	:	Rubber Research Institute of Malaysia
SE	:	Somatic embryogenesis
TE	:	Tris-EDTA

Units

µg	:	Microgram
µl	:	Microlitre
µm	:	Micrometre
µM	:	Micromolar
g	:	Gram
hr	:	Hour(s)
kDa	:	Kilo daltons
l	:	Litre
mg	:	Milligram

min	:	Minute(s)
ml	:	Milli litre
mm	:	Millimetre
mM	:	Millimolar
ng	:	Nanogram
°C	:	Degree celsius
rpm	:	Revolutions per minute
sec	:	Second(s)
U	:	Unit(s)
V	:	Volt(s)
v/v	:	Volume/volume

Chapter 1

Introduction

Plantation agriculture is a form of commercial farming where crops are grown on an extensive scale in a large continuous area of land. These crops include tea, coffee, rubber, cocoa, coconut, arecanut, oil palm, palmyrah, cashew, cinchona etc. They are high value commercial crops of greater economic importance and play an important role in the Indian economy. India is the major producer of some plantation crops, such as tea, cashew, arecanut, coconut, and rubber. Among these crops, Natural Rubber (NR) is one of the key productive assets of our country. It is a biopolymer made of poly (*cis*-1, 4-isoprene) associated with several other biological compounds (Vaysse *et al.*, 2012) which is mainly harvested in the form of latex from the rubber tree.

NR made from the latex sap of trees is one of the world's most beneficial commodity. Around 20,000 species of plants produce latex, but only 2000 species confined to 300 genera of seven family's viz., Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae have been found to contain rubber in their latex (Arias and Van Dijk, 2019; Cornish *et al.*, 1993). Out of these several thousands of laticiferous rubber bearing plant species, about 500 have been accepted as the source of NR. Yet, only a few species are currently exploited. The content of rubber differs according to different species restricting their consideration as a source of NR. In many species the rubber content is too low to be considered a source of natural rubber (George and Panikkar, 2000). Although a number of alternate NR producing species have been made available to meet the growing demand for good quality rubber, Para rubber tree remains, by any measure, the world's foremost and widely accepted commercial source of NR.

H. brasiliensis (Para rubber), which produces NR, is a deciduous tree which is basically grown in tropical humid climatic conditions. NR extracted from rubber trees in the form of milky latex made it the most economically important member of genus

Hevea. Primarily, *Hevea brasiliensis* was native to the rain forests in the Amazon regions of South America, including Brazil, Venezuela, Ecuador, Colombia, Peru and Bolivia. From their native environment, the Rubber tree plantation was later expanded to Southeast Asian countries in 1876 by the British explorer H. Wickham. Rubber seedlings introduced by him became the parent planting stock for all rubber plantations developed in present-day Malaysia and other Southeast Asian countries. From this single introduction, massive Asian rubber plantations were developed which resulted in a rubber boom in Southeast Asia. This led to the first great surge in commercial planting on a very large scale, after which the crop developed remarkably, from a wild forest tree to a major domesticated crop. Today in Asia the rubber cultivation spans for more than 12 million hectares that accounts for 93% of world's Natural Rubber production.

Globally, NR occupies the supreme position as nature's most diverse raw material. It is practically established that this wonderful material is indispensable in all movements of human life, from the most basic personal objects to modern means of transport. Chemically, NR is a high molecular weight polymer which is widely utilised in a variety of applications for the manufacture of over 50,000 products, either alone or in combination with other materials. On the Indian market, NR is used to manufacture about 35,000 different products which include tires, engineering components, household items, medical and healthcare goods, automobile goods, aviation and ship construction, agricultural machines, and various other consumer goods. In addition to this, NR is now extensively used in soil stabilisation, vibration absorption, and railroad construction. This makes rubber an undeniably essential commodity for the past 100 years. Improvements in man's mobility due to technological advancements have tremendously increased the demand of this elastomer such that the necessities could not be fulfilled with the naturally available rubber material. This shortage led to the synthesis of synthetic rubber alternatives. However, when considering growing environmental consciousness, depletion of petroleum reserves, as well as the varied uses and unique qualities of NR, it has been proven that the excellence of *Hevea* NR will never be exceeded by any other accessible synthetic product. The global demand for rubber is expected to increase over the next two decades. Despite all this, rubber production is highly constrained by

the availability of limited land, as well as biotic and abiotic stress factors which have a significant impact on the productivity of rubber plantations.

Among the biotic constrains, the most important is the incidence of various fungal diseases which cause serious crop loss. Since Asian rubber plantations originated from only a handful of seeds, all the trees are genetically very similar. Less genetic diversity means a reduced capacity to combat plant diseases. If just one tree gets affected, the disease can spread throughout the plantation, leading to its devastation (Arias and Van Dijk, 2019). Among the many diseases that affect plant growth and yield, abnormal leaf fall caused by *Phytophthora sp.*, Colletotrichum leaf spot disease caused by *Colletotrichum acutatum*, powdery mildew caused by *Oidium heveae*, and Corynespora leaf fall disease (CLFD) caused by *Corynespora cassiicola* appear on a regular basis. Abnormal leaf fall and Gloeosporium leaf spot disease appear during rainy season whereas, powdery mildew and Corynespora leaf fall diseases occur during dry season, just after the period of wintering (Manju, 2011). CLFD is currently the most important and severe disease affecting *Hevea brasiliensis.*

The causative agent of CLFD, *Corynespora cassiicola*, is a ubiquitous-fungus infecting more than 350 plant species (Farr and Rossman, 2011). Berkeley and Curtis initially described the pathogen as *Helminthosporium cassiicola* in 1868, however it has since been renamed *Corynespora cassiicola* due to various taxonomic modifications (Xavier *et al.*, 2013). Cassiicolin, the toxin secreted by the fungus, is the primary determinant of *Corynespora* pathogenicity (Breton *et al.*, 2000). The disease was reported first in Indian rubber plantations in 1958 and subsequently in Malaysia (Newsam, 1960; Ramakrishnan and Pillai, 1961), Sri Lanka, Indonesia, Thailand and African countries. In India, first severe outbreak of CLFD was observed in coastal Karnataka region and it was eventually found spreading towards traditional rubber growing regions in Kerala (Rajalakshmy and Kothandaraman, 1996). During 1999, the disease became very severe (50-70% disease intensity) in Karnataka and North Malabar regions of Kerala (Jacob and Idicula, 2004). Subsequent annual surveys in the disease endemic areas have revealed that intensity of the disease has remained moderate to severe in some locations (Manju *et al.*, 2001). CLFD spread with the introduction of new breeds. Since its first epidemic in the early 1960s, many outstanding clones, namely, RRIM 600, RRII 105, RRIC 103,

PB 260, RRIC 110 and IAR 873, succumbed to the disease. Due to the lack of a cost-effective management system, the disease affected all susceptible clones, causing defoliation and die-back. The respective governments had no alternative except to force growers to uproot their affected plantations and replant them with tolerant clones that were readily available at the time.

One of the distinguishing features of Corynespora is the development of various symptoms based on the clone type and maturity state of the plant. The CLFD appearance in Indian rubber nursery was characterized by circular, rarely irregular amphigynous leaf spots (Ramakrishnan and Pillay, 1961; Rajalakshmy and Kothandaraman, 1996). Repeated defoliation of young leaves causes the terminal portion of the shoot to dry out. Apart from railway track, fishbone, and stag horn symptoms in the affected young leaves, the disease also affects mature trees and creates similar symptoms. As a result of this varied symptom development, early disease diagnosis has become a limiting factor which is otherwise essential for effective disease management. Another challenge is the high susceptibility of the plant to CLFD, especially in the juvenile stage.

Traditional breeding approaches and proper management strategies *via* chemical control continue to play a vital role in improving *Hevea* clones. However, conventional breeding methods are cumbersome in tree crops like *Hevea*, as the process has to include many generations which take decades to grow and is expensive in terms of time, space and number of plants being handled. Owing to the requirement of repeated fungicide application, high cost of labour and environmental concerns, chemical control of CLFD is not generally advocated in many of the rubber growing countries in South East Asia.

In spite of all these measures Corynespora leaf fall still continues to be one of the major threats in natural rubber production. Thus today, attention is paid all over the world to develop clones having resistance to Corynespora leaf fall disease as the main tool to manage the disease. This initiative can be accomplished through conventional plant breeding programme which involves the development of improved clones in various rubber growing countries including India. Plant breeding and selection which is aimed at crop improvement is generally carried out

to bring together the maximum number of desirable characteristics and all beneficial traits in a particular crop. These characteristics may include disease and insect resistance; tolerance to heat, soil salinity, or frost; appropriate size, shape, and maturity time; and a variety of other general and specific traits that contribute to improved environmental adaptation, ease in growing and handling, higher yield, and improved quality (Allard, 2019). In *Hevea*, hybridization coupled with vegetative propagation and clonal selection is considered as the most important conventional breeding method. In India, *Hevea* breeding programmes were initiated during 1954. The goal of *Hevea* breeding is to create ideal clones with high production potential and desirable secondary characteristics such as early vigour, smooth thick bark with a good latex vessel system, good bark regeneration, tolerance to major diseases, tapping panel dryness and good stimulation response (Annamma *et al.*, 1990; Varghese *et al.*, 1992). In addition to maximum yield, attention has also been paid to identify clones suitable for different locations based on agroclimatic zonation.

Crop improvement of perennial tree crops in general, and *Hevea* in particular, *via* conventional breeding is a very complicated and extensive procedure. The rubber tree is highly heterozygous in nature and has got a lengthy juvenile phase of 6-7 years to achieve tappable girth. The narrow genetic base, seasonal and non-synchronous flowering pattern, high heterozygosity, long breeding and selection cycle, low fruit set, and lack of fully reliable early selection parameters are the major limitations in rubber breeding (Varghese and Mydin, 2000; Mydin, 2014). Furthermore, lack of genetic diversity of the crop due to its narrow genetic base has slowed down the genetic advancement in *Hevea* (Varghese *et al.*, 1992). In such situations, it is highly desirable to use biotechnological interventions to widen the restricted genetic basis and overcome the constraints of conventional breeding techniques. Thus scientists have developed plant tissue culture technology as a novel tool for increasing the efficiency of plant breeding process.

The tissue culture technology includes a wide range of potentially useful techniques, all of which utilize *in vitro* plant cell and tissue culture for regeneration of functional plants from tissue fragments, isolated cells, calli, protoplasts, or embryonic tissue (Poehlman, 1987). Basically, in tissue culture technology, it is necessary to understand and master the regeneration of plants from selected explants for a

particular species before the techniques can be routinely used to complement current breeding procedures. Unfortunately, within a population, cell cultures from various crop species or genotypes, or even cultures from different parts of the same plant, do not always respond uniformly to a particular culture technique. Therefore when using these culture techniques in a breeding program, it is highly essential that an efficient and reliable *in vitro* plant regeneration system has to be established first from the specific explant. Methods that use tissue culture for plant regeneration with potential utility in plant breeding include micropropagation, microcutting, somatic embryogenesis (SE), micrografting, genetic engineering, protoplast culture, mutation breeding and exploitation of somaclonal variation. Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes (Williams and Maheshwaran, 1986). Plant regeneration through somatic embryogenesis is considered as a powerful tool for plant propagation. By the end of twentieth century, Jayasree *et al.,* (1999) and Sushamakumari *et al.,*(2000b) have reported successful plant regeneration protocols in *Hevea brasiliensis via* somatic embryogenesis using anther and immature inflorescences as explants respectively.

Among several strategies developed for disease resistance breeding, which are long term methods especially for perennials, a comparatively simpler and easier method is *in vitro* selection technique of somaclonal variants based on plant tissue culture method (Ramesh *et al.*, 2010). *In vitro* selection strategy for disease resistance is a combination of biotechnological and phytopathological techniques which involves regeneration of plantlets in the culture media amended with varying concentrations of the selection agent and screening of regenerated plantlets for resistance to disease (Svabova and Lebeda, 2005). Thus, over the past few decades, cells with desirable characteristics have been isolated from *in vitro* cultures by the inclusion of fungal toxins or culture filtrate as a selection agent in the tissue culture medium. As a result, somaclonal variants resistant to various disease-causing pathogens could be regenerated. Main goal of the present study is to develop a somaclonal variant that is tolerant to *C.cassiicola* through *in vitro* selection strategy using crude culture filtrate (CCF) of *C.cassiicola* as the selection agent.

Because of the genetic gains that can be realised through selection and development of elite (clonal) lines, somatic embryogenesis (SE) offers advantages in tree breeding. It also serves as a platform for introducing genes of interest in host plant through the process of *Agrobacterium* mediated transformation via genetic engineering. *Chitinase* gene is one of the most prioritised pathogenesis related (PR) protein employed in genetic engineering processes for the development of disease-resistant transgenic plants. The reason for this priority is that overexpression of this enzymes in plants causes rapid breakdown of chitin, which is a major constituent of most fungal cell walls and thus prevent the entry of fungal pathogen into leaf tissues. Another parallel goal of this study was to attempt genetic transformation for introducing this *chitinase* gene in *Hevea* based on the importance of improving disease tolerance due to the introduction of *chitinase* gene through genetic engineering, as previously reported in other crops.

Objectives of study

- To investigate the phytotoxic effect of CCF of *C.cassiicola* on leaf tissue as well as *in vitro* generated calli from selected clones.
- To identify and evaluate various biochemical parameters responsible for defense against *Corynespora cassiicola* infection in *Hevea brasiliensis.*
- To explore the feasibility of developing CLFD tolerant plants of *Hevea* through *in vitro* selection technique by exposing or challenging *in vitro* cultures against the crude culture filtrate of *Corynespora cassiicola.*
- To clone and characterise *chitinase* gene using rDNA technology inorder to construct a *chitinase* gene expression cassette in binary vector pCAMBIA 1301.

Chapter 2

Review of Literature

Despite its remarkable properties, rubber was considered a valueless commodity until the end of eighteenth century. As per history, the Aztecs used rubber to make balls for their ceremonial ball games. However, such games perished with the Aztec civilization in the 16th century and no further practical uses of rubber were discovered until late 18th century (Muller and Strehlow, 2004).

2.1. Classification and nomenclature of *Hevea brasiliensis*

H. brasiliensis (Willd. ex A. Juss.) Muell. Arg., commonly known as the rubber tree, is a perennial dicotyledonous tree belonging to the family *Euphorbiaceae.* The taxonomic hierarchy of *Hevea brasiliensis* is as follows

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Euphorbiales
Family	Euphorbiaceae
Sub-family	Crotonoideae
Tribe	Micrandreae
Sub-tribe	Heveinae
Genus	*Hevea*
Species	*brasiliensis*

Table 2.1. Taxonomic hierarchy of *Hevea brasiliensis*

The *Euphorbiaceae* family to which rubber belongs is considered to be the fifth-largest flowering plant family, with around 300 genera and 7,500 species (Gillespie and Armbruster, 1997). Presently a total of 10 species are described under the genus *Hevea*: *H. brasiliensis, H. guianensis, H. benthamiana, H. pauciflora, H. spruceana, H. microphylla, H. rigidifolia, H. nitida, H. camporum,* and *H. camargoana* (Webster and Paardekooper, 1989; Schultes, 1990). *Hevea brasiliensis, H. pauciflora, H. spruceana* and *H. rigidifolia* originally described under the genus *Siphonia*, was however reduced under the genus *Hevea* by J. Mueller Argoviensis in 1865 (Wycherley, 1992). Murca Pires reported the last species, *Hevea camergoana*, in 1981 (Nair, 2010). Only three of the ten species, *H. benthamiana, H. brasiliensis*, and *H. guianensis*, produce rubber of acceptable quality (Schultes, 1977).

2.2. Distribution of genus *Hevea*

The NR originated in parts of the Amazon Basin, Matto Grosso (Upper Orinoco) and the Guianas. Geographically, wild and semi – wild *Hevea* is widespread in the Northern part of South America from Brazil to Venezuela and from Columbia to Peru and Bolivia (CABI, 2021). Different species of *Hevea* prefer different habitats. Seven species have been found in Colombia. Peru has four, whereas Venezuela has five. However, all ten species can be found in Brazil (Wycherley, 1992). Half of the genus *Hevea brasiliensis* was found mostly in the southern Amazon, extending to the Brazilian states of Acre, Mato Grosso, and Parana, as well as areas of Bolivia and Peru, North of the Amazon to the west of Manaus, and South of Colombia. Currently, rubber is grown extensively in the tropical regions of Asia, Africa, and South America, in countries, such as Malaysia, India, Indonesia, SriLanka, China, Thailand, Myanmar, Philippines, Vietnam, Cambodia, Bangladesh, Singapore, Nigeria, Democratic Republic of Congo, The Republic of Cameroon, Ivory Coast, Zaire, Ghana, Brazil, Liberia and Mexico. However, tropical Asia accounts for the majority of total production of rubber (Nair, 2010).

2.3. History of *Hevea*

The origin of the term rubber is quite intriguing. In 1493, the explorer Christopher Columbus was the first European to come across rubber during his second journey to Central America. Later in 1735, Charles Marie de La Condamine

had been sent to South America by the French Royal Academy of Science on an expedition to determine the shape of the globe where he became aware of certain primitive uses of rubber without seeing any rubber trees. His paper to the Academy in 1745 attracted the attention of Francois Fresneau, an engineer working in Cayenne at the time and it is to Fresneau that credit must be given for making the first systematic observations on rubber (Jones and Allen, 1992). When English chemist Joseph Priestley discovered it could be used to rub out pencil traces, he gave it the term "rubber" in 1770 (Nair, 2010). Jean Baptiste Fusee Aublet, a French botanist, gave the first botanical description of the genus *Hevea* and of the type species *H. guianensis* in 1775. L.C. Richard proposed the generic name Siphonia in 1779 as the equivalent of Aublet's *Hevea*, and Gmelin proposed *Caoutchoua elastica* in 1781 to replace *H. guianensis,* however both names were not published until 1791 (Wycherley *et al.*, 1992). Since then many considerable changes occurred in the taxonomy of genus *Hevea*. The name itself is a Latinized form of the Ecuadorian Indian name, *Hheve*, and there was some earlier competition with other possible names such as *Siphonia* and *Caoutchoua* (Jones and Allen, 1992). Despite the fact that Charles Macintosh was the first to develop NR for industrial use in 1823, it was Charles Goodyear's invention of vulcanization in 1839 that revealed the full potential of NR. With the discovery and development of tyres by Dunlop in 1888, Michelin in 1895, and Ford in 1910, NR became ever more linked with transportation (Saha and Priyadarshan, 2012). As a result of the increased use of rubber, large-scale commercial cultivation and trading of rubber trees occurred.

Prior to the commercial cultivation of *H. brasiliensis*, the main sources of natural rubber were *Ficus elastica* and *Castilla elastica*, which grew wild in the woods of Central and South America, India, Africa, Madagascar etc. In India, the major contribution was from *Ficus elastic. Manihot glaziovii* (Ceara rubber), *Manihot dichotoma* (Jeque rubber), *Funtimia elastic* (Lagos rubber), *Landolphia kirkii* (Landolphia rubber), *Cryptostegia grandiflora* (Palay rubber), *C.madagascariensis* (Madagascar rubber), *Castilla elastica* (Panama rubber), *Ficus elastica* (India rubber), *Parthenium argentatum* (guayule rubber), *Taraxacum kok-saghyz* (Russian dandelion) were other alternate sources of rubber (Priyadarshan, 2011). Despite the

fact that all of these sources contribute to the production of rubber, none of them can compete with *H.brasiliensis* in terms of efficiency.

The real success story of rubber in Southeast Asia began in the late 1800s, when it was promoted as a plantation agricultural product. Sir Henry Wickham gathered 70,000 seeds from the Rio Tapajoz valley (Amazon, Brazil) and carried them first to Kew Botanical Gardens (London) in June 1876, paving the way for domestication of *H.brasiliensis*. Only 2700 of them appear to have germinated. According to Kew records, 1900 seedlings were sent to the Botanic Gardens in Colombo, where 90% of them survived; 18 were transferred to the Botanic Gardens in Bogor, Indonesia, where two survived; and 50 were sent to Singapore, where most likely none survived (Jones and Allen, 1992).

During the same year Robert Cross sailed from England to Brazil in search of another collection of *H. brasiliensis*. He returned back after five months with 1000 rubber seedlings collected from the lower Amazon. In June 1877, 22 seedlings not specified either as "Wickham" or "Cross" were sent from Kew to Singapore, which were distributed in Malaya forming the prime source of 1000 tappable trees found by Ridley during 1888. In September 1877, 100 *Hevea* plants specified as "Cross material" were sent to Ceylon (Saha and Priyadarshan, 2012) while 400 seedlings were retained at Kew Gardens. The subsequent fate of these is a mystery; the general thing seems to be that none of Cross's material survived, however Bulkwill thinks that "some small admixture of Cross genetic material cannot be entirely ruled out." This matter is significant since the Wickham and Cross collections were obtained from different parts of the Amazon, suggesting that their genetic makeup would undoubtedly differ (Jones and Allen, 1992). Wickham collection of Ceylon seedlings were also distributed worldwide. In India, rubber was first received from Sri Lanka in 1878. In fact, by then Sri Lanka had established itself as a hub of early activity, with the Heneratgoda Botanic Gardens serving as a key source of rubber seeds for both local and foreign markets. Whatever the case may be, it is apparent that the rubber trees that span millions of hectares in Southeast Asia are descended from a small number of Wickham's original stock obtained from the banks of Tapajoz, one of the Amazon's greatest streams (Imle, 1978).

2.4. General description of *Hevea brasiliensis*

Hevea brasiliensis is a deciduous tree that grows quickly, reaching heights of 25 m (in plantations) to 43 m (in the wild). The fact that planted rubber trees are tapped for latex harvesting is the reason for their tiny stature. The trunk of the tree is straight, with light grey bark. Branches are typically formed to form an open leafy crown. The style of branches and crown shape differ among clones. The leaves are organised in groups or storeys. A cluster of spirally organised, trifoliate, glabrous leaves is formed from each storey (Premakumari and Saraswathyamma, 2000). Because the tree is deciduous, it sheds its leaves over the winter, a behaviour known as wintering. The winter season in South India lasts from December through February.

Hevea brasiliensis is a fast growing deciduous tree, with a height range of 25 m (in plantations) to 43 m (in the wild). The fact that planted rubber trees are tapped for latex harvesting is the cause for their low stature. The tree has a straight trunk with light grey bark. Branches are usually developed to form an open leafy crown. The branching habit and crown shape vary among clones. The leaves are arranged in groups or storeys. From each storey, a cluster of spirally arranged, trifoliate, glabrous leaves are produced (Premakumari and Saraswathyamma, 2000). The tree being deciduous shed the leaves, the phenomenon being termed as wintering. South India's winter season runs from December through February. During this time, the trees may drop all or some of their leaves. New leaf flushes and inflorescences appear sooner or later. The flowers are fragrant and have a short stalk. Both male and female flowers are seen on the same inflorescence which is a pyramid shaped particle (George *et al.*, 1967).

2.5. Area, production and consumption

The world's largest producers of NR are Thailand, Indonesia, Malaysia, Vietnam, China, and India. Global NR production and consumption are now around 12.40 and 12.60 million tonnes, respectively. The top NR consumers are China, India, the United States, Japan, Thailand, Indonesia, and Malaysia. The first rubber plantations in India were established in 1895 on the hill slopes of Malabar and Travancore in Kerala. Commercial rubber cultivation, on the other hand, began in

1902. Other plantation areas include Tamil Nadu, Nicobar Islands, North Eastern states of Tripura, Assam, Odisha, Karnataka, Maharashtra and West Bengal.

Currently, India is the world's sixth largest NR producer with the highest productivity (6,94,000 tonnes in 2017-18). This production rate increased by 9.4% in 2019-20, reaching upto 7,12,000 tonnes. In addition, with an annual consumption of around 1.1 million tonnes, India is the world's second-largest consumer of NR. In view of current domestic deficit, more than 40% of the demand for NR has to be satisfied by imports.

2.6. Economic importance of rubber

Between the 16th and 17th century, a commercial recession was experienced in use of rubber in Europe. To combat this, French scientists rediscovered rubber in the middle of the seventeenth century. As part of this process, several modifications and discoveries were made in utilisation of rubber. As a result, in 19th century, rubber got transformed from a mere curiosity into an important commercial and industrial product. In addition to this, the rapid growth of the automotive industry in the nineteenth century further propelled the demand for NR. Rubber is now being utilised in the fabrication of a wide range of industrial and household items. Despite competition from other forms of synthetic rubbers, it remains an indispensable raw material in the manufacture of a wide variety of products. (Nair, 2010). According to Saha and Priyadarshan, (2012), NR is a renewable ("green") elastomer being used mainly in tire sector (70%), latex products (12%), and many other industrial applications. Additional products derived from *H. brasiliensis* include oil seeds and wood. Rubber wood represents a relatively sustainable alternative to tropical woods harvested from natural forests. Furthermore, rubber wood has proven to be very versatile in its use in the manufacture of furnitures and the wood-based panels industry. A profitable sector has been created by rubber wood in Malaysia, Thailand, India, Vietnam, Indonesia, Cambodia and Nigeria. Its unique and excellent physical properties along with the natural pale cream colour make it an ideal material for flooring, household furniture, boards and packing boxes. Because of the high value of this commodity, multiple superior latex-timber clones have been developed (Rahman *et al.*, 2013). Rubber seed oil is a semi-drying light yellow oil extracted from rubber

seeds that could be used in a variety of applications such as biolubricants, detergents, paints & coatings, and so on (Hong *et al.*, 2018). It also has better potential for use as alternative diesel fuel in compression ignition engines (Ikwuagwu *et al.*, 2000; Ramadhas *et al.*, 2005).

2.7. *Corynespora cassiicola*- A major threat to rubber cultivations

Oidium heveae, *Colletotrichum spp.*, *Phytophthora spp.*, *Microcyclus ulei*, and *Corynespora cassiicola* are some fungal pathogens that affect NR production in *Hevea.* Out of these pathogens, currently, *Corynespora cassiicola* is responsible for the most destructive leaf fall disease in *Hevea* in Asian and African countries (Umoh and Fashoranti, 2018). *Corynespora cassiicola*, a fungus of the family Ascomycetes, was first reported as *Helminthosporium cassiicola* by Berkeley and Curtis in 1868 which subsequently underwent several taxonomic modifications and was reclassified to its current nomenclature by Wei, (1950). *Corynespora cassiicola* is a highly diverse plant pathogen with a broad host spectrum. More than 500 crop species belonging to 50 families in tropical, subtropical, and temperate countries are affected by this fungus (Fernando *et al.*, 2012). The host plants include several economically important crops such as cotton (Jones, 1961), cowpea (Olive *et al.*, 1945), cucumber (Blazquez, 1967), eggplant (Onesirosan *et al.*, 1974), sesame (Stone and Jones, 1960), soyabean (Seaman and Shoemaker, 1965), tobacco (Fajola and Alasoadura, 1973), tomato (Mohanty and Mohanty, 1955), rubber (Ramakrishnan and Pillay, 1961). Corynespora infection on these crops causes various diseases known as blotch disease of cucurbits; fruit spot of tomato; leaf spot (of cotton, tobacco, tomato); stem and fruit spot (of eggplant, papaya); target spot (of cucurbits, soybean) and so on. The disease symptoms most frequently appear on leaves, however stems, fruits, and roots are also susceptible to disease (Jones and Jones, 1985). The devastating leaf defoliation (Corynespora Leaf fall Disease) in infected rubber trees has become a threat to the natural rubber plantation industry by limiting its growth and productivity level. The highly complex nature of this pathogen and the presence of multiple physiological races are important reasons for the rapid increase in disease intensity (Pernezny *et al.*, 2002; Da Silva *et al.*, 2006).

2.7.1 History of Corynespora Leaf Fall Disease

C.cassiicola infection has apparently been reported in *Hevea* plants grown in different places throughout the world. *C. cassiicola* was initially isolated from rubber trees in Sierra Leone in 1936, followed by India and Malaysia in the early 1960s. Since then, the disease has spread rapidly over most rubber-producing countries in Asia and Africa, causing severe sporadic outbreaks and significant losses in natural rubber yields. (Deon *et al.*, 2012).

CLFD was first recorded in India in 1958 at the Experimental Station of RRII, Kottayam. Later the same disease was identified in different places of Kerala (Mundakayam, Kanjirapally, Kalaketty, Thodupuzha, Chalakudy, Trichur and Vithura) and Tamil Nadu (Kaliyil and Nagercoil) (Ramakrishnan and Pillay, 1961). It then spread to mature rubber trees in some localities of India such as Kodumon in 1969, Chittar in 1970, Shaliacary, Kallar and Cheruvally in 1976 (George and Edathil, 1980). The first incidence of epidemic form of the disease was reported in India in 1996 in mature rubber plantations of *Hevea* breeding substation at Nettana, South Karnataka. Rajalakshmy and Kothandaraman, (1996) reported the epidemic of this disease in main field on RRIM 610, RRIM 622 and Tijr 1 seedlings. The severity of this disease subsequently spread to commonly cultivated clones like RRII 105, PCK 2, RRII 118, RRII 300, RRII 305, PCK 1, RRIM 600, PB 86, PB 235, PB 255, PB 260, PB 311, PR 107, GL 1 and Tijr 1 (Jacob, 1997). Later in 1999, the disease became more severe in the Subramanya, Sullia, Puthur, Madikeri and Kanhangad regions of Karnataka (50-70% of the disease severity). As a result, an eradication campaign was launched with the support of the World Bank assisted rubber project, in which more than 10, 000 ha were sprayed with copper or mancozeb fungicide, resulting in the suppression of the inoculum (Jacob and Idicula, 2004). Subsequent surveys conducted in coastal Kamataka and the North Malabar region of Kerala during the 1998-2000 disease seasons indicated that disease incidence and intensity of infection varied between mild to severe in all locations. (Manju *et al.*, 2001). Later on, the severity of the disease increased and new incidents started occuring every year.

In late 1985, pathologists at the Rubber Research Institute in Sri Lanka identified this disease in a nursery on the Dortonfield Estate in Sri Lanka. As a result

of this disease, rubber plantations (more than 4000 hectares) with clones of RRIC 103 were severely affected, and RRIC 103 trees in large areas were significantly destroyed (Liyanage *et al.*, 1989). This high yielding rubber clone 103 was developed by the Rubber Research Institute of Sri Lanka (RRISL) in 1958 and released to the rubber growers in 1978 after extensive evaluation. Kuruvilla Jacob, the Indian rubber researcher wrote in this situation that "the occurrence of the disease on the new clone shattered the confidence of growers in the new clone developed in Sri Lanka and inorder to retain their confidence it was necessary for RRISL to recommend destruction of all the plantations of RRIC 103 and for the Sri Lankan government to pay compensation for growers which amounted to over Rs. 60 million". After that the high yielding clone RRIC 110 was cultivated which appeared to be highly promising at the time, but ten years later, they also became susceptible to Corynespora disease.

The incidence of Corynespora disease was first reported in Thailand by Kajornchaiyakul, (1987). The disease was observed on *Hevea* clones RRIC 107 and KRS 21, grown in the international clone exchange trials at Surat Thani Rubber Research Centre, Thailand, resulting in 2% tree mortality. The disease was reported to be prevalent in Thailand's south, east, and northeast areas according to a survey conducted in 1999, and susceptible clones included Songkhla 36, PR 255, PR305, and RRIT 251 (Chanruang, 2000).

During 1960, CLFD was first detected in Malaysia, in budwood nursery plants which were weak due to iron deficiency (Newsam, 1960). The disease became more common and started spreading to new areas in Malaysia since 1975. In 1975, rubber trees of clone RRIC 725 planted in the main field were reported to be infected with the disease, which later spread to a few more clones. A disease incidence survey conducted in 1990 found that several sites in Johore and Terengganu showed high disease incidence. The results of the subsequent survey in 1993 indicated that the disease had spread to new areas like as Perak and Kedah (Kamar, 1994).

In Indonesia, Corynespora disease incidence on *Hevea* clones was detected for the first time in 1980 at Sembawa experimental station in Sumatra. It was later found that the disease spread to Central and West Java (Soepena, 1986). Subsequent observations revealed the spread of the disease to many other rubber growing regions of

the country. During 1980, nearly 1200 ha was badly affected of which 400 ha had to be uprooted, causing an economic loss of Rs. 200 billion (Sinulingga and Soepena, 1996).

The disease was first detected in Vietnam in August 1999 at Laikhe Rubber Experimental Station. In January 2000, a severe outbreak of disease was reported from Locninh where complete defoliation of more than 200 ha of immature plantation of RRIC 104 occured. Severely infected clones in Vietnam include LI 1 88/372, RRIC 103 and RRIC 104. In an effort to eliminate susceptible trees, 221 trees were removed from the Laikhe Experimental Station. In addition to this, over 3000 vulnerable trees were also removed from various estates in Vietnam to minimise the possibility of disease transmission from infected trees (Dung and Hoan, 2000).

In Cote d'Ivoire, CLFD on *Hevea brasiliensis* was detected for the first time in 1989. On clone RRIC 103, the disease became severe, and the clone was eradicated as a result. Mild infection was observed on other clones like PB 28/59, PB 260, RRIC 110 and some IRCA clones. *Corynespora cassiicola* has been found for the first time in South China on *Hevea* rubber tree during a survey in 2006 (Jinji *et al.*, 2007).

2.7.2. Disease symptoms

Corynespora disease is particularly severe during the refoliation period (December to April) of rubber trees (Reshma *et al.*, 2016). Eventhough the disease is mainly confined to the nurseries and young immature plants, it also affects mature trees, causing severe defoliation of newly matured leaves produced during a dry period following an earlier wintering in December (Ogbebor and Adekunle, 2005) thereby reducing latex production to less than 45% (Ogbebor, 2010). The climatic factors favourable for *C. cassiicola* include humid weather with moderate rainfall throughout the day and temperatures ranging from 26-29^{0}C (Manju, 2011).

Though it affects leaves of all stages, leaves in the light green immature stage appear to be more susceptible. The symptoms of the disease vary with the clones, locality and maturity state of the plant (Jacob, 1997; Umoh and Fashoranti, 2018). Also the incidence of infection is not of the same intensity on all clones. Some clones are severely infected and defoliated while others adjacent to these remain disease free or with mild infection (Ramakrishnan and Pillai, 1961). In addition, *C. cassiicola*

pathogenicity and genetic diversity within the same agroclimatic region (Darmono *et al.*, 1996; Jayasinghe and Silva 1996; Breton *et al.*, 2000; Atan and Hamid 2003; Romruensukharom *et al.*, 2005; Dixon *et al.*, 2009; Qi *et al.*, 2009). The disease causes circular, amphigynous spots which are 1-8 mm in diameter. Sometimes irregular lesions are also formed. As a result of the development of these lesions, young leaflets may exhibit shrivelling and drying of the terminal part (Ramakrishnan and Pillai, 1961). A dark reddish brown ring surrounds each spot, which has a brown or white papery centre. The ring is surrounded by a yellow halo. Usually, the main vein or small veinlets adjacent to the spots become dark coloured giving rise to 'fishbone' or 'railway-track' appearances. The shot hole effect is sometimes noticed on leaves due to the disintegration of the centre of the spots (Jinji *et al.*, 2007). Severe infection of the midrib causes leaf blight. The tissues in the immediate vicinity become yellow and then brown. Even a single lesion on the midrib or base of the leaf is sufficient to cause leaf abscission. Affected leaves abscise prematurely leading to tree defoliation. A severe infection causes the die back of shoots. Defoliation of diseased trees on a regular basis eventually kills them (Jacob, 1997).

The main factors accelerating the damage caused by *C.cassiicola* are survival rate and extent of spread. Results of the studies conducted to investigate the survival mechanism of the pathogen in rubber plantations revealed that the pathogen survived in crop debris for roughly two years (Pernezny and Simone, 1993). It has also been claimed that it can survive in the field in root debris and stem during the wet season (Boosalis and Hamilton, 1957; Seaman *et al.*, 1965). Another finding was that the pathogen was able to survive on infected leaves of the plant, in leaf litter (for up to 11 days) and also under the bark of dried infected twigs as thick dark brown dormant mycelium (Manju *et al.*, 2016).

2.7.3. Alternate hosts

Corynespora cassiicola causes disease in a wide range of plant species all over the world. The fungus *C. cassiicola* is primarily found in the tropics and subtropics, and is widely diverse in substrate utilization and host association (Dixon *et al.*, 2009). In its asexual condition, the necrotrophic fungus *C. cassiicola* functions as a pathogen, saprophyte, and endophyte. It has a wide host range, affecting plants in 252

genera, representing 79 plant families (Schlub *et al.*, 2009). These diverse host species include both economically important plants as well as several weeds. In North America and Brazil, *C. cassiicola* has caused serious problems in soyabean (*Glycine max*), cotton (*Gossypium hirsutum*) pawpaw (*Carica papaya*), hydrangea (*Hydrangea macrophylla*), cucumber (*Cucumis sativus*), tomato *(Solanum lycopersicum)* and blueberry (*Vaccinium corymbosum*) (Seaman *et al.*, 1965; Jones, 1961; Bird *et al.*, 1966; Sobers,1966; Blazquez, 1967; Volin *et al.*, 1989; Onofre *et al.*, 2016). In Asia and Africa, the fungus was mainly responsible for epidemic outbreaks in rubber plantations affecting thousands of hectares of rubber trees (Deon *et al.*, 2012). Other susceptible hosts in India, China and Sri lanka are cassava (*Manihot esculenta*), castor bean (*Ricinus communis*), brinjal (*Solanum melongena*), cotton (*Gossypium hirsutum*), croton (*Codiaeum variegatum*), cassava (*Manihot esculenta*), sweet basil (*Ocimum basilicum*), sweet potato (*Ipomoea batatas*), eucalyptus, chilli and cucurbit crops including watermelon, muskmelon (Roy, 1965; Karan,1966; Sarma and Nayudu, 1971; Lakshmanan *et al.*, 1990; Jayasuriya *et al.*, 2009; Liu *et al.*, 2010; Vishwakarma *et al.*, 2011; Xu *et al.*, 2016; Phan *et al.*, 2015; Suresh *et al.*, 2011; Wang *et al.*, 2013 and Wei, 1950). In Korea the fungus seriously affected cultivations of pepper (Kwon *et al.*, 2001) and cotton rose (Kwon *et al.*, 2003). Also mild infections were reported in various other crops such as beach vitex (Park *et al.*, 2013); Korean raspberry (Kwon *et al.*, 2012) and rose of sharon (Seo *et al.*, 2013).

2.8. *In vitro* plant cell and tissue culture

Gottlieb Haberlandt presented his hypothesis on the intrinsic ability of isolated plant cells to live autonomously in 1902, which marked the beginning of *in vitro* plant cell and tissue culture research (Feher, 2019). The intrinsic ability of a plant's cell to give rise to a whole plant is called cellular totipotency, a capacity that a cell retains even after undergoing final differentiation in the plant body. A differentiated cell must first "dedifferentiate" and then "redifferentiate" in order to express its totipotency. The phenomenon in which mature cells return to the meristematic state and form undifferentiated callus tissue is called "dedifferentiation", and the ability of dedifferentiated cells to form whole plant or plant organs is called redifferentiation. During *in vitro* culture of the explants, which involves isolation and wounding of the explanted tissue, exposure to new signals from the medium, and the formation of new

gradients, a series of molecular and physiological events are triggered, resulting in dedifferentiation, unorganised cell division and growth, and the formation of callus. When new gradient form and different signals function, reorganization begins with better co-ordinated cell division and the development of meristematic growth centres. Organized morphogenetic expression and redifferentiation occur when new correlations among cells in the meristem are formed (Ziv, 1999). However, somatic embryogenesis is a preferred pathway, because it offers better potential for multiplication (Yantcheva *et al.*, 1998).

2.8.1. Somatic embryogenesis

Embryogenesis is the process by which embryo formation is initiated, either from a zygote (zygotic embryogenesis, ZE) or from somatic cells (somatic embryogenesis, SE) (Mendez-Hernandez *et al.*, 2019). A morphogenetic event where somatic cells have the ability to produce embryos without gamete fusion is termed somatic embryogenesis. It is used as a technique for plant mass propagation and consists of six well defined steps such as induction, expression, development, maturation, germination and plant conversion, each of which is characterized by distinct physiological, morphological and molecular events (Garcia *et al.*, 2019). Plant regeneration by somatic embryogenesis from cultured cells was originally observed in carrot by Steward *et al* in 1975. During somatic embryogenesis, somatic cells develop by division to form complete embryos analogous to zygotic embryos. The bipolar structure of the somatic embryo consists of both shoot and root meristems. As the embryos develop, they proceed through the following morphological and developmental stages: globular, heart, torpedo and cotyledonary stages globular, heart, torpedo and cotyledonary stages (Philips *et al.*, 1995). Two general patterns of embryogenic development of *in vitro* embryogenesis are recognized (1) Direct embryogenesis, in which embryos emerge directly from tissues without callus proliferation. Direct embryogenesis initiates from cells that have already been determined for embryogenic development, i.e. pre-embryogenic determined cells (PEDCs), which require only growth regulators or favourable conditions to enable cell division and embryogenesis expression. (2) Indirect embryogenesis, in which an intervening callus phase precedes embryo development. Indirect embryogenesis involves the redetermination of differentiated cells, the proliferation of calli, and the

development of the embryogenically determined state. For these induced embryogenically determined cells (IEDC's), growth regulators are required not only for re-entry into mitosis but also for determination of the embryogenic state (Sharp, 1980; Williams and Maheswaran, 1986).

2.8.2. Somatic embryogenesis in *Hevea*

Immediately after the first description of somatic embryogenesis in carrot cell cultures, this process has been reported in different plant varieties like eggplant (Gleddie *et al.*, 1983), conifers (Tautorus *et al.*, 1991) and woody plants (Jain *et al.*, 2000). During the last few decades, somatic embryogenesis was well recorded in almost all plant species. But the tissue culture of woody perennial plants was found to be recalcitrant due to its complex seasonal cycles and life cycles (McCown, 2000). However, limited success has been achieved in tissue culture of angiosperm and gymnosperm woody plants which became quite noteworthy in the past decade (Jain *et al.*, 2000). Research on *Hevea* tissue culture for physiological studies was initiated for the first time by Bouychou of the Institut Francais du Caoutchouc in 1953, with the aim of utilising calli to obtain convenient material for the study of the laticiferous system. This area of research was again taken up by Chua, (1966) followed by Wilson and Street, (1975) and later by Audley and Wilson, (1978). In the early 1970's a Chinese team at the Rubber Cultivation Research Institute (Baodao) and a Malaysian team at the Rubber Research Institute of Malaysia worked simultaneously to develop somatic embryogenesis as an *in vitro* propagation technique (Nayanakantha and Senèviratne, 2007). In 1972, researchers at Rubber Research Institute of Sri Lanka succeeded in initiating *Hevea* callus cultures and later, in 1984, they expanded their study on somatic embryogenesis. Moreover at the same time, the institute also developed the first subcultureable anther-derived callus (Satchuthananthavale and Irugalbandara, 1972). Paranjothy achieved root induction and differentiation of embryoids for the first time from anther wall derived calli. Eventhough complete plant regeneration could not be achieved by this method he finally succeeded in shoot development of embryos derived from the same explant (Paranjothy; 1987). After the development of the first three pollen plants by anther culture in 1977 by some researchers at Baoting Institute of Tropical Crops, Hainan, China, Chen *et al.*, (1979) described the process of obtaining pollen plants of *Hevea brasiliensis*. Thereafter

plantlets could be successfully developed from various cell cultures raised from different explants such as integumental tissues of immature fruit (Carron, 1982), stamens (Wang and Chen, 1995), immature anther (Jayasree *et al.*, 1999) and immature inflorescences (Sushamakumari *et al.*, 2000b). Wang and Chen developed 130 normal plantlets from high-production Haiken 2 and SCATC 88-13 clones in 1980. The same research team then successfully transplanted a total of some fifty *in vitro* cultured plantlets developed from *Hevea* anther wall callus to the soil for the first time in 1984 (Wang *et al.*, 1984). In 1979, the French Rubber Research Institute, IRCA, also initiated work in the same field and successful plantlet development via somatic embryogenesis could again be achieved from anther wall derived callus by Carron (1980). Shortly after this, Carron and Enjarlic, (1985) showed the embryogenic potential of a new explant, the inner integument of seed, which functions as mother tissue in the same way that the anther wall does. According to the above mentioned authors, the whole process of somatic embryogenesis involves four successive stages callogenesis, differentiation of embryos, multiplication of embryos and germination of embryos and plantlets. The growth, anatomy and morphogenetic potential of callus and cell suspension cultures of *Hevea brasiliensis* have also been studied. Callus cultures initiated from stem explants of young *Hevea* plants failed to yield a growing cell suspension when transferred to agitated liquid MS medium (Wilson and Street, 1975).

Several investigators worked extensively on somatic embryogenesis to learn the effect of various factors on somatic embryogenesis and to improve the frequency of somatic embryo induction and plant regeneration in *Hevea.* Also studies were conducted to optimise culture conditions, nutritional requirements, and hormonal requirements during somatic embryogenesis. The supply of arginine and polyamines during a primary phase of callus growth resulted in an increase in the polyamine content of the calli and in the percentage of embryogenic-type structures (El Hadrami *et al.*, 1989). After a while in 1991, he illustrated in his studies that decreasing the concentration of auxin (3, 4-D) and cytokinin (BAP) has little effect on callogenesis in *H. brasiliensis* but greatly promoted the tendency of calli towards the formation of somatic embryos. The production of embryogenic callus was eventually stabilized by progressive modifications in culture conditions, such as timing of subculturing (Michaux-Ferrier and Carron, 1989), culture atmosphere (Auboiron *et al.*, 1990),

water status of the medium and explant (Etienne *et al.*, 1991a), mineral and carbohydrate nutrition (Etienne *et al.*, 1991b), and consideration of how hormone balances affect embryogenic potential. Influence of growth regulators, sucrose, calcium and various culture media on callus friability as well as somatic embryogenesis (Montoro *et al.*, 1993), effect of polyethylene glycol and abscisic acid on embryo induction (Veisseire *et al.*, 1994; Linossier *et al.*, 1997) and the role of different carbohydrates in somatic embryo induction (Blanc *et al.*, 2002) have also been evaluated. Te-Chato and Chartikul, (1993) reported somatic embryo induction and subsequent plantlet regeneration by culturing integument of seed on basal MS medium. Recurrent embryogenesis was used to establish a long-term embryogenic line, which was then maintained on hormone-free medium for three years by transferring selected proembryogenic masses every ten days (Cailloux *et al.*, 1996). Shijie *et al.*, (1990) developed 1700 plants from 52,896 embryoids by inoculating 31,584 anthers of 13 different clones in tubes containing culture medium. Out of these they could effectively transplant and establish 539 plants. However, there was a large difference among clones in terms of induction frequency. When the concentration of one growth factor (3,4-D or kinetin) was reduced from 4.5μM to 0.45 μM during the first culture, or when high sucrose or calcium levels (351 mM and 12 mM, respectively) were maintained during subcultures, callus friability was enhanced in clone PB 260 (Montoro *et al.*, 1993). Zeyun and Xiongting, (1995) experimentally illustrated that temperature is an important factor in rubber stamen culture and somatic plant regeneration. They optimized the temperatures for callus induction, embryogenesis and plant regeneration as 26°C, 24-25°C, and 26-27°C respectively.

Somatic embryogenesis, which was formerly considered to be difficult, is now successfully applied in the majority of the clones studied (particularly PB 260, PB 235, PR 107, RRIM 600, GT 1), resulting in a high frequency of embryogenic calli (Carron *et al.*, 1995). About 100 somaplants developed from three of these clones (PR 107, PB 260, and RRIM 600) were planted for field trials in order to compare them with classical budded clones. In field trials, growth and yield of these *in vitro* plants proved to be significantly more vigorous than the mature budded control (Carron *et al.*, 2007). Engelmann *et al.*, (1997) demonstrated excellent survival, rapid regrowth, and somatic embryo formation from cryopreserved calli of a commercial *Hevea* clone,

where cryopreservation was done using two protocols, one by means of a traditional freezing process and the other by employing a simple freezing process. Carron *et al.*, (2000) compared the root system architectures in seedlings and *in vitro* plantlets of *Hevea brasiliensis*, in the initial years of growth in the field. The *in vitro* plantlets had a structure similar to that of seedlings with a well-developed taproot and lateral root system. In 2010, Zhou *et al* established a system for callus induction and plant regeneration via somatic embryogenesis from root explants of *Hevea* clone Reyan 87-6-62.

Since 1990, the Rubber Research Institute of India (RRII) team has been striving hard to establish an effective *in vitro* plant regeneration system through somatic embryogenesis, especially for Indian *Hevea* clones. A reliable regeneration system for Indian rubber clones have been developed via somatic embryogenesis using immature *Hevea* anthers as explant by Jayasree *et al.*, (1999). According to them, optimum callus induction was obtained on modified MS medium supplemented with 2.0 mg/l 2, 4-D and 0.5 mg/l KIN and better somatic embryo induction was noticed on medium with 0.7 mg/l KIN and 0.2 mg/l NAA. A hormone-free media was used for further development of these embryos into plantlets. Sushamakumari *et al.*, (1999) were successful in forming multiple shoots from germinating somatic embryos of *Hevea brasiliensis.* They were able to induce an average of 3.45 micro-shoots per explant by manipulating the amount of BA and TDZ in the medium. In the following year, Sushamakumari *et al.*, (2000b) reported another efficient and reproducible plant regeneration pathway through somatic embryogenesis. This study once again investigated the effect of various growth regulators and sucrose at various stages of this pathway. Higher plant regeneration frequency was noticed in BA and GA fortified medium, a higher sucrose level was essential for effective embryo induction and maturation, and a lower sucrose level was beneficial for plant regeneration, according to their reports. In the same year Sushamakumari *et al.*, (2000a) made a remarkable achievement in protoplast culture in *Hevea*. They were successful in developing for the first time plantlets from embryogenic cell suspension derived protoplasts of rubber. Experiments were earlier conducted by researchers on isolation and culture of protoplasts in *Hevea* (Cazaux and d'Auzac, 1995). Eventhough they were successful in isolating protoplasts, callus induction could not be obtained from these isolated protoplasts. Kumari Jayashree, (2001) improved the efficiency of

somatic embryo induction and germination by optimising culture conditions and other parameters. For promoting embryo induction efficiency, they supplemented embryo induction medium with 200 mg/l glutamine and 400 mg/l casein hydrolysate. Kumari Jayasree and Thulaseedharan, (2004) succeeded in maintaining embryogenic cultures for over three years without losing their embryogenic potential by employing suitable MS medium containing proline (100 mg/L) and charcoal (0.1g/L) with regular subculturing at an interval of 50 days. Since then, there has not been much new research on somatic embryogenesis and plant regeneration, with the exception of studies by Kala *et al.*, (2009) on leaf explants of clone RRII 105 and Sushamakumari *et al.*, (2014) on root explants whereby somatic plants could successfully be regenerated from these two explants.

Despite the fact that *Hevea brasiliensis* is a difficult species to culture *in vitro*, there have been many success stories in *Hevea* micropropagation. Montoro *et al.*, (2012) concluded in his review that primary somatic embryogenesis is a technique that can be applied for clone rejuvenation of a large number of cultivated *H. brasiliensis* clones like PB 260, PR 107, 557, RRIM 600, PB 235, RRIM 703, IRCA 109, PB 254 and PB 310 in the CIRAD laboratory, clone BPM 24 at RRIT in Thailand, and clones PR 107, BPM 24, PB 254, IRCA 109, IRCA 317, PB 260, RRIM 703, RRIM 600, PB 217 and IRCA 41 in the CPN Michelin laboratory. Using anther calli, RRII in India, CATAS in China, RRIM in Malaysia and IBRIEC in Indonesia have developed similar techniques on clones RRII 105, Haiken 1, Haiken 2, GL1, Dafeng 95, Reyan 7-33-97, Wenchang 217, Yun Yan 77-2, GL1 and PR 300. Embryogenesis associated rejuvenation process enables vegetative multiplication of elite trees, genetic transformation and genome editing (Mignon and Werbrouck, 2018). Although the efficiency of regeneration *via* somatic embryogenesis has greatly improved and encouraging results have been obtained, the system still have some setbacks such as frequent callus browning (Housti *et al.*, 1991, 1992), low rates of germination and plant conversion (Cailloux *et al.*, 1996; Linossier *et al.*, 1997), reliable somatic embryo formation limited to only a few genotypes (Jayasree *et al.*, 1999) and occurrence of abnormal embryos (Sushamakumari *et al.*, 2000b). As a whole the extensive efforts implemented in studying somatic embryogenesis turned out to be quite intriguing for the *Hevea* industry.

2.9. *In vitro* screening methods

Diseases caused by different phytopathogens have a substantial impact on the production and yield of the majority of crops, resulting in massive economic losses. Conventional breeding strategies always played an important role in crop improvement to overcome these losses. However, a lack of success and failure to generate the desired outcome resulted in limited usage of these strategies. In view of this, a combination of biotechnological and phytopathological techniques that provided an alternate approach to traditional resistance breeding methods has been utilised since 1980, in parallel with the progress in plant biotechnology (Svabova and Lebeda, 2005).

In order to design a suitable method for early screening and selection for disease resistance a basic understanding of the biology of the causative agent and its relationship with the host plant is highly essential (Russell, 1978). There are several methodological approaches available for detecting resistant genotypes and selecting plants with enhanced resistance, among which *in vitro* screening is one of the most high-throughput and efficient methods (Svabova and Lebeda., 2005). *In vitro* culture techniques are currently being employed in the production of disease-free plants, rapid multiplication of rare plant genotypes, plant genome transformation, and production of plant-derived metabolites of significant commercial value (Espinosa *et al.*, 2018). These techniques can be classified based on their purpose (screening for stress tolerance, biochemical studies of host-pathogen interactions, selection of resistant/tolerant lines) and the type of selection agent (pathogen, modified pathogen, culture filtrate, phytotoxin/pathotoxin, elicitor) when used in disease resistance selection.

An ideal model of *in vitro* disease resistance selection should include: (1) an explant culture with a high ability to regenerate resistant/tolerant plants; and (2) an easily obtained selection agent that trigger reactions similar to the responses of the plant to the pathogen. It is necessary to develop a suitable technique for *in vitro* cultivation of the desired explant. The principles and strategies of these techniques along with the basic methods for aseptic cultures of plant organ or plant tissues have been described in detail by Bhojwani and Razdan, (1983). In general, whole plant or various parts of the plant grown under aseptic conditions can be subjected to the

selection pressure of a suitable agent *in vitro*, for developing disease resistance. Thus cultures of excised organs and/or isolated cotyledons, fruits, leaves, stems, shoots and roots were often used for *in vitro* resistance screening in crop-pathogen interactions. Callus cultures (Storti *et al.*, 1992; Fernandez *et al.*, 2000; Rao and Ramgoapl, 2010; Zhang *et al.*, 2012), shoot cultures, cell suspension cultures (Kramer *et al.*, 1988; Ishida and Kumashiro, 1988; Koike and Nanbu, 1997; Pedras and Biesenthal, 2000; Mishra *et al.*, 2021) protoplast cultures (Sjodin and Glimelius, 1989; Koike *et al.*, 1993), anther and pollen cultures (Ye *et al.*, 1987) fall under frequently used cultures in *in vitro* screening techniques. Various selection agents include natural pathogen isolates (Rines and Luke, 1985; Chawla and Wenzel, 1987; Vidhyasekaran *et al.*, 1990; Hammerschlag, 1990; modified fungal cultures/culture filtrate (Cvikrova *et al.*, 1992; Jayasankar and Litz, 1998; Chen and Swart, 2002; Singh *et al.*, 2003; Yusnita *et al.*, 2005; Rao and Ramgoapl, 2010; Savita *et al.*, 2011), phytotoxin (Fuson and Pratt, 1988; Rines *et al.*, 1985; Chand *et al.*, 2008), partially purified toxins (Jayasankar *et al.*, 1999; Mezzetti *et al.*, 1994), toxic extracts and fungal cell-wall components, so-called elicitors of resistance response (Scala *et al*, 1985; Storti *et al.*, 1992; Koike and Nanbu, 1997; Biton *et al.*, 2001; Porat *et al.*, 2003).

Before employing a selection agent in disease resistance screening test, a preliminary experiment needs to be demonstrated, where a suitable concentration range allows for a comparison of the toxic effects on susceptible and tolerant germplasm. This is done to figure out the precise dosage of selection agent that is optimal for screening resistant material. The impact of the selection agent on cultures can be assessed using various parameters such as inhibition of culture growth, reduced callus growth and cell viability, percentage of regenerating or necrotic explants, colour or morphological changes evaluated using computer image analyses, cell viability measurement by colorimetric assays (Kodama *et al.*, 1991; Hollmann *et al.*, 2002; Ramulifho *et al.*, 2019), and staining of polysaccharides, callose, polygalacturonases and β-glucanases (Storti *et al.*, 1992; Simoni *et al.*, 1995; Li *et al.*, 1999).

With the advent of the production and extraction of fungal exudates accompanied by the progressive toxin research, many studies demonstrated the role of various fungal toxins in pathogenesis. As a result, during the last three decades,

researchers have investigated the effect of various selection agents on *in vitro* cultures of host plants, as well as applications of agents in regeneration systems, resulting in lines with increased variability in disease resistance or susceptibility. The feasibility of *in vitro* selection was first demonstrated by Carlson, (1973) who obtained tobacco plants from callus cultures which were insensitive to the toxin of *Pseudomonas syringae* pv *tabaci.* Since then, many researchers have extrapolated this technique for the generation of disease resistant varieties. Generation of disease resistant plants by *in vitro* selection using appropriate selective agents has been rarely applied to woody species. Neverthless, successful results for selection of resistant lines have been well reported in fruit crops such as papaya against *Phytophthora* wilt (Sharma and Skidmore,1988), guava against wilt (Vos *et al.*, 1988; Bajpai *et al.*, 2005), lemon against mal secco disease (Gentile *et al.*, 1992), banana against black Sigatoka disease (Okole and Schulz, 1997), peach against *Xanthomonas* (Hammerschlag, 1988), mango against anthracnose (Jayasankar *et al.*, 1999), apple against black spot (Raman and Goodwin, 2000), strawberry against wilt (Orlando *et al.*, 1997), abaca against fusarium wilt (Purwati and Harran, 2007) and citrus against *Phytophthora parasitica* (Savita *et al.*, 2011). Many other investigators have apparently obtained successful selections in other several systems such as alfalfa (Arcioni *et al.*, 1987); maize (Gengenbach *et al.*, 1977); soybean (Song *et al.*, 1994); bean (Fernandez *et al.*, 2000) and potato (Behnke, 1979) where crude culture filtrate was used as the selection agent. Svabova and Lebeda, (2005) investigated and comprehensively reviewed the potential role of *in vitro* selection methods in screening and selecting various crops for improved disease tolerance. The effect of various selection agents extracted from about 40 plant pathogens on 30 plant species was covered in this review. Thus with all these insights in the past few decades, *in vitro* selection strategy has been developed as an alternative for conventional breeding approach to accelerate the development of disease resistant plants.

Plant tissue culture experiments incorporating fungal toxin or crude culture filtrate represents a useful technique for studying plant-pathogen interactions (Daub, 1986; Van Den Bulk, 1991). Furthermore use of fungal toxins as screening agent is considered as an indirect procedure for assessing disease resistance wherein the sensitivity of the concerned plants to fungal exudates was tested rather than to the

fungus itself and so this method proved to be advantageous by eliminating the risk of introducing new fungal strains into environment. The symptom diversity attributed to *C.cassiicola* under different environmental conditions, sporadic occurrence of the disease and the possible confusion with some other leaf diseases are some of the bottlenecks limiting early disease identification and scoring under natural field conditions in *Hevea*. Also the studies that assessed the resistance/susceptibility of rubber clones in fields and nurseries (Liyanage *et al.*, 1986; Manju *et al.*, 2010) were of course confined to smaller localities, thus making extrapolations uncertain. All of these limitations could be successfully mitigated while employing *in vitro* screening approach for the selection and screening of disease resistant varieties.

The toxin cassiicolin secreted by the pathogen *Corynespora cassiicola* is believed to be responsible for the incidence of Corynespora leaf fall disease. Cassiicolin is a glycosylated cysteine rich small secreted protein of 27 amino acids that was discovered by Onesirosan *et al.*, (1975), then purified and characterised by De Lamotte *et al.*, (2007). A study incorporating this toxin was conducted on *Hevea* clones for the first time by Breton *et al.*, in 2000. Accordingly, fifty one *Hevea* clones were screened for their sensitivity to the purified cassiicolin toxin, obtained from an aggressive isolate of *C.cassicola* from Philippines (CCP), using leaf wilt assay. Results of this assay were later compared with the susceptibility results of the same *Hevea* clones inoculated with CCP conidial suspension. The profiles obtained in both cases were in close correlation showing that cassiicolin was essential for pathogenicity and can be considered as the primary determinant to *Corynespora cassiicola* pathogenesis (Breton *et al.*, 2000); but laboratory assessment and field observations seem to provide different rankings of the cultivated clones. It is still difficult to propose tolerant clones with a good level of confidence, and the need to choose tolerant clones strongly reduces the diversity of the clones that can possibly be used in affected areas.

In vitro selection involves regeneration of plantlets in the culture media amended with varying concentrations of the selection agent and screening of regenerated plantlets for resistance to disease. Thus during the last few decades, cells with desirable characteristics have been selected from *in vitro* cultures by

incorporating fungal toxins or culture filtrates as a selection agent in the tissue culture medium, resulting in the identification of somaclonal variants.

.10. Plant defense mechanisms

Plants, as sessile organism, are often encountered by a vast variety of microorganisms over the course of their lives. These interactions of plant with the microorganisms can be either beneficial or detrimental, resulting in mutualistic or pathogenic interactions, respectively. The pathogenic interaction with one or more of plant's key physiological or biochemical systems result in disease conditions or symptoms. Eventhough a variety of management strategies are available for effective disease management, most of them have their own limiting factors making them less acceptable than they would otherwise be. That is, pesticides provide effective protection against these diseases, but their potential application may be compromised by negative environmental impacts and by the emergence of resistant pathogen strains. Chemical controls are often beyond what farmers in developing countries can afford. Because of all these reasons, much effort has been put to understand the innate resistance mechanism of plants.

Plants have evolved diverse array of defense mechanisms so as to achieve resistance against pathogenic infections.The activation of defense responses in plants is initiated by host recognition of pathogen-encoded molecules called elicitors (e.g., microbial proteins, small peptides, and oligosaccharides etc) (Yang *et al.*, 1997). The hypersensitive reactions of a plant to a pathogen are one of the most efficient and effective defense mechanisms in nature (Stintzi *et al*:, 1993). In hypersensitive response, cells immediately surrounding the infection site die rapidly, depriving the pathogen of nutrients and thus blocking its spread (Taiz and Zeiger, 2002). Or in other words, after a successful hypersensitive response, an intrinsic senescence program called programmed cell death (PCD) is activated by individual cells leaving a small region of dead tissue at the site of the attempted invasion (Gilchrist, 1998) sparing the rest of the plant from infection. This response is connected with a coordinated and integrated set of alterations that aid in preventing further pathogen invasion as well as strengthening the host's ability to restrict subsequent infection by other types of pathogens. Certain plant species respond to fungal or bacterial invasion by

producing lignin or callose (Hernandez-Blanco *et al.*, 2007; Chowdhury *et al.*, 2014) which causes cell wall rigidification and serves as a barrier against pathogen attack. A novel, rapid and related stress response is the cell wall protein modification. After treatment of soyabean plant cells with fungal elicitor or glutathione, certain preexisting (hydroxy) proline rich structural proteins in the cell wall become insolubilized by H_2O_2 mediated oxidative cross-linking (Bradley *et al.*, 1992).

Production of phytoalexin (Darvill and Albersheim, 1984; Ebel, 1986) is perhaps another best studied plant response to bacterial or fungal invasion. Phytoalexins are low molecular weight antimicrobial metabolites which are chemically diverse. Different plant families, however, produce different types of secondary products as phytoalexins. Isoflavonoids, for example, are common phytoalexins in the legume family, whereas various sesquiterpenes are produced as phytoalexins in plants of the potato family (*Solanaceae*), such as potato, tobacco, and tomato.

An array of hydrolytic enzymes collectively named as pathogenesis-related (PR) proteins, which target the pathogen's cell wall (Caruso *et al.*, 1996; Datta *et al.*, 2001; Jain and Khurana, 2018) is triggered by fungal invasion. PR's have been defined as 'proteins encoded by the host plant but induced only in pathological or related situations' (Antoniw *et al.*, 1980). Pathogenesis-related (PR) proteins were detected for the first time in tobacco leaves in early 1970's after infection with *Tobacco Mosaic Virus* (Gianinazzi *et al*, 1970; Van Loon and Van Kammen, 1970). Among various PR proteins chitinases and β-1, 3 glucanases are the two important hydrolytic enzymes that are abundant in many plant species and are induced as the first line of defense against pathogens. Both these enzymes play a major role in defense against fungal pathogens by degrading their prime cell wall components which are generally chitin and β-1,3 glucans (Sharma, 2013). The presence of chitinase was described for the first time in 1911 by Bernard who found a thermosensitive and diffusible antifungal factor in orchid bulbs. Since then, upon fungal infection, enhanced levels of chitinases could be observed in many plants such as pea (Mauch *et al.*, 1988); grapes (Derckel *et al.*, 1998); tobacco (Yun *et al.*, 1996); rice (Velazhahan *et al.*, 2000); chick pea (Giri *et al.*, 1998) etc. Several studies have also shown that when plants are attacked by phytopathogens, chitinase expression and

induction are higher in resistant varieties in comparison to susceptible varieties. Sugar beet (Nielsen *et al.*, 1993), wheat (Anguelova *et al.*, 2001), and tomato (Lawrence *et al.*, 2000) are among some examples.

2.10.1. Reactive oxygen species and response of antioxidants as ROS scavengers

Hypersensitive response is often preceded by generation of reactive oxygen species (ROS) (Vanacker *et al.*, 2000; Gessler *et al.*, 2007). Reactive oxygen species are highly unstable molecule initially recognised as normal cellular metabolic by product. In recent years, it has become apparent that ROS serves as cell signalling molecules for regular biological processes such as growth, development and especially response to biotic and abiotic environmental stimuli. The major members of ROS family include a number of reactive molecules and free radicals formed by the reduction of molecular oxygen, including the superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (•OH), as well as non radical molecules like singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2)and so forth. The hydroxyl radical is the strongest oxidant of these active oxygen species and can initiate radical chain reactions with a range of organic molecules (Lamb and Dixon, 1997). In plants, different cellular compartments such as chloroplast, mitochondria, and peroxisomes serves as the primary sites of ROS production, whereas endoplasmic reticulum, cell membrane, cell wall, and apoplast serves as the secondary sites (Das *et al.*, 2014). Increased ROS production under environmental stress conditions threatens cells by lipid peroxidation, protein oxidation, nucleic acid damage, enzyme inhibition, and activation of the programmed cell death (PCD) pathway, eventually leading to cell death (Sharma and Dubey, 2005; Maheshwari and Dubey, 2009; Srivastava and Dubey, 2011; Meriga *et al.*, 2004). Inspite of their destructive nature, ROS are well-described as key signaling molecules in a variety of cellular processes including tolerance to environmental stresses (Neill *et al.*, 2002; Yan *et al.*, 2007). The delicate balance between ROS production and scavenging determines whether ROS will act as a damaging or signalling molecule. Because of the multifunctional roles of ROS, it is more important for cells to precisely manage ROS levels in order to minimise oxidative harm, than just entirely eliminating them. So to maintain an adequate balance between the production and detoxification of ROS, plants have evolved an efficient antioxidant machinery (Mittler, 2002) comprising of two components, the non enzymic as well as enzymic antioxidants. The

enzymatic antioxidants include super oxide dismutase (SOD), catalase (CAT), peroxidase (POX), enzymes of ascorbate glutahione (AsA-GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). Nonenzymic antioxidants in the cell include ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics (Sharma *et al.*, 2012). These two components work collaboratively to scavenge ROS. In several plant-microbe interactions, an increase in the antioxidant activity was seen during infection, which should be assumed to be associated with the elimination of ROS. Several researchers have demonstrated enhanced activity of numerous enzymes of the antioxidant defense system in plants to resist the oxidative stress caused by diverse environmental conditions. (Zaefyzadeh *et al.*, 2009).

Environmental stimuli such as UV, pathogen attack, and gravity can induce rapid changes in hydrogen peroxide (H_2O_2) levels, leading to a variety of physiological responses in plants (Heinze and Gerhardt, 2002). Catalase, the first antioxidant enzyme to be discovered and characterised is the major H_2O_2-scavenging enzyme ubiquitously present in all aerobic cells (plants, animals and microbes). It is a tetrameric haeme containing metalloenzyme that functions as a cellular sink for hydrogen peroxide thereby detoxifying it into water and oxygen. Plants have a number of H_2O_2-degrading enzymes, including catalases, which are unique in that they do not require a cellular reducing equivalent to function. Despite the fact that they are found in all major sites of H_2O_2 production in the cellular environment, such as peroxisomes, mitochondria, cytosol, and chloroplast of higher plants (Sharma and Ahmad, 2014) peroxisomes are regarded as the key reservoirs of catalases. H_2O_2 is a reactive molecule that is produced under almost all stress conditions. When cells are stressed for energy and are rapidly generating H_2O_2 through catabolic processes, this H_2O_2 is degraded by catalase in an energy-efficient manner (Mallick and Mohn, 2000). Though it is evident that there is a general rise in activities of various antioxidant enzymes and non-enzymatic components following exposure to various environmental stresses, yet a declination is noted in some antioxidant responses depending on the intensity, duration, and type of the stress (Sharma and Dubey, 2005; Han *et al.*, 2009; Moussa and Abdel-Aziz, 2008). Likewise, catalase activity is either

enhanced or depleted during plant pathogen interactions. In maize plants, infection with *Aspergillus flavus* demonstrated a significant increase in catalase activity in the resistant lines than the susceptible lines (Magbanua *et al.*, 2007). Cassava plant leaves showed increased catalase activity in response to attempted infection by African cassava mosaic virus when compared to uninfected leaves. (Amoako *et al.*, 2015). Transgenic tobacco plants with ~10% wild-type catalase activity under elevated light stress accumulated oxidized glutathione and a 4-fold decrease in ascorbate, indicating that catalase is critical for maintaining the redox balance during oxidative stress. Also stress analysis revealed increased susceptibility of these catalase-deficient plants to paraquat, salt and ozone, but not to chilling (Willekens, 1997). Seed-specific overexpression of antioxidant genes (Mn-superoxide dismutase, catalase, and homogentisate phytyl transferase) in Arabidopsis enhances oxidative stress tolerance during germination and early seedling growth (Xi *et al.*, 2010).

Peroxidase, glutamate dehydrogenase and glucose-6-phosphate dehydrogenase are other enzymes that are activated in plants in response to various environmental stresses (Lee *et al.*, 1976; Van Assche *et al.*, 1988; Weigel and Jager, 1980). Peroxidases are ferric heme-containing monomeric glycoproteins that catalyse the oxidation of a substrate by using hydrogen peroxide or organic peroxide. They exist as isoenzymes in different plant species, and each isoenzyme has different amino acid sequences and expression profiles, implying that they are involved in different physiological processes. Papaiah and Narasimha, (2014) measured peroxidase and polyphenol oxidase activities in healthy and virally infected sunflower (*Helianthus annuus* L.) leaves, and found that virus-infected sunflower leaves had higher peroxidase activity than healthy sunflower plants.

2.10.2. Electrolyte leakage

Another hallmark of stress response in intact plant cells is electrolyte leakage (Demidchik *et al.*, 2014) where electrolytes leak out of the cell into the surrounding tissues. Electrolyte leakage is observed very instantly following the introduction of a stress element and lasts for a few minutes to several hours. A simple way to quantify such electrolytes leaked from a tissue is to measure the increase in electrolytic conductivity of water that contains the tissue with dying cells (Hatsugai and Katagiri,

2018). Electrolyte leakage bioassays are quick and easy to perform, requiring only a conductivity metre as the only machine or equipment. The pioneering studies on electrolyte leakage measurements were carried out by Dexter *et al* in 1932. According to his hypothesis, the degree of injury from controlled freezing treatments might be determined by the extension of measurement of exosmosis of electrolytes and other materials. The outward diffusion of such electrolytes can readily be estimated by conductivity measurements. Later studies suggested that ion leakage has been employed as an efficient parameter for monitoring the damage that affects the integrity of membranes, the consequences of which may be identified quickly by measuring the increase in electrolyte conductivity in the solution in which the tissues were immersed. (Duke and Kenyon, 1993). These findings were supported by Lee and Zhu, (2010) who reported that electrolyte leakage can be used as a parameter to evaluate plant tolerance to salt, drought, or freezing stress by measuring the degree of cell damage after stress exposure. Besides this it also served as an additional index to identify disease resistant varieties (Mohanraj *et al.*, 2003a).

The phenomenon of ion leakage is found to be triggered by various abiotic stress responses such as salt stress (Nassery, 1975 ; Shabala *et al.*, 2006; Sreenivasulu *et al.*, 2000), heavy metals (De and Mukherjee, 1996; Murphy and Taiz, 1997), low (Coursolle *et al.*, 2000; Vainola and Repo, 2000) and high temperatures (Ismail and Hall, 1999; Saelim and Zwiazek, 2000), drought (Blum and Ebercon, 1981; Shcherbakova and Kacperska, 1983) and even in response to biotic stresses such as pathogen attack (Atkinson *et al.*, 1985; Blatt *et al.*, 1999). It was demonstrated in subsequent studies that electrolyte leakage measurements are linked with a variety of physiological and biochemical parameters, with the accumulation of reactive oxygen species being the most consistent process accompanying it (Demidchik *et al.*, 2010). Accumulated evidence also shows that electrolyte leakage is mainly related to K^+ efflux from plant cells, which is mediated by plasma membrane cation conductances. The efflux of K^+ and so-called counter ions (Cl^-, HPO_4^{2-}, NO_3^-, $citrate^{3-}$, $malate^{2-}$) that move to balance the outflow of positively charged potassium ions causes specific alterations in membrane semi-permeability (Demidchik *et al.*, 2014). Potassium is not replaceable in cytoplasmic functions and the plant most likely needs to maintain the cytoplasmic concentration of K^+ in the range of 100-200 mM (Leigh and Wyn Jones,

1984). But some stresses induce a dramatic loss of K^+ thereby dropping this cytoplasmic concentration to around 10-30 mM.

Electrolyte leakage was found to be significantly increased in all infected leaf tissues when compared to healthy tissues in all plant infections. This is evident in the studies of Dewir *et al.*, (2015), where Phytoplasma infected stem tissues of *Euphorbia coerulescens* and *Orbea gigantean* showed significant increase in leakage of electrolytes as compared with healthy tissues. Similar to plant pathogens, phytotoxins can also induce disease reactions accompanying electrolyte leakage. The red rot phytotoxin caused increased electrolytic leakage on leaf tissues in susceptible sugarcane varieties and higher levels of phytoalexins in resistant sugarcane varieties. The findings of this study also suggested that the phytotoxin induced changes and disease reaction could possibly be used as an additional index to rapidly identify red-rot resistance in sugarcane varieties (Mohanraj *et al.*, 2003b). Exposure of wheat leaves (line ND495) to *Pyrenophora tritici-repentis* (Ptr) toxin showed enhanced electrolyte leakage depending upon the toxin concentration and exposure time. Furthermore, all toxin-sensitive wheat varieties showed increased electrolyte leakage, and in contrast, the insensitive ones did not show any increase in the leakage of electrolytes (Kwon *et al.*, 1996).

2.11. Genetic transformation

The advent of a variety of genetic engineering techniques has made it possible to incorporate foreign genetic information into plant genome. Plant genetic transformation is the introduction of foreign DNA into target plant genome and regeneration of the complete plant from the resulting transformed cells. The advancements of plant transformation technology over the last two decades has enabled the creation of numerous transgenic crops, some of which have been released for commercial use across the world. Eventhough several methods such as particle bombardment, electroporation, PEG mediated transformation are used for genetic engineering of plants *Agrobacterium*-mediated DNA transfer using the Ti plasmid of *Agrobacterium tumefaciens* is the most popular as well as efficient means of plant genetic transformation.

Agrobacterium tumefaciens is a soil phytopathogen that naturally infects plant wound sites and causes crown gall disease via delivery of transferred (T)-DNA from bacterial cells into host plant cells (Hwang *et al.*, 2017). When *Agrobacterium* infects a plant cell, it transfers a small segment of its own DNA termed T DNA, present on Ti plasmid into the plant cell nucleus, where it is eventually integrated into the plant genome. The T-DNA genes code for enzymes synthesizing opines and phytohormones which serve as a source of carbon and energy for the bacterium. To exploit this natural system in genetic engineering, Ti plasmids must be disarmed, which entails removing all genes from T DNA, rendering the strain non-oncogenic, and replacing them with the desired gene (Hellens *et al.*, 2000). A large number of plant species have been genetically modified using this amazing technique. In most cases, the process requires incubating the target tissue with an *A.tumefaciens* strain that has been previously disarmed and has a Ti plasmid containing the gene of interest to be transferred. After gene transfer, plants are generated from the tissue that has come into close contact with *Agrobacterium.* Vectors employed in plant transformation have distinct characteristics. For an effective transformation to happen, the important requirements that the vector used should possess are multiple cloning site (MCS) for the insertion of the gene of interest, presence of selectable markers to identify and select the transformed plant cells, as well as an appropriate promoter and terminator to make sure that the expression of the desired gene occurs in plant. CaMV, 35S and nos are examples of constitutive and non-specific promoters that have been widely used.

Genetic engineering appears to be a feasible option for *Hevea* genome improvement. The feasibility of genetic transformation in rubber trees was first investigated in 1991, when a strain of *Agrobacterium tumefaciens* was used to produce tumor tissues on the stems of *in vitro* and *in vivo* propagated rubber seedlings (Arokiaraj and Rahaman, 1991). To date, the simplest and most effective method for genetic transformation of *Hevea* has been *Agrobacterium tumefaciens* (Montoro *et al.*, 2000; Coomber *et al.*, 1996; Sobha *et al.*, 2003; Jayashree *et al.*, 2003). A reliable method for regenerating an entire plant from a transformed cell is a basic requirement for all genetic manipulation systems. Transformation is usually possible with explants that produce actively dividing cells. Seedling explants, shoot apices, embryos, cell

suspensions, anthers and young seedling leaves could all be used to make these. The most suitable target tissue for transformation studies in *Hevea* appears to be dedifferentiated calli (diploid) obtained from anthers that are 38 to 41 days old (Arokiaraj, 2000).

The *Agrobacterium*-mediated transformation of *Hevea brasiliensis* was started by testing the virulence of several wild-type strains of *Agrobacterium tumefaciens*. As a result, different responses were found among various *Agrobacterium* strains used on *Hevea* (Arokiaraj, unpublished). So far, EHA101, EHA105, LBA 4404, pGV2260, and pGV3850 *Agrobacterium* strains have been used for *Hevea* transformation. These strains have been shown to be highly efficient for genetic transformation in *Hevea* (Coomber *et al.*, 1996; Arokiaraj *et al.*, 1998; Sobha *et al.*, 2003; Jayashree *et al.*, 2003; Jayashree *et al.*, 2013; Kala *et al.*, 2014). Marker genes (*gus* and *npt II*) have successfully been integrated into the genome of regenerated plantlets from transformed cells in *Hevea* using the *Agrobacterium*-mediated transformation system (Arokiaraj *et al.*, 1998) or by using particle gun method (Arokiaraj *et al.*, 1994). The resulting transgenic rubber plants express the desired protein, and these plants can be vegetatively propagated using the budding system in large numbers. The transgene for the desired trait will then be carried by the propagated buddings, shortening the lengthy process of traditional breeding techniques. From this it is made clear that successful *Hevea* transformation by using genes of agronomic importance can improve the crop quality and plant characteristics as well as the recombinant protein production in the laticiferous system for diagnostic and therapeutic purposes (Yeang *et al.*, 1998; Sunderasan and Shuib, 2017).

Because of the importance of the *hmgr* gene in rubber biosynthesis, attempts were made in the late twentieth century to overexpress a member of the *hmgr* gene (*hmg1*) in *Hevea* by means of transformation. As a result of this, the HMGR activities of transformed *Hevea* anther callus ranged from 70 to 410 percent of wild type control, while activity in transformed embryoids was between 250 and 300 percent (Arokiaraj *et al.*, 1996). But unfortunately they were unable to produce transgenic plantlets from the transformed embryoids. Superoxide dismutase (SOD) was another gene of interest studied because it could improve plant cell tolerance to oxidative stress by scavenging free radicals. The genetic transformation of *Hevea* anther calli

using the *Hevea* Mn-SOD (*HbSOD*) gene construct under the control of the CaMV 35S promoter yielded a transformation frequency of 4% (Jayashree *et al.*, 2003). Simultaneously Sobha *et al.*, (2003) carried out genetic modification of *Hevea* anther calli using the *Hevea* Mn-SOD (*HbSOD*) gene construct controlled by the FMV 34S promoter where they got transformation frequency of about 50%. The physiological performance of one year old bud grafted MnSOD transgenic plants developed by Jayashree *et al* was evaluated in a dry sub humid environment by withholding irrigation and then rewatering them.Transgenic plants outperformed non-transformed controls in terms of water relations and photosynthetic parameters during drought and drought recovery period (Sumesh *et al.*, 2014). These genetically modified (GM) rubber plants were recently field planted on the outskirts of Guwahati, Assam to check their stress tolerance potential under actual field conditions. Genetic transformation of *Hevea* to incorporate *ipt* gene was attempted by Kala *et al.*, (2003). However, the embryos displayed developmental abnormalities, and the majority of the transformants were severely deformed.

Leclercq *et al.*, (2012) created transgenic *Hevea* plant lines that overexpressed *Hevea brasiliensis*' cytosolic CuZnSOD gene (*HbCuZnSOD*). After subjecting the plants to water deficit treatments, they investigated the physiological parameters associated with drought tolerance. Lower stomatal conductance and proline content were observed in transgenic lines. Overexpression of the *HbCuZnSOD* gene, as well as activation of all ROS-scavenging enzymes, suggested that the transgenic line was more effective at ROS protection. Osmotin is another stress-responsive multifunctional protein that provides plant with osmotolerance (Husaini and Abdin, 2008). Rekha *et al.*, (2013) attempted to transfer the gene encoding tobacco osmotin (*Tb osm*) to *Hevea* anther callus tissues *via Agrobacterium*-mediated gene transfer, yielding a transformation frequency of 48%. Later, the same tobacco osmotin gene was introduced into *Hevea* embryogenic calli induced from immature zygotic embryos in order to achieve abiotic stress tolerance and could achieve a transformation frequency of around 44.8 percent (Rekha *et al.*, 2014). *In vitro* stress tolerance studies with these transgenic *Hevea* callus lines revealed positive signs of drought tolerance, as indicated by increased proline accumulation under water stress and active callus proliferation under salinity stress (Rekha *et al.*, 2016). The

stacking of two genes, manganese superoxide dismutase (MnSOD) for abiotic stress tolerance and 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgrI*) for enhanced latex yield, was also achieved by repeated genetic transformation (Sobha *et al.*, 2014). Recently, efforts were made to introduce the sorbitol-6-phosphate dehydrogenase gene, which was isolated from apples, into the *Hevea* genome in order to confer drought tolerance. Sorbitol functions as a compatible solute in conditions of decreasing water, an anti-freezing agent for chilling, and a free radical scavenger. The target tissue for this *Agrobacterium*-mediated gene transformation was embryogenic callus derived from immature anther and plantlet regeneration was also achieved (Jayasree *et al.*, 2015).

In the current Indian context, fungal infections are a serious concern affecting the total rubber cultivation. Conventional procedures suffer from a variety of constraints. Hence genetic engineering appears to be a feasible option. Among the various natural disease resistant genes identified and isolated so far, PR (pathogenesis related) genes stand out as truly beneficial genes against fungal pathogens due to their particular ability to inhibit fungal cell wall synthesis. Significant progress has been achieved in increasing oxidative stress-induced tolerance in agricultural crops by generating transgenic lines with varying amounts of PR proteins such as chitinase and β-1, 3-glucanase. Transformation of a winter-type oilseed rape (*Brassica napus*) with a hybrid *endochitinase* gene resulted in transgenic plants that showed increased tolerance to three different fungal diseases (*Cylindrosporium concentricum*, *Phoma lingam*, and *Sclerotinia sclerotiorum*) (Grison *et al.*, 1996). The expression of the tobacco *chitinase* gene in peanut genome enhanced the resistance of these plants to the fungal pathogen *Cercospora arachidicola*, the causative organism of the peanut leaf spot or Tikka disease (Rohini and Rao, 2001). In greenhouse trials, transgenic tobacco plants expressing high amounts of the *Serratia marcescens chitinase* gene (*chi A*) demonstrated substantial resistance to fungal infection caused by *Rhizoctonia solani* (Dunsmuir *et al.*, 1993). Transgenic plants with increased fungal tolerance have been produced by integrating chitinase gene in the genome of a variety of plants (Antony and Ignacimuthu, 2012). Saiprasad *et al.*, (2009) developed transgenic tobacco plants expressing *Trichoderma harzianum endochitinase* encoding gene which conferred antifungal activity on the growth of fungus *A.alternata*. The same

Trichoderma harzianum endochitinase gene construct was discovered to be effective against *Alternaria porri* induced onion purple blotch (Mythili *et al.*, 2018).

From all these studies it can be inferred that transgenic plants harboring genes for broad spectrum resistance against fungal pathogens could be an economically viable strategy for reducing crop damage caused by these pathogens. Increased expression of *chitinase* gene has been shown to significantly improve plant defense against a wide range of pathogens. Therefore this defense responsible gene is widely used to produce fungal disease resistant transgenic plants. This can be done by overexpressing this gene in host plant by means of transformation.

Chapter 3

In vitro screening as an early detection tool for sensitivity towards Corynespora leaf fall disease in *Hevea brasiliensis*

3.1. Introduction

Hevea brasiliensis recognized as the sole commercial source of natural rubber is a recently domesticated perennial, tropical tree crop. As with any other agricultural crop, incidence of diseases increased with domestication and so far more than hundred pathogens have been identified as capable of attacking the rubber tree. In India, the adverse climatic conditions like cloudy weather, low temperature, very high humidity as well as more unpredictable rainfall patterns triggered the outbreak and rapid spread of many diseases. *Corynespora cassiicola* is a devastating fungal pathogen causing Corynespora leaf fall disease (CLFD) in *Hevea brasiliensis*. CLFD is listed as the fourth most serious leaf disease of rubber trees of South East Asia which occurs regularly during refoliation and its severity varies among different clones of *Hevea* (Manju *et al.*, 2014). Though the pathogen affects leaves of all stages, young leaves in the light green stage appear to be the most susceptible (Umoh and Fashoranti, 2018). Incidence of this disease may lead to a delay in maturation of young rubber trees and yield reduction of mature rubber trees (Ogbebor, 2010) which may even end up in plant death of susceptible clones.

Hevea clones that evolved in various rubber growing regions showed varying degrees of susceptibility to pathogenic diseases (Narayanan and Mydin, 2012). Mostly all clones in India are affected by CLFD (Mathew, 2006). Among the different clones studied by Manju *et al* in 2001, it was found that RRII 105, the most popular and high yielding clone, recorded the highest susceptibility towards CLFD, while GT 1 was the least susceptible, whereas the clones PB 217, PB 235 and PB 260 showed moderate infection to CLFD. Among RRII 400 series clones, RRII 414 and 430 were found to be comparatively less infected by the pathogen under the assistance of prophylactic fungicidal spray (Varghese *et al.*, 2009). Another in *vitro* study using conidial

suspension of *Corynespora cassiicola* proved the highly susceptible nature of clone PB 260 while the clone GT 1 was found to be resistant (Breton *et al.*, 1997).

In order to develop a suitable method for early screening and selection for disease resistance a basic knowledge about the biology of the causal agent and its relationship with the host plant is highly essential. The toxin cassiicolin secreted by the pathogen *Corynespora cassiicola* is believed to be responsible for the incidence of CLF disease (Breton *et al.*, 2000). Cassiicolin is a glycosylated cysteine rich small secreted protein of 27 amino acids that was discovered by Onesirosan *et al.*, (1975), then purified and characterised by De Lamotte *et al.*, (2007). In the case of *Hevea*, the symptom diversity attributed to *C.cassiicola* under different environmental conditions, sporadic occurrence of the disease and the possible confusion with some other leaf diseases are some of the bottlenecks limiting early disease identification and scoring under natural field conditions. Also the studies that assessed the resistance/ susceptibility of rubber clones in fields and nurseries (Liyanage *et al.*, 1986; Manju *et al.*, 2010) were of course confined to smaller localities thus making extrapolations uncertain.

With the advent of the extraction of fungal exudates accompanied by the progressive toxin research, many studies demonstrated the role of various fungal toxins in pathogenesis. Use of fungal toxins as screening agent is considered as an indirect procedure for assessing disease resistance wherein the sensitivity of the concerned plants to fungal exudates was tested rather than to the fungus itself and this method proved to be advantageous by eliminating the risk of introducing new fungal strains into environment. Such a study incorporating fungal toxin was conducted on *Hevea* clones for the first time by Breton *et al* in 2000. Accordingly fifty one *Hevea* clones were screened for their sensitivity to the purified cassiicolin toxin, obtained from an aggressive isolate of *C.cassicola* from Philippines (CCP), using leaf wilt assay. Results of this assay were later compared with the susceptibility results of same *Hevea* clones inoculated with CCP conidial suspension. The profiles obtained in both cases were in close correlation showing that cassiicolin was essential for pathogenicity and can be considered as the primary determinant to *Corynespora cassiicola* pathogenesis (Breton *et al.*, 2000).

Plant tissue culture experiments incorporating fungal toxin or crude culture filtrate (CCF) represents a useful technique for studying plant-pathogen interactions (Daub, 1986; Van Den Bulk, 1991). In these studies CCF was used as the selection agent for *in vitro* screening of cultures such as calli or cell suspension cultures of host plants. A number of investigators have obtained apparently successful selections in systems where CCF was used to select resistant material of soybean, alfafa and potato (Jin *et al.*, 1996; Hartman *et al.*, 1984; Behnke, 1979).

Primary objective of this study is to investigate the phytotoxic effect of CCF of *C.cassiicola* on leaf tissue as well as on *in vitro* generated calli from selected clones and to explore the possibility of employing it as a tool for early screening of *Corynespora* tolerance in potential pipeline clones and germplasm material. As a preliminary step towards this end, experiments were conducted to find out whether the *in vitro* cultures of *Hevea* clones show similar sensitivity towards *Corynespora cassiicola* as that observed under field conditions.

3.2. Materials and methods

3.2.1. Plant material

Eight prominent clones of *Hevea* of which four identified as susceptible and four as tolerant to *Corynespora cassiicola,* from previous studies and field reports, were selected for studying the effect of toxin under *in vitro* conditions. The selected clones were grouped as follows.

Susceptible clones – RRII 105, RRII 203, PB 217 & PB 260

Tolerant clones –RRII 414, RRII 430, GT 1 & FX 516

3.2.2. Preparation of CCF

The *C.cassiicola* used in this study was isolated from the diseased rubber tree leaves collected from the rubber plantations of RRII and purified by single conidium isolation. The mycelium was cultivated on PDA (Potato Dextrose Agar) (Fig. 3.1 a) in a 90 mm Petridish at 25°C in dark and were routinely transferred to a fresh medium every 4 weeks. For CCF production, 100 ml of modified Czapeck liquid medium (De Lamotte *et al.*, 2007) was inoculated with 3 fungal mycelial plugs (5 mm) from a 7 days old culture on PDA at 25°C and incubated without agitation at 25°C for 12 days

(Fig. 3.1 b & c). The fungal cultures on liquid media were filtered through Whatmann No.1 filter paper to remove spores and mycelia (Fig. 3.1 d & e). This CCF was used for further experiments.

3.2.3. Sensitivity test through leaf wilt bioassay using whole leaves

In this assay, six day old healthy leaflets were excised from all the clones belonging to both susceptible and tolerant groups. In each leaf, a fresh cut was made at the end of the petiole keeping it under water and the exuding latex was removed completely using a tissue paper. Immediately after this, one set of leaves from each group were exposed to the toxin by immersing the cut end of the leaves in McCartney bottles containing the CCF (1 ml of crude culture filtrate diluted with 4 ml sterilized water). The other set of leaves whose cut ends were immersed in 5ml sterile water in McCartney bottles served as control. All the samples were incubated at room temperature and extent of wilting was assessed at regular time intervals. Three replications were carried out with each sample.

3.2.4. Sensitivity test using leaf discs

Vacuum infiltration of leaf segments with CCF

Healthy immature leaves (six day old) of both susceptible and tolerant clones were collected and excised into small pieces which were then subjected to vacuum infiltration with 2 ml of CCF for 15 min (Fig. 3.1f). These leaf samples were transferred to moistened filter paper in Petri dishes and kept at room temperature. Leaf pieces vacuum infiltrated with autoclaved distilled water for 15 min served as the control. The effect of vacuum infiltration of the leaf segments with CCF was assessed through observation of the necrotic damage using a stereo microscope. Moreover the colour difference between necrotic and non necrotic tissue was made further clear using eye dropper tool, for which the samples were scanned with an HP Laser Jet 3052 Scanner and observed in Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). These observations were taken at different time intervals of 24, 36 and 48 hrs of the treatment. The experiment was performed with three replicates for each clone.

Measurement of electrolyte leakage of leaf discs

In this experiment six day old disease free leaves were collected from both susceptible and tolerant plants grown in the field. Circular leaf discs of size 1 cm diameter were then punched out, rinsed thoroughly with sterile distilled water followed by air drying. After this, five leaf discs from each clone were immersed in 2 ml of CCF followed by vacuum infiltration for 15 min. Another set of five leaf discs from each clone were subjected to vacuum infiltration by keeping in 2 ml of autoclaved distilled water and these served as the control. All these samples were kept at 25°C for 24 hrs, after which the leaf discs were washed quickly for three times with distilled water. Later each set of discs were soaked in 5 ml of autoclaved distilled water in separate glass tubes and kept for 12 hrs in the dark, for leaching of electrolytes. The content of each tube was shaken well and the initial electrical conductivity of both control and treated sets was measured as C1 and T1 respectively using conductivity meter. The leaf tissues were then killed by autoclaving for 15 min to release all ions from the tissues, cooled to 25°C after which the final electrical conductivity was measured as C2 and T2. The same experiment was repeated with two more treatment durations (48 and 72 hrs). In each case the percentage of electrolyte leakage (EL %) was calculated using Sullivans formula, % Injury = {1-[1-(T1/T2)/1-(C1/C2)]} x 100. Three replicates were included for this experiment. Statistical analysis (ANOVA) at the 5% level was performed for all parameters.

3.2.5. *In vitro* screening using callus cultures

Callus cultures were raised from all the eight clones under study, using immature inflorescence as the initial explant, according to the method already standardised (Sushamakumari *et al.*, 2000b). Fresh, proliferated calli were exposed to fungal exudates / toxin by transferring them to proliferation medium fortified with different volumes of filter sterilized CCF (0.5, 1.0, 2.0 and 3.0 ml/100 ml medium). Before transferring, these calli pieces were gently macerated for uniform exposure of the cells to the toxin. Calli inoculated on toxin free proliferation medium were used as control. The cultures were incubated under dark conditions at 25±2°C for three weeks in culture, then the effect of CCF was assessed through visual evaluation of various parameters such as percentage of necrosis, colour and morphological changes of the callus.

3.3. Results

Before performing an *in vitro* screening experiment for selecting disease resistant varieties, it is desirable to check whether the results of *in vitro* laboratory experiments are in agreement with the field observations regarding sensitivity to the particular disease. Accordingly, a total of eight clones belonging to two groups, susceptible and tolerant, with four clones in each group were selected and subjected to different Corynespora leaf fall disease sensitivity experiments. The results of sensitivity studies of these selected clones towards phytotoxic CCF have been found to be well in accordance with the already recorded field observations.

3.3.1. Leaf wilt bioassay using whole leaves

In this experiment the two groups, susceptible and tolerant clones, responded differently towards the treatment of CCF as evidenced in Fig. 3.2 which shows the wilting symptoms of these various clones at 24 hrs of treatment. Leaves of the susceptible clones RRII 105, RRII 203, PB 217 & PB 260 [Fig. 3. 2 (e-h)] immersed in the CCF showed wilting symptoms, within 24 hrs of treatment, characterized by browning and curling up of leaves, initially at the tip which then proceeded gradually towards the leaf base and complete wilting was noticed by about 48 hrs. On the contrary, the control set of leaves immersed in sterile distilled water remained intact without any wilting signs until 48 hrs. Leaves of the tolerant clones RRII 414, RRII 430, FX 516 and GT 1 [Fig. 3.2 (a-d)] showed no signs of wilting and remained intact until 48 hrs of treatment just similar to the control set of leaves belonging to this group.

3.3.2. Sensitivity test using leaf segments

In the vacuum infiltration experiment of leaf segments with CCF, the two groups responded differently in terms of the damage occurred due to the toxic effect of the CCF (Fig. 3.3). In the case of susceptible clones (RRII 105, RRII 203, PB 217 & PB 260), onset of necrotic symptoms started appearing by 24 hrs of treatment. Intensity of necrosis increased considerably with increase of exposure time, showing dark brown lesions followed by partial and complete collapse of tissue by 48 hrs of treatment. Among the members of the tolerant group, clones RRII 430, GT 1 and FX 516 remained green without any necrosis even after 48 hrs of CCF treatment,

indicating better inherent tolerance of these clones towards the toxin. In the case of RRII 414, slight necrosis was noticed at 24 hrs of treatment. However, there was only marginal increase in necrosis with increase in exposure time. Control samples of both the groups remained intact without any necrosis.

3.3.3. Quantification of plant cell injury by electrolyte leakage measurement

In this method, quantification of the impact of fungal exudates on detached leaves is carried out by measuring the induced electrolyte leakage (EL%). In the present experiment, the measured EL% values of the clones were found to be inversely proportional to the *Corynespora* tolerance of those clones. The susceptible clones showed much greater electrolyte leakage ($P<0.05\%$) than the tolerant ones (Fig. 3.4). The susceptible *Hevea* clones that developed necrosis in response to toxic exudates in the crude culture filtrate exhibited enhanced electrolyte leakage and the tolerant clones that failed to develop necrosis did not show that much electrolyte leakage. The maximum EL% value was observed with an incubation period of 48 hrs for all the clones tested. The lower incubation period of 24 hrs appeared insufficient, especially for tolerant clones such as FX 516 and GT 1. With a longer incubation period of 72 hrs, the EL% values were lower. The susceptible clone RRII 105 showed the maximum electrolyte leakage (64.68 %) in response to CCF treatment at 48 hrs incubation period, whereas the tolerant clone GT 1 showed the lowest electrolyte leakage (22.04 %) at the same incubation hours. Other clones PB 217, PB 260, RRII 203, RRII 414, RRII 430, FX 516 exhibited an EL% of 50.02%, 45.89%, 39.47%, 34.57%, 30.4% and 24.01% respectively at 48 hrs of incubation.

Our study involved eight clones and three time points, creating an eight-by-three matrix with a total of 24 different treatment combinations. In the variance analysis (ANOVA) applied to the data set, the effects due to the clone x treatment interaction were highly significant ($p=0.00018$). On comparing all possible pairs of treatments, significant difference in mean EL% values could be noticed among the clones categorized as tolerant or sensitive. The significance of clone x treatment effects was compared separately for each clone at different treatment durations using Duncan Multiple Range Test (DMRT) calculated on the basis of mean EL% values. Analysis of variance on the DMRT data showed that tolerant and susceptible clones

differed significantly with regard to electrolyte leakage in response to CCF treatment. In addition, these values have shown good coherence with the known susceptibility of clones. That is, the EL% response was found to be proportional to the already established field sensitivity ranking of tested clones.

Statistical Analysis

Data presented are means ± standard error for independent experiments. Two factor ANOVA followed by Duncan's Multiple Range Test ((DMRT), both carried out using SPSS (version 12.0) software packages, were used for expressing the statistical significance.

3.3.4. *In vitro* screening of callus against cassiicolin

Inclusion of CCF of *Corynespora cassiicola* in the culture medium adversely affected the survival and growth of calli, owing to the toxicity of the CCF. As the concentration of CCF in the medium increased, the inhibitory effect on the cultured calli also increased. It was also observed that sensitivity of calli developed from different clones under study was different towards the CCF contained in the medium, as evidenced by the per cent survival of calli from different clones (Fig. 3.5).

Calli derived from all the four susceptible clones showed dose dependent necrosis of calli. In these clones extensive cellular damage as indicated by brown discoloration followed by necrosis could be observed. Even the lowest level of CCF (0.5% (v/v)) was lethal to PB 217 and PB 260 as observed in [Fig. 3.5 (1 & 2)]. Calli raised from the clones RRII 105 & RRII 203, showed slight browning and necrosis at a CCF concentration of 1% (v/v), the degree of necrosis increasing gradually with increase in CCF level, as shown in [Fig.3.5 (3 & 4)]. Also no further growth of callus could be observed in these susceptible clones.

On the contrary, growth and survival of intact callus was observed in the tolerant group. Calli derived from these clones remained intact without browning even at a CCF level of 2% (v/v). Clones FX 516 & GT 1 proved to be the most tolerant among these four clones, being unaffected even at the highest level 3% (v/v) of the CCF [Fig.3.5 (7 & 8)]. Calli from clones 414 and 430 were not affected up to a level of 2% (v/v) above which a slight browning could be noticed [Fig.3.5 (5 & 6)]. Further

proliferation of the toxin exposed calli could be obtained from all the four clones. Control callus cultures belonging to both the groups were cultured over CCF free medium did not show any discolouration or necrosis.

Fig.3.1. ***Coynespora cassiicola*- pathogen isolation, culture filtrate preparation and vacuum infiltration a) *Corynespora cassiicola* colony on PDA medium after 10 days b) Czapeck liquid medium with *C.cassiicola* mycelial plugs c) Mycelial mat formation d) & e) Vacuum filtration of culture through Whatmann filter paper f) Vacuum infiltration of the leaf segments with the culture filtrate**

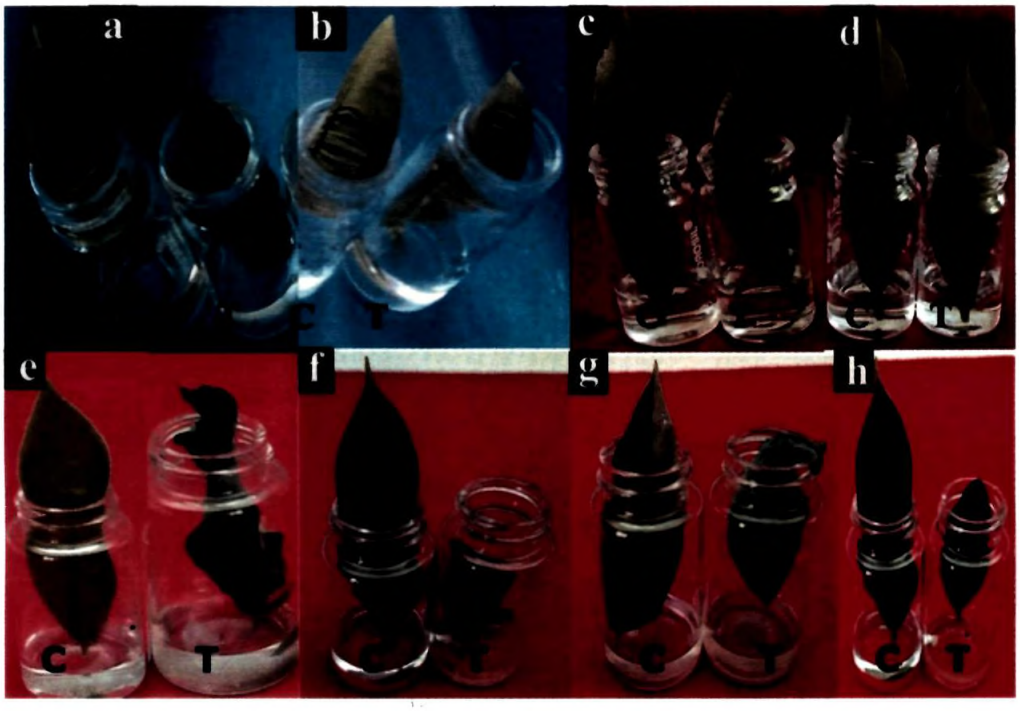


Fig.3.2. Leaf wilt bioassay of susceptible and tolerant clones: a) RRII 414 b) RRII 430 c) GT 1 d) FX 516 e) RRII 105 f) RRII 203 g) PB 217 h) PB 260. C- Control, T-Treated

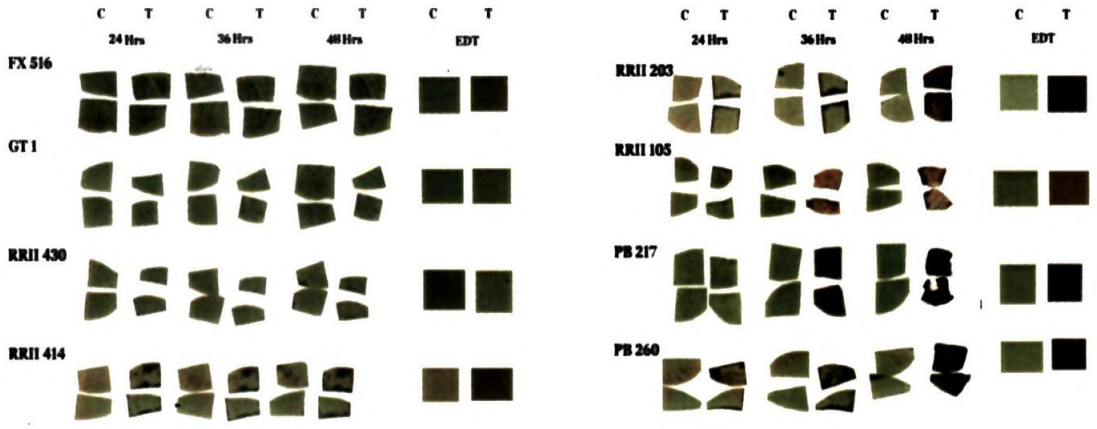
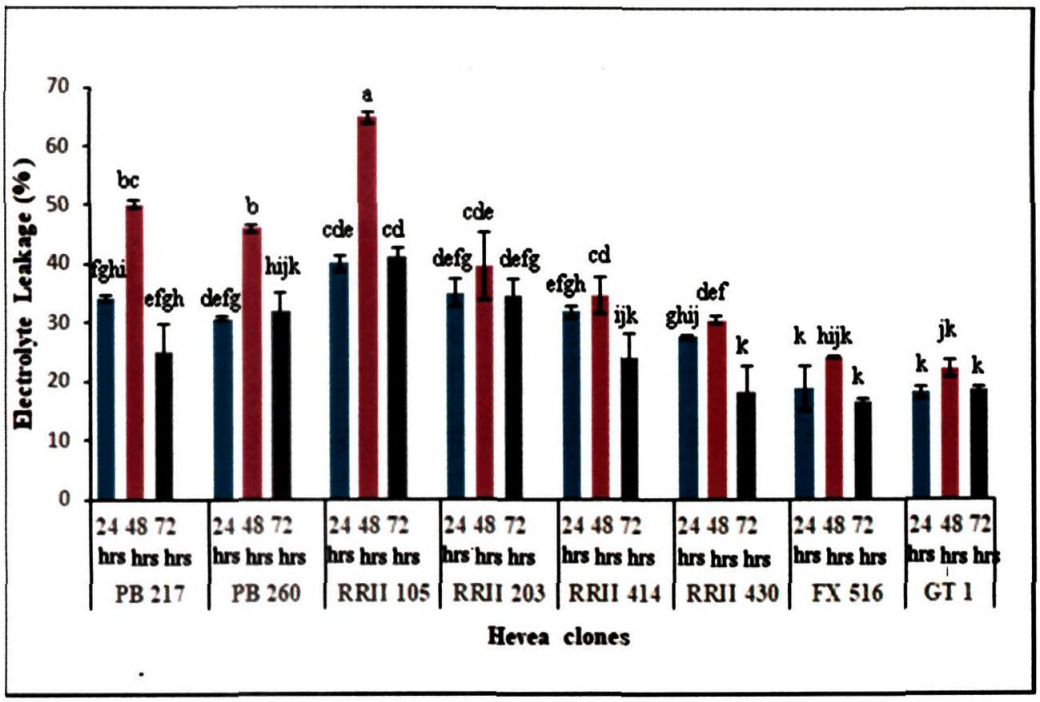


Fig.3.3. Response of leaf segments of different clones of *Hevea brasiliensis* towards vacuum infiltration with CCF of *Corynespora cassiicola*. C- water treated control, T- toxin treated, EDT- eye dropper tool at 48 hrs



CD (treatment x time) =7.15, p≤ 0.05

Fig.3.4. Electrolyte leakage in leaf discs of different clones at different treatment durations. Top letters on each bar indicate the significance of the difference between treatments

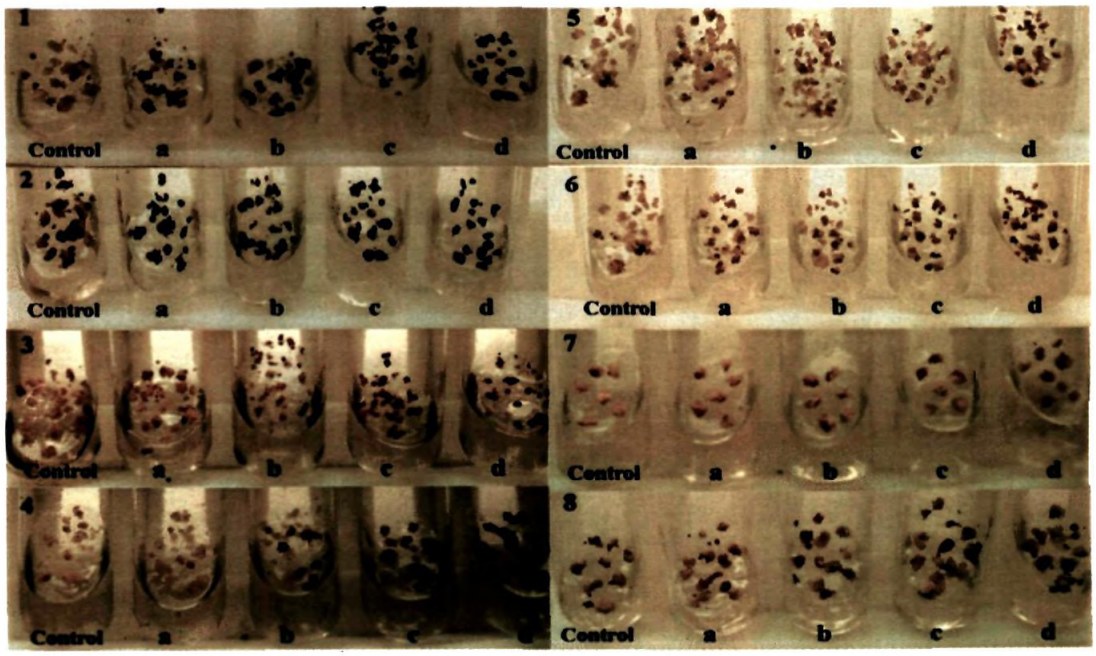


Fig.3.5. Response of calli from different *Hevea* clones (1- PB 217, 2- PB 260, 3- RRII 105, 4- RRII 203, 5- RRII 414, 6- RRII 430, 7- FX 516 , 8- GT 1) on exposure to CCF at different levels (a-d) a- 0.5 %, b -1 %, c - 2 % & d -3 % of CCF

3.4. Discussion

In our study, culture filtrate was found to infect detached *Hevea* leaves and induce disease symptoms similar to those caused by the pathogen *Corynespora cassiicola* in the field. The results of sensitivity studies towards CCF have been found to be well in accordance with the already recorded field observations. Progressive wilting of leaves of CLFD susceptible *Hevea* clones after CCF treatment demonstrated the phytotoxicity of CCF. A gradual increase in the intensity of wilting symptoms was also observed over time after CCF infection. Breton *et al*, (2000) was the first to demonstrate the phytotoxicity of a culture filtrate of *Corynespora cassiicola* isolate (CCP) on the leaves of *Hevea* clones. In this experiment, the leaves of susceptible *Hevea* clones wilted after being exposed to culture filtrate, whereas no disease symptoms were observed on the leaves of resistant *Hevea* clones. Breton, (1997) also demonstrated for the first time the purification and biochemical characterisation of cassiicolin, a phytotoxic compound secreted by *C.cassiicola* in culture filtrate. Thus, if we combine the explanation for pathological wilting of leaves with what Breton *et al* has demonstrated, the wilting of *Hevea* leaves in response to CCF application in the current study can be attributed to the presence of cassiicolin toxin in the crude culture filtrate.

Results of the sensitivity tests of leaf segments using vacuum infiltration technique also revealed the same trend as that in the leaf wilt bioassay of whole leaves. The leaf segments of susceptible clones had severe necrosis with dark brown lesions, whereas the leaf segments of tolerant clones remained intact without any necrosis. Necrosis and development of dark brown lesions in leaf segments of CCF infiltrated susceptible *Hevea* clones demonstrate the pathogenicity of CCF. These findings also suggest that CCF can mimic disease symptoms caused by the fungus *Corynespora cassiicola*, making it an excellent choice for *in vitro* selection studies.

In vitro screening using culture filtrate has been proven to be a safe technique since this method does not use the real pathogen. The filtrate of the culture medium in which the pathogen has grown is used as the selection agent. The ability of culture filtrate to induce necrotic symptoms on susceptible host plants demonstrates its pathogenicity, and this can be used as a criterion for selecting a phytotoxin-containing

culture filtrate in *in vitro* selection studies. The toxicity of culture filtrates might be attributed to the production of certain toxic metabolites and/or enzymes in the culture medium (Saadabi and El-Amin, 2007). According to the hypothesis of Adair, (1996) the metabolic byproducts of the pathogen are at least partially responsible for symptom expression. On account of this similarity in inducing disease symptoms, crude culture filtrate appeared to be an ideal model for the study of host parasite interactions.

Several studies investigated the phytotoxicity of cell-free culture filtrates on plant leaves. In response to artificial inoculation with culture filtrates of *Colletotrichum lindemuthianum* (the causal agent of anthracnose disease) the leaves of anthracnose-susceptible bean cultivar 'Collacia' showed dark brown lesions on the lower surface of its veins (Fernandez *et al.,* 2000). *Fusarium solani* isolates obtained from different hosts (asuki bean, common bean, cowpea, alfalfa, garden bean, lima bean) caused leaf chlorosis and/or necrotic spots on soybean seedlings, which are the symptoms of Sudden Death Syndrome (Hartman *et al.*, 2004). Necrotic spots appeared on the leaves of 30-day-old pineapple seedlings after a 35-hrs treatment with the *F. subglutinans* culture filtrate (Borras-Hidalgo and Bermudez, 2010). Pathological wilting of plants is caused by vessel plugging (blockage of xylem) and systemic toxicity (membrane injury and water leakage caused by fungal toxin) (Adair, 1996; Sun *et al.*, 2017; Wang *et al.*, 2014). The culture filtrate produced by *Stemphylium solani*, the causative agent of new leaf blight of cotton, was capable of eliciting the disease symptoms on cotton cotyledons as observed under field conditions, and no clear-cut distinction could be made between leaf symptoms produced by the culture filtrate and by the pathogen itself. The first toxic symptoms of leaf blight disease appeared on the leaves of cotton seedlings, 24 hrs after infiltration of culture filtrate as sunken spots of irregular sizes, while fully developed symptoms appeared 4 days after culture filtrate infiltration (Mehta and Brogin, 2000).

Pathogenic microorganisms use stomata to enter the intercellular spaces/leaf tissues during infection (Liu *et al.*, 2015). According to reports, a few processes allow access to the inner leaf cells *via* these stomata for the experimental application of different types of fluid from the outside (Leuzinger *et al.*, 2013; Molisch, 1912). Vacuum infiltration is one such forced infiltration process in which different liquid

substances are introduced into the plant tissue using vacuum pump by generating a pressure gradient between the leaf surface and its intercellular spaces (Chincinska, 2021). Detached leaves of the black pepper cultivar Kottanadan-1 infiltrated with crude culture filtrate of *Fusarium solani* f. sp. *piperis*, the causative agent of black pepper root rot and stem blight, displayed the first signs of toxic metabolite uptake 48 hrs later, in the form of discoloured secondary veins (Maria de Lourdes and Archer, 2003).

Alterations observed in the cell wall permeability of all selected *Hevea* clones in the present study is an indication of phytotoxicity induced by CCF. The highest EL% values in response to CCF treatment was recorded in the leaves of CLFD susceptible *Hevea* clones whereas the tolerant varieties recorded lower EL% values. The higher levels of electrolytic leakage in susceptible varieties suggest that, this leakage plays a role in the development of Corynespora leaf fall disease symptoms such as necrosis and brown discolouration. There was also a significant difference in EL% values between clones within each group. A similar response in electrolyte leakage was elicited in other cultivars also. Evaluation of the phytotoxic effects of culture filtrate of *F. subglutinans* on pineapple varieties assessed using electrolyte leakage measurement revealed a highly significant electrolyte leakage in 'Smooth Cayenne' (susceptible) cultivars as well as absence of significant electrolyte leakage in 'Perolera' (resistant) cultivars (Borras-Hidalgo and Bermudez, 2010). The red-rot toxin caused electrolyte leakage in leaves of different sugarcane varieties, with susceptible varieties exhibiting significantly higher electrolyte leakage than resistant varieties (Mohanraj *et al.*, 2003a).

The plasma membrane is one of the most sensitive parts of the cell, as it is the first subcellular component to come into contact with the toxin (Batchvarova *et al.*, 1992). A change in membrane permeability, which is a rapid and general response of susceptible plants or tissue to toxin treatment, is typically detected as solute efflux or electropotential depolarisation across the plasma membrane (Dunkle and Wolpert, 1981). Wheeler and Hanchy, (1968) proposed altered permeability as the initiating phase of triggering host physiology in plant infections. The penetration of *Fusarium oxysporum f. sp. cubense* culture filtrate to detached leaf discs of banana clones *via* vacuum infiltration resulted in an increase in ion leakage in treated leaf tissues than

the untreated ones (Morpurgo *et al.*, 2010). According to Bollard and Matthews, (1966), many fungal parasites appear to produce some substance or substances that increase permeability upon entry into the host tissue. Those substances called pathogen toxin(s) appear to act ahead of the pathogen by impairing the semi-permeability of the host plasma membrane, allowing the pathogen to advance through the 'door of the larder.' According to this, our results also suggest that CCF contains cassiicolin toxin as demonstrated by Breton *et al.*, (2000). The electrolyte leakage values obtained by infiltration of leaf tissues of *Hevea* clones GT 1 (CLFD tolerant) and PB 216 (CLFD susceptible) using cas1, the cassiicolin toxin secreted by *Corynespora cassiicola* isolate CCP, were consistent with the clones' previously known susceptibility to Corynespora infection. Not only that, the susceptible clone PB 217 had higher EL% values in response to toxin treatment than the tolerant clone GT 1. The EL% values were found to be proportional to the toxin incubation time. However at 72 hrs, the electrolyte leakage values dropped sharply, which is claimed to be due to the degradation of electrolytes caused by the prolonged action of toxin (Tran *et al*; 2016). In our current study as well, the same pattern was observed in the percentage of electrolyte leakage values measured at different times after CCF treatment in selected *Hevea* clones.

In vitro screening of callus against the CCF supplemented in the proliferation medium also revealed necrotic damage in the cultures raised from susceptible clones whereas growth and survival of intact callus was observed in the callus belonging to the tolerant group. As observed in the present study, many researchers have reported differences in CCF sensitivity of calli developed from different clones (susceptible and tolerant), as well as the development of brown discolouration and necrosis of calli depending on the dose of CCF in the growth medium, in a variety of crops subjected to culture filtrate treatment. Influence of culture filtrate on callus cultures of different species such as bean (Fernandez *et al.*, 2000), wheat (Ahmed *et al.*, 1996), soyabean (Jin *et al.*, 1996) and corn (Van Asch *et al* .,1992) are examples of such reports. The adverse effects on callus growth observed when they were exposed to culture filtrate challenged medium suspect the presence of toxic metabolites secreted by the pathogen, as previously reported by Dehgahi *et al.*, (2014); Anderson, (1978) and Arcioni *et al.*, (1987). The findings of *in vitro* screening of callus cultures mentioned

in our study also emphasise the possibility of using culture filtrate as an effective agent to screen for fungal toxicity or select resistant mutants in culture.

As a whole the results of all *in vitro* screening experiments revealed that the resistance/ susceptibility ratings of all selected *Hevea* clones were well in accordance with the field observations regarding sensitivity to CLFD.

Chapter 4

Biochemical assay of parameters responsible for defense mechanism in *Hevea brasiliensis* against infection with crude fungal exudates of *Corynespora cassiicola*

4.1. Introduction

Corynespora Leaf fall disease (CLFD) caused by the fungus *Corynespora cassiicola* has emerged as a major foliar disease of rubber in South East Asia. Eventhough chemicals provide effective protection against this disease, environmental concerns, high cost of labour and chemicals made it beyond the means of farmers in developing nations. For these reasons, numerous studies are being conducted to investigate plant's innate immune systems, with the goal of increasing plant resistance in a variety of ways. Most of the plant diseases are caused by fungal and insect pathogens. To counter these pathogen infections, plants have evolved various methods such as the production of antimicrobial agents and a type of programmed cell death known as the hypersensitive response, as well as the formation of hydrolytic enzymes belonging to the pathogenesis related (PR) protein group. Among the various PR proteins, chitinase is a key hydrolytic enzyme found in abundance in many plant species that acts as a first line of defense against pathogens. This enzyme plays a major role in defense against fungal pathogens by degrading chitin, which is a major constituent of most fungal cell wall (Sharma *et al.*, 2013).

Reactive oxygen species (ROS) is a natural byproduct of plant cellular metabolism. However, when plants are exposed to various environmental stresses, ROS species are produced in excess, resulting in progressive oxidative damage and eventually, cell death. Different types of environmental stresses that cause the production of ROS in plants include salinity, drought, high or low temperature, high light, pathogen infection, and nutrient deficiency. The delicate balance of ROS production and scavenging determines whether ROS act as signalling molecules or

cause oxidative damage to tissues. If the increase in ROS is not significant, the inherent antioxidant capacity is adequate to maintain the original balance between ROS production and scavenging. However, if the ROS level increases by 3 to 10 times, they certainly behave as extremely reactive molecules resulting in cellular damage. Therefore, in order to assess whether cellular injury has occurred under stress conditions, it is sufficient to examine the presence of ROS accumulation in stress exposed plants. NBT Staining technique is used to identify ROS such as superoxide ($O2^{\bullet-}$). In the presence of superoxide, Nitroblue tetrazolium (NBT) gets reduced to diformazan, a dark blue insoluble precipitate.

Excess ROS are scavenged or detoxified by an effective antioxidative system that includes both nonenzymic and enzymic antioxidants. The enzymatic antioxidants include super oxide dismutase (SOD), peroxidase (POX), catalase (CAT), enzymes of ascorbate glutahione (AsA-GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). Nonenzymic antioxidants in the cell include glutathione (GSH), ascorbate (AsA), phenolics, tocopherols, and carotenoids (Sharma *et al.*, 2012). An increase in antioxidant activity during pathogenic infection in plants is thought to be related to ROS elimination. Many researchers have demonstrated increased activity of numerous antioxidant enzymes in the plant defensive mechanism to resist oxidative stress caused by a variety of environmental conditions. A plant with strong antioxidant capacity reveals its increased tolerance to environmental factors. As a result, measuring an affected plant's antioxidant enzyme activity can be used to determine whether or not cellular injury occurred to that particular plant under stress conditions, and if so, to what extent.

The objective of this study is the biochemical analysis of various parameters such as activity of the enzymes chitinase, catalase and peroxidase as well as accumulation of ROS in various clones of *Hevea* before and after treatment with crude culture filtrate (CCF) of *C. cassiicola.*

4.2. Materials and Methods

4.2.1. Plant material

The clones selected for this study were the same as described in Chapter 3. Crude culture filtrate (CCF) of *C.cassiicola* was also prepared as per microbiological standardised procedures as described in Chapter 3.

Leaf enzyme activity was measured in all selected clones using the following samples

a) Control samples– Fresh leaves collected directly from the field grown plants

b) Toxin treated samples– Application of CCF by leaf puncture technique, retaining the leaves on the plant itself for 15 hrs after which the leaves were harvested

c) Water treated control– Sterile water was applied instead of CCF and the leaves were kept on the plant itself for 15 hrs before harvesting

Comparison of enzyme activity in the control samples was carried out in order to see whether there is any difference in the activity between the susceptible and tolerant groups. Similarly the enzyme activity after CCF treatment was assayed in all the clones to determine the impact of exposure to CCF on individual clones. Inorder to nullify the effect of leaf puncturing a water treated control was also included along with the samples. Also a comparison between the control and toxin treated samples of each clone was performed along with a water treated control.

4.2.2. Chitinase assay

The crude culture filtrate (CCF) of *Corynespora cassiicola* was applied onto the pale green leaves of selected *Hevea* clones by leaf puncture technique where the leaves were pierced with a needle to make a few perforations. Strips of Whatmann No.1 filter paper soaked in CCF were pressed on either sides (adaxial and abaxial surfaces) of these perforated leaves, and the leaves and strips were wrapped with cling film. The leaves were then retained on the respective plants for a specified period of upto 15 hrs afterwhich they were harvested from the plant. After removing the ribs in the middle the remaining leaf tissues were homogenised in 0.1 M sodium citrate buffer of pH 5.0 (1.0 ml / 0.5 g fresh weight) using a pre-chilled mortar and pestle.

The crude homogenate was then centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant thus obtained was referred to as the crude extract, for the estimation of chitinase activity.

The reagents used for the assay are chitin powder, hydrochloric acid (HCl), dinitrosalicylic acid (DNS), phenol, sodium sulphite, sodium hydroxide, rochelle salt solution, N- acetyl-D-glucosamine (GlcNAc) and 50 mM phosphate buffer (pH 7.0). The chitinase activity in the leaf extract was estimated using colloidal chitin as the substrate and the activity was determined using dinitrosalicylic acid (DNS) method which tests for the presence of free carbonyl group, the so-called reducing sugars (Miller, 1959). Colloidal chitin was prepared from the chitin flakes (CDH, India) by the modified protocol of Murthy and Bleakley, (2012). The chitin flakes were manually crushed to a fine powder using a mortar and pestle. 5g of this chitin powder was taken in a beaker and 40 ml of concentrated HCl was slowly added to this beaker with continuous stirring under cooling conditions (on ice). The mixture was then incubated at 37°C in a water bath to reduce its viscosity. This warm solution was then mixed with 2 liters of sterile distilled water and allowed to stand still over night at a temperature of 4°C. The supernatant was carefully decanted the next day and the residue was collected on a Whattmann No 1 filter paper. This residue (chitin cake) was thoroughly washed with sterile distilled water to attain a neutral pH. The colloidal chitin thus obtained in the form of a paste was stored at 4°C until further use.

DNS reagent which contained 1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide and 1 ml of 40% Rochelle salt solution was prepared according to modified protocol of Miller, (1959). DNS reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at wavelength 540 nm which could be detected by their colour intensities using a spectrophotometer. The amount of reducing sugar released during this assay was estimated by comparison to a standard calibration curve prepared with the reducing sugar of interest which is N- acetyl-D-glucosamine (GlcNAc).

Standard curve of GlcNAc was prepared with minor modifications in the protocol of Vinusha *et al.*, (2013). The standard solution of GlcNAc was prepared by dissolving 100 mg of GlcNAc in 100 ml of distilled water. Different dilutions of

standard GlcNAc solutions of known concentrations were prepared in a series of test tubes as shown in Table 4.1. The absorbance of each dilution was measured using a spectrophotometer. The measured absorbance values along with its GlcNAc concentrations of different dilutions were entered in an Excel sheet. Using this data, a scatter chart was created with the help of chart tools. A linear regression trendline was then added to this scatter chart which represents the calibration curve of GlcNAc. The linear regression equation and r^2 value of the line was displayed on the chart by using format trendline options. The unknown concentrations of GlcNAc (mg/ml) liberated in each test sample during enzyme assay could be obtained by substracting the y-intercept from the measured absorbance and dividing it by the slope. The concentrations of GlcNAc liberated in mg/ml were converted to moles by using the formula Mole = Mass / Molar Mass.

Sl.No	GlcNAc standard solution(ml)	Distilled water (ml)	DNS (ml)
1 (Blank)	0.0	1.0	3.0
2	0.2	0.8	3.0
3	0.4	0.6	3.0
4	0.6	0.4	3.0
5	0.8	0.2	3.0
6	1.0	0.0	3.0

Table 4.1. Preparation of N-acetyl-D-glucosamine standard curve

Chitinase assay was carried out according to some modifications in method of Zarei *et al.*, (2011). The reaction mixture containing 0.9 ml of 1% w/v colloidal chitin prepared in 50 mM phosphate buffer (pH 7.0) and 0.1 ml enzyme extract was incubated at 45°C for 45 min. The volume was made upto 1 ml with distilled water. The reaction was stopped by the addition of 3 ml DNS reagent followed by boiling in water bath at 100°C for 5 min. After cooling, the absorbance was measured at 540 nm using UV spectrophotometer along with blanks. The amount of enzyme required to

produce 1 micromole of reducing sugar (GlcNAc) per minute is defined as one unit of enzyme activity (U).

Enzyme activity can be calculated using the equation

$$\text{Enzyme activity (U/ml)} = \frac{\text{Moles of liberated GlcNAc}}{\text{Reaction volume (ml) x Time of reaction (minutes)}}$$

4.2.3. Detection of ROS by NBT staining

To understand whether cell damage occurred due to generation of superoxide oxygen anions in selected *Hevea* clones, in response to the infection with the crude fungal culture filtrate, histochemical stain NBT is used. Yellow water soluble NBT is reduced by superoxide radicals to blue, water soluble formazan. NBT staining of treated leaves was performed with minor modifications in the method of Liu *et al.*, (2007). The CCF was applied onto pale green leaves by leaf puncture technique, sustaining the leaves on the plant itself for 15 hrs. Treated leaves were harvested and leaf discs of size 1 cm diameter were punched out of the treated leaves (excluding the punched areas), vacuum infiltrated with NBT at 1mg/ml in staining buffer (10 mM KPO_4, 10mM NaN_3) for 30 minutes and further stained overnight at room temperature. The leaves were then destained overnight in 95% ethanol and the produced ROS were observed macroscopically. Untreated leaves served as the control.

4.2.4. Assay of antioxidants

Preparation of extract

The CCF was applied onto the leaves by leaf puncture technique, sustaining the leaves on the plant itself for 15 hrs. Untreated leaves served as the control. The leaf tissue (1 g), with mid ribs removed, was collected after treatment with the CCF. These leaf tissues were then homogenised in 1 ml of solution containing 50 mM potassium phosphate buffer (pH- 6.8), 0.1 mM EDTA and 2% PVPP using a pre-chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was used as the crude enzyme extract for determining the antioxidant assays.

4.2.4.1. Catalase assay

The CAT activity was determined following the method proposed by Cakmak and Marschner, (1992). The reaction was initiated following the addition of 250 µl of the crude enzyme extract to 750 µl of a reaction mixture containing 25mM potassium phosphate buffer (pH- 7.0) and 10 mM H_2O_2. The determination of CAT activity was based on the rate of H_2O_2 decomposition measured in the spectrophotometer at 240 nm for 3 min at 25^0C. An extinction coefficient of 39.4 $M^{-1}cm^{-1}$ was used to calculate the CAT activity.

4.2.4.2. Peroxide assay

The POX activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar and Mishra, (1976). The reaction was started after the addition of 15 µl of the crude enzyme extract to 985 µl of a reaction mixture containing 25 mM potassium phosphate (pH- 6.8), 20 mM pyrogallol and 20 mM H_2O_2.The POX activity was measured by the absorbance of purpurogallin at 420 nm for 1 min at 25^0C. An extinction coefficient of 2.47 $mM^{-1}cm^{-1}$ was used to calculate the POX activity.

4.2.5. Statistical Analysis

Data presented are means ± standard error for independent experiments. Two factor ANOVA followed by Duncan's Multiple Range Test ((DMRT), both carried out using SPSS (version 12.0) software packages, were used for expressing the statistical significance.

4.3. Results

4.3.1. Estimation of chitinase activity

A graph of the standard curve was plotted between the concentration of N-acetyl-D-glucosamine and optical density values. The colour change of DNS reagent from orange yellow to dark orange red confirmed the chitinase activity of the plant extract.

It has been observed that there was significant difference in the intrinsic chitinase activity of susceptible and tolerant clones, as evidenced in Fig.4.1. Intrinsic chitinase activity was much lower in all the four susceptible clones whereas all the

clones belonging to the tolerant group exhibited significantly higher levels of chitinase. Among the tolerant clones, FX 516 showed the highest chitinase activity of 0.14 U followed by GT 1 (0.13 U), RRII 430 (0.12 U) and RRII 414 (0.11 U). Among the susceptible clones, RRII 105 exhibited the least enzyme activity (0.07 U) followed by PB 217 (0.076 U), PB 260 (0.082 U) and RRII 203 (0.083 U).

The same trend was observed among the different samples after CCF treatment, tolerant clones exhibiting higher enzyme activity than the susceptible ones. Comparison of chitinase activity within one clone before and after treatment revealed that treatment with the fungal culture filtrate significantly increased the enzyme activity in the tolerant clones FX 516 and GT 1. The increase was found to be higher in GT 1 (0.035 U) indicating this clone to be more tolerant towards the culture filtrate followed by FX 516 (0.03 U) and RRII 430 (0.021 U). Clone RRII 414, grouped under the tolerant category, however showed only a slight increase by 0.012 U thereby exhibiting a final activity of 0.12 U after treatment. The rise in chitinase activity in these clones ranged from 0.012 U - 0.035 U. Susceptible clones also indicated an increase in chitinase activity following CCF treatment, but not as high as tolerant clones. The increase was found to be higher in clone PB 217 (0.017 U), followed by PB 260 (0.016 U), RRII 105 (0.015 U) and RRII 203 (0.014 U). The rise in chitinase activity in these clones ranged from 0.014 - 0.017 U.

4.3.2. Detection of ROS

ROS produced in the leaves under the stressful conditions of infection was detected macroscopically. In the presence of superoxides, NBT is reduced to dark purple or blue coloured spots of formazan deposit which represent ROS production. The leaf discs of susceptible clones RRII 105 and PB 217 showed very high levels of formazan precipitate after NBT staining, as evidenced by intense blue coloration covering almost their entire leaf lamina [Fig. 4.2(a-d)]. However, in the case of the other two susceptible clones PB 260 and RRII 203, the blue coloration diffused across the leaf lamina was found to be less, indicating a reduction in ROS production. Regarding the tolerant clones, no colour formation was observed after NBT staining in clones RRII 430, GT 1 and FX 516, indicating the absence of ROS production [Fig. 4.2(e-h)]. In clone RRII 414, on the other hand, small traces of blue formazan staining

could be observed on the leaf discs, indicating a low level of ROS production. These findings indicate that the cells of the tolerant clones were less damaged by CCF treatment, whereas cells of susceptible clones become infected with crude fungal exudates and suffered extensive cell damage during treatment.

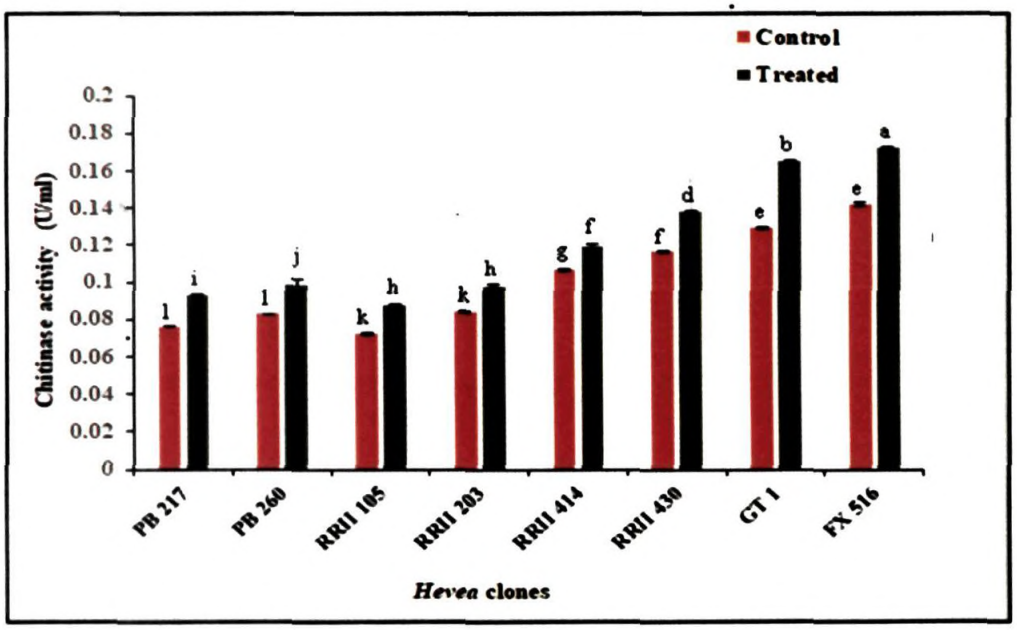
4.3.3. Catalase assay

There was a substantial difference in the inherent catalase activity profile between the leaf samples of the clones belonging to the two groups under study. Compared to the respective controls, increase in CAT activity was shown by all treated clones belonging to both the groups. Of these, susceptible clones showed the highest CAT activity. The increased peroxidase formation is intended to remove the ROS produced in these clones as a result of infection. After treatment, the mean values of CAT activity progressively increased over the controls. Differences between mean values of treated samples and untreated controls were considered significant at $p<0.05$ (Fig. 4.3). Among the susceptible clones, the highest statistically significant increase (140%) was observed in case of clone RRII 105 while the lowest statistically significant increase (116.66 %) was noticed in clone PB 217. The other clones PB 260 and RRII 203 showed an increase in activity by 133.33% and 120% respectively. In case of tolerant clones, after treatment, statistically significant increase in mean values of CAT activity could only be noticed in clone RRII 414 (40%) whereas the increase in enzyme activity in other tolerant clones was found to be insignificant.

4.3.4. Peroxide assay

There was a substantial difference in the POX activity profile between the leaf samples of the clones belonging to the two groups under study. Compared to the respective controls, increase in POX activity was shown by all treated clones belonging to both the groups. Of these, susceptible clones showed the highest POX activity. The increased peroxidase formation is intended to remove the ROS produced in these clones as a result of infection. After treatment, the mean values of POX activity progressively increased over the controls. Differences between mean values of treated samples and untreated controls were considered significant at $p<0.05$ (Fig. 4.4). Among the susceptible clones, the highest statistically significant increase (107.96 %) was observed in case of clone RRII 105 and PB 217 while the lowest

statistically significant increase (89.96 %) was noticed in clone RRII 203. The other clone PB 260 showed an increase in activity by 98.96%. In case of tolerant clones, after treatment, statistically significant increase in mean values of POX activity could only be noticed in clone RRII 414 (89.96%) and RRII 430 (71.97%) whereas the increase in enzyme activity in other tolerant clones was found to be insignificant.



CD (clone x treatment) = 0.007 with $p \leq 0.05$

Fig.4.1. Comparison of chitinase activity in leaves of different *Hevea* clones before and after CCF treatment

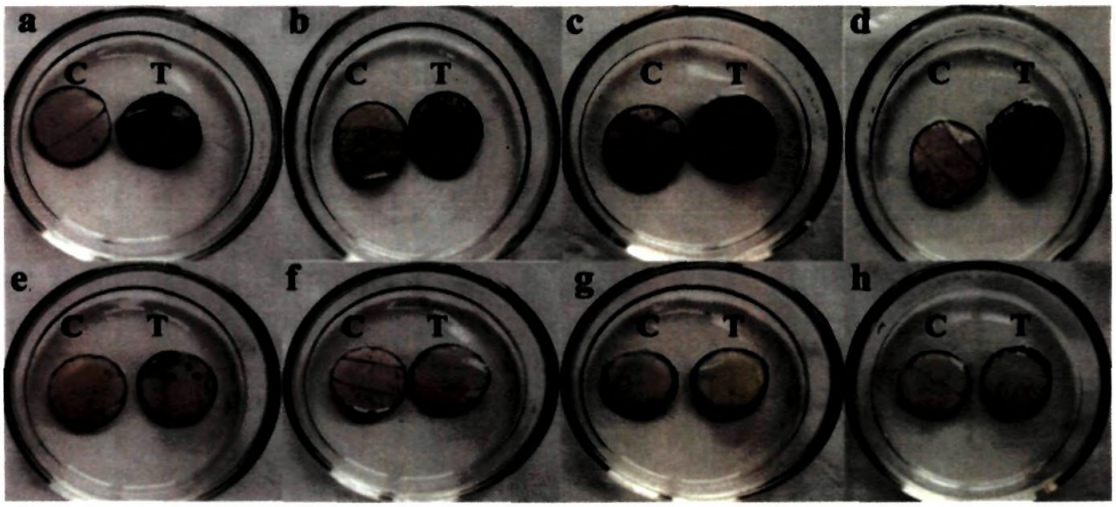
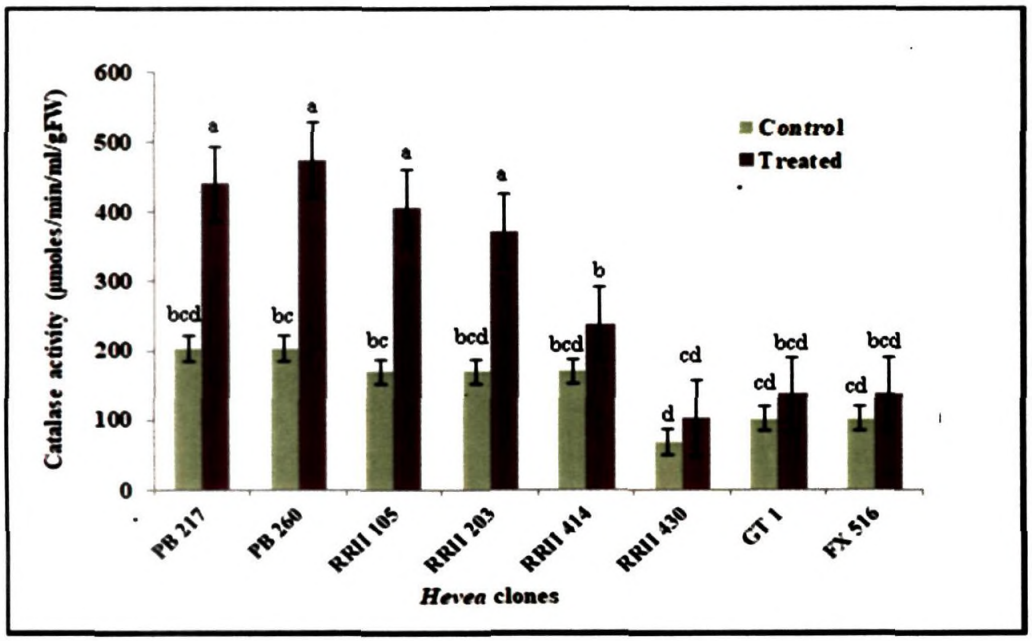
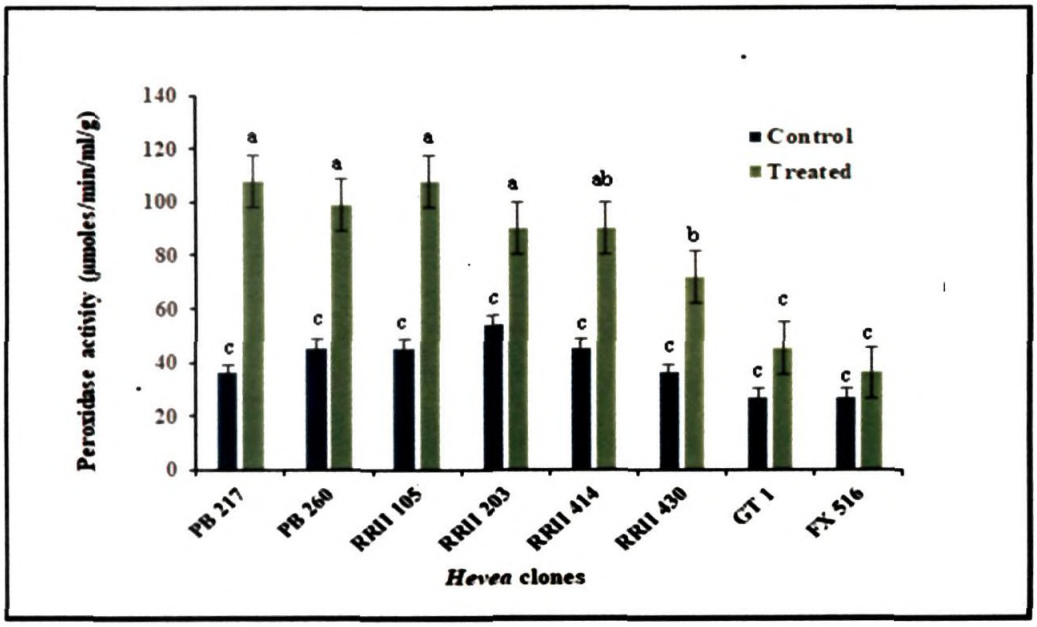


Fig. 4.2. Detection of ROS by NBT staining in the leaf discs of the *Hevea* clones under study. ROS production is visualized as dark blue coloration whereas absence of dark blue-colored formazan deposit indicates no ROS production a) PB 217 b) PB 260 c) RRII 105 d) RRII 203 e) RRII 414 f) RRII 430 g) FX 516 h) GT 1. C- Control, T-Treated



CD (clone x treatment) = 0.001 with $p \leq 0.05$

Fig 4.3. Comparison of catalase activity in leaves of different *Hevea* clones before and after culture filtrate treatment



CD (clone x treatment) = 0.001 with $p \leq 0.05$

Fig 4.4. Comparison of peroxidase activity in leaves of different *Hevea* clones before and after culture filtrate treatment

4.4. Discussion

Pathogen-related proteins are a group of proteins associated with a disease condition or other biotic stresses. They are induced in a coordinated manner, accumulate systemically and locally, and are associated with the development of systemic acquired response (SAR), induction of hypersensitive response (HR) and accumulation of salicylic acid (Gupta *et al.*, 2013). Activation of plant defense pathways that restrict the entry or spread of the pathogen, leads to the induction of PR proteins. PR proteins are found both intracellularly and in the intercellular spaces of plant cells, especially in the cell walls of different tissues (Agrios, 1997). PR proteins are classified into 17 families based on their amino acid sequence data and biochemical functions, which include chitinases, β-1,3-glucanases, ribosome-inactivating protein defensins, peroxidases, thaumatin-like proteins, thionins, oxalate oxidase, nonspecific lipid transfer proteins and oxalate-oxidase-like proteins (Oliveira *et al.*, 2016; Mahesh *et al.*, 2017). Chitinase may be produced at low or undetectable levels in plants and is significantly induced in response to a variety of biotic and abiotic stresses, including mechanical wounding, infection (viral, bacterial and/or fungal), ethylene, chitosan, salicylic acid, fungal cell wall fragments, and insect/hematode feeding. *In vitro* studies on the growth of chitin-containing fungi as a component of the cell wall show that plant chitinase inhibits fungal growth by hydrolyzing this chitin (Boller, 1985; Mauch *et al.*, 1988). Another situation in which the defense system of plants is activated is when the host plant detects the signal metabolites of a pathogen known as elicitors. (Van't Slot and Knogge, 2002). These elicitor molecules bind to receptors on plant cell plasma membranes and trigger signalling events that initiate the defense response (Umemoto *et al.*, 1997). Treatment with substances such as chitin oligosaccharides, culture filtrates, fungal cell wall fragments or salicylic acid, can act as elicitors, triggering general plant defense responses (Zhang, 1995).

A previous study demonstrated that *Penicillium simplicissimum* GP17-2 culture filtrate is just as effective as living inocula at inducing systemic resistance against bacterial leaf speck pathogen, *Pseudomonas syringae pv.tomato* DC3000 (*Pst*) in *Arabidopsis thaliana* by the activation of multiple defense signals (Hossain, 2007). A similar induction in the defense response to CCF-treated *Hevea* clones was

observed in the current study, which is evident by an increase in chitinase activity. Regardless of their sensitivity to CLFD, all selected *Hevea* clones showed a significant increase in activity of chitinase after CCF treatment as compared to their untreated control plants. Similar results were reported by Shrestha *et al,* (2008) in rice; Varma *et al,* (2009) in black pepper and Zur *et al,* (2013) in winter triticales. In the present study, CCF treatment significantly increased chitinase activity in tolerant *Hevea* clones compared to susceptible ones. The findings of o study show that resistant *Hevea* clones respond actively to infection by increasing their chitinase activity dramatically. Immediate activation response of the *Hevea* clones to the CCF appears to be more important than the rate of production. For the expression of resistance, the speed and magnitude of the mechanism's activation appear to be important.

Similar differences in the induction of chitinase activity have been noticed among resistant and susceptible varieties in many crops upon infections with various agents. The activity of chitinase was found to be induced fivefold and threefold in susceptible and resistant barley leaves 7 days after inoculation with *Erysiphe graminis* f.sp. *hordei* (powdery mildew fungus) (Kragh *et al.*, 1990). Despite the fact that studies on chitinase activity in leaves and pods of resistant chickpea cultivars infected with *Ascochyta rabiei* revealed a fivefold increase, susceptible cultivars revealed a lower induction of chitinase (Nehra *et al.*, 1994). In response to *Phoma lingam* infection, chitinase activity was induced in both resistant and susceptible cultivars of oil seed rape. Resistant cultivars showed a rapid and large increase in chitinase activity, whereas susceptible cultivars did not show an increase in activity until 24 hrs after infection (Rasmussen *et al.*, 1992). Inoculation of onion plants with leaf blight pathogen *Alternaria palandui* resulted in the accumulation of the chitinase defense enzyme in leaf tissues of both resistant and susceptible cultivars, with the accumulation being greater in resistant cultivars than susceptible ones (Karthikeyan *et al.*, 2005).

Under normal growth conditions, plants produce very little ROS. However; in response to various stress factors, ROS levels in plants increase dramatically, upsetting the normal balance of •OH, O_2•− and H_2O_2 with in cellular environment (Sharma *et al.*, 2019). ROS production in plants as a result of pathogenic infections is

a well-documented phenomenon, as pathogens/pathogenic agents is found to be the major and familiar stress factor. (Daub *et al.*, 2013). In response to different pathogenic infections, increased levels of ROS species have been reported in various plants like rice (Chittoor *et al.*, 1997), bean (Radwan *et al.*, 2010), arabidopsis (Simon *et al.*, 2013), wheat (Foley *et al.*, 2016), tobacco (Rossi *et al.*, 2017) and so on. Our findings revealed that due to pathogen tolerance differences, the production of ROS in selected *Hevea* clones in response to CCF differs among clones, with susceptible clones producing more ROS than tolerant clones. Similar effects have been previously reported in other crops that have been subjected to different stresses. One example is the difference in H_2O_2 production under salt stress in wheat genotypes with varying tolerance to water stress, as reported by Sairam *et al.*, (1998). Induced accumulation of O2•− in higher levels in wheat that is more sensitive to Ptr ToxA (a host selective toxin of *Pyrenophora tritici-repentis*) than Ptr ToxA insensitive wheat is yet another example. The O2•− accumulated in Ptr ToxA sensitive wheat reacted with NBT to form blue formazan precipitate (Manning *et al.*, 2009). NBT has been reported to detect superoxides because it has been suggested that this reagent is capable of reacting with different ROS and, thus, is probably better for measuring oxidative stress (Halliwell, 2006). The presence of dense blue formazan spots on the leaves of CLFD susceptible *Hevea* clones in response to CCF treatment in the present study indicates increased superoxide production. The absence/lack of superoxide generation in CLFD tolerant *Hevea* clones after CCF treatment suggests that the plant's response to the culture filtrate was strong enough, owing to the plant's resistance to Corynespora infection. According to previous reports (Rossi *et al.*, 2017; Liu *et al.*, 2007; Zurbriggen *et al.*, 2009), plant ROS production in response to stress is also considered important in the development of necrotic lesions. Based on these findings, it can be inferred that the increased accumulation of ROS produced as a result of CCF infiltration may also contribute to the development of necrotic lesions in the leaves of CLFD susceptible *Hevea* clones. The absence or lack of superoxide generation in tolerant *Hevea* clones after CCF infection suggests that the plant's response to the culture filtrate was strong enough, owing to the plant's resistance to Corynespora infection. High concentration of ROS causes cellular damage or even hypersensitive cell death (Gechev *et al.*, 2010; Bhattacharjee, 2005) whereas low

concentration of ROS functions as signalling molecule, mediating a variety of plant responses. (Kovalchuk, 2010; Gill and Tuteja, 2010).

In the current study, both CAT and POX activity significantly increased in CCF-treated leaves of all CLFD susceptible *Hevea* clones compared to untreated controls, indicating that these clones produced more H_2O_2 to defend themselves from CCF stress. On the other hand, the increase in CAT and POX activity in response to CCF treatment was significantly lower in CLFD tolerant *Hevea* clones RRII 430, FX 516 and GT 1, indicating a lower amount of H_2O_2 production in these clones which was sufficient to defend the minor damage caused by CCF stress. ROS levels are usually associated with successful disease- resistant responses (Torres *et al.*, 2006). Eventhough increased CAT and POX levels were found to be associated with CLFD susceptible *Hevea* clones, however, they did not provide resistance to CCF, possibly because catalase and peroxidase expression did not meet the threshold level required to inhibit the action of toxin/toxic metabolites present in the CCF.

Overall, the findings of the present study indicate that chitinase plays an important role in the interactions between *Hevea* and crude culture filtrate of *Corynespora cassiicola*. The induced levels of chitinase highlight the critical role of these enzymes in the resistance of *Hevea* clones to *C. cassiicola* infection and confirm their participation in the defense strategy of the resistant *Hevea* clones. This study also showed that, in response to infection, there was an increase in ROS production in susceptible clones, which was accompanied by an increase in the antioxidants catalase and peroxidase.

Chapter 5

Development of Corynespora tolerant plants of *Hevea brasiliensis* clone RRII 105 through *in vitro* selection against crude culture filtrate of *C. cassiicola*

5.1. Introduction

The production of Natural Rubber (NR) continues to be adversely affected due to various biotic and abiotic stresses. Fungal diseases are the major biotic stress factors on rubber trees, causing significant crop failure and drastically reducing NR production around the world. (Clement-Demange *et al.*, 2007). Different *Hevea* clones displayed varying levels of susceptibility towards different pathogenic diseases (Narayanan and Mydin, 2012). In India, almost all clones were reported to be affected by Corynespora leaf fall disease (CLFD) (Mathew, 2006). In 2010, Manju *et al* reported the susceptibility and distribution of CLFD in South India, according to which the most promising *Hevea* clone RRII 105 recorded the highest CLFD susceptibility while the clone GT 1 was found to be least susceptible. Earlier surveys conducted by Manju *et al*, (2001) to assess the intensity and spread of CLFD in coastal Karnataka and North Malabar regions of Kerala also revealed similar reports regarding susceptibility of clone RRII 105.

Traditional breeding approaches and proper management strategies *via* chemical control continue to play a vital role in improving *Hevea* clones. Owing to the requirement of repeated fungicide application, high cost of labour and environmental concerns, chemical control of CLF is not generally advocated in many of the rubber growing countries in South East Asia. However, conventional breeding methods are cumbersome in tree crops like *Hevea*, as the process has to include many generations which take decades to grow, failed to provide desirable results and is expensive in terms of time, space and large number of plants being handled. Hence *in vitro* selection strategies for disease resistance which is a combination of biotechnological and phytopathological techniques is considered as an alternative tool

for addressing the critical problems of conventional disease resistance breeding (Svabova and Lebeda, 2005).

Since 1958, *Corynespora* leaf fall disease on *Hevea* caused by *Corynespora cassiicola* has been studied considering it as an epidemiologic disease. Developing disease resistant plants is one of the cheapest and efficient control measures of plant diseases (Borras-Hidalgo and Bermudez, 2010). However, *Hevea* clones with high yield and at the same time showing tolerance to *Corynespora cassiicola* have not been yet identified or developed. Therefore it is necessary to find various approaches to obtain high yielding *Hevea* clones tolerant to this pathogen. There is a wide range of different methodological approaches available to detect resistant genotypes and to select plants with improved resistance (Dhingra and Sinclair, 1986; Kiraly *et al.*, 1974; Trigiano *et al.*, 2004) among which *in vitro* screening is one of the most high-throughput and efficient methods (Svabova and Lebeda, 2005). *In vitro* selection involves regeneration of plantlets in a culture media amended with varying concentrations of the selection agent and screening of regenerated plantlets for resistance to disease (Ramesh *et al.*, 2010). For the past three decades, the potential role of *in vitro* selection method in screening and selecting various crops for improved disease tolerance has been recognised and comprehensively reviewed by Svabova and Lebeda, (2005).

Daub, (1986) has clearly mentioned in his review about the unique advantage of using tissue culture technique to generate plants resistant to diseases that proved challenging in the sense that no sources of resistance have been identified, or where current sources provide only a low level of resistance. The most common approach for *in vitro* selection of disease resistant cultures is to use toxic culture filtrates or pathogen toxins as the selection agent in culture medium since they are the primary determinants in pathogenesis and induce typical disease symptoms in the absence of pathogen (Goodman *et al.*, 1960). The key reason for using culture filtrate in *in vitro* selection studies is the observation that culture filtrate contain some toxic compounds produced by pathogens and that when these filtrates were used for *in vitro* selection studies, the plants developed had better resistance to such toxic compounds (Remotti *et al.*, 1997). In addition, the crude culture filtrates, which contain a combination of

fungal metabolites, were also able to induce disease like symptoms and trigger elicitation of various defense responses (Dehgahi *et al.*, 2014).

The feasibility of *in vitro* selection was demonstrated for the first time by Carlson in 1973, when they obtained tobacco plants resistant to the toxin of *Pseudomonas syringae* pv *tabaci* from toxin challenged callus cultures. Since then, many researchers have extrapolated this technique for the development of disease resistant varieties. Development of disease resistant plants by *in vitro* selection using appropriate selective agents has been rarely applied to woody species. Neverthless, successful results for selection of resistant lines have been well reported in fruit crops such as papaya against causing *Phytophthora* wilt (Sharma and Skidmore,1988), guava against wilt (Vos *et al.*, 1998; Bajpai *et al.*, 2005), banana against black Sigatoka disease (Okole and Schulz, 1997), mango against Anthracnose (Jayasankar *et al.*, 1999), strawberry against wilt (Orlando *et al.*, 1997), peach against *Xanthomonas* (Hammerschlag, 1988) and apple against black spot (Raman and Goodwin, 2000). And not just this, according to Miah *et al.*, (2013), research on the development of disease tolerant plants through existing or novel methodologies has become more important. Thus with all these insights in the past few decades, *in vitro* selection strategy has been developed as an alternative for conventional breeding approach to accelerate the development of disease resistant plants.

Onesirosan *et al.*, (1975) reported the first evidence of a toxin produced by *C.cassicola* when the culture filtrate of this fungus was found to be toxic to the excised tomato leaves. Liyanage *et al.*, (1986) and Purwantara, (1987) also noticed similar results on *Hevea* leaves infected with *Corynespora cassiicola.* Later, studies under controlled conditions were focused on the role of a small host selective phytotoxic protein involved in determining CLFD in rubber trees. Leaf wilting bioassay of *Hevea* clones using *C.cassiicola* culture filtrate confirmed the presence of this host selective toxin in the culture filtrate (Breton *et al.*, 2000). This toxin named cassiicolin is a glycosylated cysteine rich small secreted protein of 27 amino acids purified and biochemically characterised for the first time by Breton, 1997 and later by De Lamotte *et al.*, (2007). Breton *et al.*, (2000) suggested that this host selective toxin, cassiicolin produced by *Corynespora cassiicola*, can be used directly for screening the clones in the laboratory; but laboratory assessment and field

observations seem to provide different CLFD sensitivity rankings of the cultivated clones. It is still difficult to suggest tolerant clones with high confidence, and the necessity to select tolerant clones limits the diversity of clones that can ultimately be used in affected areas.

Besides using purified or partially purified toxins (Wedge and Tainter, 1997), crude toxins have also been used as effective screening agents in attempts to select for disease tolerance. Callus cultures are often employed in *in vitro* selection strategies using toxins, inorder to obtain disease insensitive lines (Arai and Takeuchi, 1993; Jayasankar and Litz, 1998; Kintzios *et al.*, 1996; Mohanraj *et al.*, 2003b; Gentile *et al.*, 1992; Rines and Luke, 1985; Wolf and Earle, 1990). Hammerschlag, (1988) screened embryogenic callus derived from immature zygotic embryos of peach against the culture filtrate produced by the bacterial pathogen *Xanthomonas compestris pv.pruni* and reported that regenerated plants were more resistant to the pathogen. Gentile *et al.,* (1992), regenerated mal secco resistant lemon by selecting nucellar embryogenic cultures for resistance to a partially purified phytotoxin produced by the fungus *Phoma tracheifila.* Fusarium wilt disease resistant plants were regenerated from abaca embryogenic calli on medium fortified with *Fusarium oxysporum* f.sp. *cubense* culture filtrate (Purwati *et al.*, 2007). *Phytophthora* resistant cultures of *Citrus jambhiri* were recovered by exposing cotyledon derived calli to culture filtrate obtained from the causal agent *Phytophthora parasitica* (Savita *et al.*, 2011).

Taking into account the above mentioned *in vitro* selection studies, we explored the feasibility of developing CLFD tolerant plants of *Hevea brasiliensis* through *in vitro* selection technique by exposing or challenging cultures of embryogenic calli against the crude culture filtrate of *Corynespora cassiicola.* The clone chosen for this study was RRII 105, the most popular and extensively cultivated *Hevea* clone which at the same time is highly susceptible to CLFD.

5.2 Materials and Methods

5.2.1. Plant material and culture media

Immature inflorescences collected from field grown mature trees of clone RRII 105 served as the source of explant for the initiation of callus. Segments of these

inflorescences were surface sterilized and used for callus induction as described by Sushamakumari *et al.*, (2000b). Basal medium used in this study was MS (Murashige and Skoog, 1962) supplemented with B_5 vitamins (Gamborg *et al.*, 1968), 5% coconut water and 3% sucrose. The different growth regulators added for callus induction were 2, 4-D (4.5 µM), Kinetin (2.3 µM) and NAA (2.7 µM). The induced callus was proliferated and cultured over embryo induction medium. Modified MS basal containing increased levels of ammonium nitrate (1000 mg/l) and potassium nitrate (1500 mg/l) and fortified with GA_3 (2.9 µM), BA (4.4 µM), 2,4-D (1.0 µM), 5% sucrose and 0.5% agar was employed for embryo induction. The emerging embryogenic callus was used as the initial material for toxin challenge.

5.2.2. Preparation of Crude Culture Filtrate (CCF)

Crude culture filtrate (CCF) of *C.cassiicola* was prepared as per microbiological standardised procedures as described in chapter 3.

5.2.3. *In vitro* screening and selection of tolerant lines

For the preparation of toxic medium, the pathotoxic culture filtrate was filter sterilized by passing through a 0.22 µm syringe filter unit (Millipore). To attain different treatment concentrations, various volumes of this filtrate were dispensed into culture tubes containing the embryo induction medium precooled to 35 – 45 °C after autoclaving. The different volumes of toxin added were 0.1, 0.2, 0.5, 0.8 and 1 % (v/v) designated as T_1, T_2, T_3, T_4 & T_5 media respectively. Friable embryogenic calli were placed onto the toxin media and maintained at 25°C in the dark. After 6 weeks, the surviving calli were transferred to toxin free medium for proliferation where it was kept for another 3 weeks under the same culture condition. This process of selection on toxin medium and subsequent multiplication on toxin free medium was repeated once again, after which the surviving calli were maintained in the toxin free medium for embryo induction. Simultaneously, a control culture was initiated without toxin treatment and maintained over embryo induction medium.

Induced embryos were subcultured for further developments like embryo maturation, germination and plant regeneration. Protocols as well as the media combinations for these different developmental stages were as described in an earlier report on plant regeneration through somatic embryogenesis in *Hevea* (Sushamakumari

et al., 2000b). Embryo maturation was attempted in modified MS medium supplemented with different concentrations (0.2-2.0 μM) of the growth regulators Kinetin and IBA along with 0.1μM Abscisic acid (ABA). Mature embryos obtained were transferred for germination and plant regeneration into modified MS medium with different levels (1.0-10.0 μM) of BAP, Kinetin and GA_3. Other additives in these media comprised of sucrose (5%), charcoal (0.2 %), phytagel (0.3 %) and organic supplements including coconut water (5%) and banana powder (800 mg/l). The cultures were incubated at 25±2^{0}C in culture room provided with Philips white fluorescent light regulated for 16/8 hr light and dark photoperiod. The regenerated plantlets were subsequently transferred to half strength regeneration medium with decreased levels of sucrose (3%) and phytagel (0.25%), as a preparatory step for acclimatisation. The pH of all media was adjusted to 5.6 with 1 N KOH or 1 N HCl prior to autoclaving (121°C and 1.06 kg cm^{-2} pressure for 15 min).

Regenerated plantlets with mature leaves were subjected to acclimatisation by transferring to earthenware pots containing presterilized potting medium consisting of a mixture of sand and Soilrite in the ratio 1:1 and keeping under diffused sunlight at 25±2 °C. In order to ensure a microclimate with high humidity which is suitable for the plantlets, the pots were initially covered with polythene bags. The humidity was gradually reduced by putting holes in the covers. After attaining atmospheric humidity the covers were removed. The plants which survived this initial phase of hardening were transferred to big polybags filled with garden mixture and kept in the shade house under partial shade. Later the plants with at least two whorls of mature leaves were established in the field and maintained with regular watering and manuring for further foliage development.

5.2.4. Laboratory level confirmation of tolerance of the regenerated plants towards CLFD

In order to confirm whether the plantlets regenerated from the culture filtrate habituated cultures have acquired the desired tolerance towards Corynespora infection, different laboratory level experiments were performed. The experimental group of plants consisted of one plant P_1 raised from the treatment T_2 and three plants (P_2, P_3 and P_4) raised from the treatment T_3. One plant (P_0) raised from cultures not exposed to CCF treatment was selected as control. The confirmation experiments

include leaf wilt bioassay, vacuum infiltration of leaf segments, measurement of electrolyte leakage of leaf discs, detection of ROS and assay of enzymes chitinase, catalase and peroxidase. Each experiment was performed with three replicates.

5.2.4.1.Leaf wilt bioassay

A sensitive enough leaf wilt bioassay was employed for testing the disease tolerance of the field established somatic plants raised from CCF challenged callus in comparison to the control plants raised from the unchallenged calli. In this assay, healthy young leaflets of light green stage were excised from both groups of plants. Two sample sets were prepared as a) test sample where leaves from both the groups were immersed in the CCF and b) control sample where leaves from both the groups were kept in sterile water. In the detached leaves, a fresh cut was made at the end of the petiole keeping it under water and the exuding latex was removed completely using a tissue paper. Immediately after this, the cut end of the leaves belonging to the test set were immersed in McCartney bottles containing the toxin (1 ml crude toxin diluted in 4 ml sterilized water) while those belonging to the control set were immersed in McCartney bottles containing 5ml sterile water. The leaflets were incubated at room temperature and wilting intensity was assessed after 24 hrs.

5.2.4.2.Vacuum infiltration of leaf segments

In this bioassay, leaf segments were subjected to CCF treatment *via* vacuum infiltration. Healthy young leaves of the same stage as those used in the leaf wilt bioassay were collected from both groups of plants and cut into small pieces. One set of leaf pieces from both the groups were subjected to vacuum infiltration with 2 ml of CCF for 15 min and these served as the test samples. Another set of leaf pieces from both groups were subjected to vacuum infiltration with autoclaved distilled water for 15 min and these served as the control samples. Leaf pieces were then transferred to moistened filter paper in Petri dishes and kept at room temperature. The effect of vacuum infiltration of the leaf segments with CCF was assessed through observation of the necrotic damage using a stereo microscope. Moreover the colour difference between necrotic and non necrotic tissue was made further clear using eye dropper tool, for which the samples were scanned with an HP Laser Jet 3052 Scanner and observed in Photoshop (Adobe Systems Incorporated, San Jose, CA, USA).These

observations were taken at different time intervals of 24, 36 and 48 hrs of the treatment.

5.2.4.3.Electrolyte leakage of leaf discs

In this experiment six day old disease free leaves were collected from both the groups of plants. Circular leaf discs of size 1 cm diameter were then punched out, rinsed thoroughly with sterile distilled water followed by air drying. One set of five leaf discs from both the groups were subjected to vacuum infiltration with 2 ml of CCF for 15 min and these served as the test samples. Another set of five leaf discs from both groups were subjected to vacuum infiltration with autoclaved distilled water for 15 min and these served as the control samples. All these samples were kept at 25°C for 24 hrs, after which the leaf discs were washed quickly for three times with distilled water. Later, each set of leaf discs were soaked in 5 ml of autoclaved distilled water in separate glass tubes and kept for 12 hrs in the dark, for leaching of electrolytes. The content of each tube was shaken well and the initial electrical conductivity of both control and treated sets was measured as C1 and T1 respectively using a conductivity meter. The leaf tissues were then killed by autoclaving for 15 min to release all ions from the tissues, cooled to 25°C after which the final electrical conductivity was measured as C2 and T2. The same experiment was repeated with two more treatment durations (48 and 72 hrs). In each case the percentage of electrolyte leakage (EL %) was calculated using Sullivans formula, % Injury = {1-[1-(T1/T2)/1-(C1/C2)]} x 100. Three replicates were included for this experiment. Statistical analysis (ANOVA) at the 5% level was performed for all parameters.

5.2.4.4.Chitinase assay

Chitinase activity was measured in the two groups of plants using the following samples

a) Control samples – Fresh leaves collected directly from field established plants belonging to both the groups

b) Toxin treated samples – Application of CCF by leaf puncture technique, retaining the leaves on the plant itself for 15 hrs after which the leaves were harvested

c) Water treated control - Sterile water was applied instead of CCF and the leaves were kept on the plant itself for 15 hrs before harvesting

After 15 hrs, all leaf samples were harvested from respective plants and their midribs were removed. Leaf extract was prepared from each sample as follows. 0.5 g of leaf tissue was homogenised in 1.0 ml of 0.1 M sodium citrate buffer (pH 5.0) using a pre-chilled mortar and pestle. The crude homogenate was then centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant thus obtained was referred to as the crude extract, for the estimation of enzyme activity of chitinase. The activity of chitinase in all leaf extracts was estimated using colloidal chitin as the substrate and the activity was determined using dinitrosalicylic acid (DNS) method.

5.2.4.5. Detection of ROS by NBT staining

The CCF was applied onto pale green leaves of both groups of plants by leaf puncture technique, retaining the leaves on the plant itself for 15 hrs. Leaf discs of untreated pale green leaves from both the groups served as the control sample. Treated leaves were harvested and leaf discs of size 1 cm diameter were punched out of the treated leaves (excluding the punched areas), vacuum infiltrated with NBT at 1mg/ml in staining buffer (10 mM KPO_4, 10 mM NaN_3) for 30 min and further stained overnight at room temperature. The leaves were then destained overnight in 95% ethanol and the produced ROS were observed macroscopically.

5.2.4.6. Antioxidant assays

Catalase and peroxidase activities were measured in the two groups of plants using three samples as mentioned above for chitinase assay.

After 15 hrs, all leaf samples were harvested from the respective plants and their midribs were removed. Leaf extract was prepared from each sample as follows. 1 g of leaf tissue was homogenised in 1 ml of solution containing 50 mM potassium phosphate buffer (pH- 6.8), 0.1 mM EDTA and 2% PVPP using a pre-chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was used as the crude enzyme extract for determining the antioxidant assays. The CAT activity was determined following the method proposed by Cakmak and Marschner, (1992). The POX activity was assayed following the colorimetric

determination of pyrogallol oxidation according to Kar and Mishra, (1976). The procedures for these two assays are as described in Chapter 4.

5.2.5. Multiplication and field evaluation of *in vitro* raised plants

Field established plants were allowed to grow till grafting stage, for six months. Standard field management practices were done throughout this period. Simultaneously stock plants (root stocks) were developed from assorted seeds. Once the collar diameter of stock seedlings achieved 25 cm circumference, buds were collected from both control and experimental plants and patch buddings were done. Green budding was carried out using the buds from these plants. After 21 days, budding success was assessed. One week after this, each plant was cut back from 1cm above the bud patch and buds were allowed to sprout and maintained for further growth.

5.3. Results

5.3.1. *In vitro* screening and selection of tolerant lines

It was observed that supplementation of toxin in the medium interfered with further development of the embryogenic callus. Growth and proliferation rate of the callus decreased significantly as the toxin concentration in the medium increased. As evidenced in Fig.5.1a, callus growth and proliferation on T_1 medium was not at all affected, indicating that 0.1% toxin was inadequate for inhibiting the callus growth. Also, these calli retained the slight creamy colour of the embryogenic callus initially cultured over this medium. On the contrary, the growth of calli on T_5 medium containing the highest toxin level (1.0%) was completely inhibited as a result of which these calli turned brown and gradually dried up. The high concentration of toxin in this medium could have destroyed the viability and regenerative capacity of the callus thereby leading to the tissue death. In the intermediate levels of toxin (T_2, T_3 & T_4), even though overall growth of the callus was badly affected, a few surviving calli could be retrieved which were transferred to toxin free medium for proliferation.

The selected surviving calli proliferated well on CCF free medium. These calli were chosen visually based on their ability to grow in the first toxin level and on an appearance suggesting that this selected calli would differentiate if placed on

regeneration medium without CCF. In the second cycle of selection, on tox medium, about 20% of these proliferated calli died whereas the toxin insensitive ca responded well yielding more embryogenic calli.This embryogenic calli, when cultur again on toxin free medium, gave rise to clusters of globular proembryos, initiati embryo induction. Thus upon prolonged culture in the toxin free medium for about 6 weeks, embryo induction was acheived in the callus emerged from all the thr treatments T_2, T_3 & T_4, with a frequency of 50% (Fig. 5.1b). Embryos at differe stages of embryogenesis organization from globular, heart to cotyledonary stages cou be observed simultaneously. Embryo maturation was obtained in medium fortified wi Kinetin (1.35 µM), IBA (2.0 µM) & ABA (0.1µM). Some of the mature cotyledona embryos exhibited bipolar differentiation followed by root and shoot development upo transfer to medium containing BAP (6.75 µM), Kinetin (5 µM) and GA_3 (3 µM), whi a few others showed abnormalities in development. Some became dormant, son shootless with only roots, some with distorted or multiple cotyledons which final failed to grow into complete plantlets. Normal germinating embryos regenerated in full plantlets in the medium containing growth regulator combination of BAP (4.5 µM Kinetin (2.5 µM) and GA_3 (6 µM) (Fig. 5.1c).

Even though embryo induction was obtained in all the three treatments T_2, and T_4, there was considerable difference in further development of the embryos fro the different treatments. Germination and plant regeneration could be obtained on from the embryos of T_2 & T_3 whereas the embryos developed in T_4 showed sever abnormalities whereby further growth was suspended and finally those embryos g dried up. The comparatively higher toxin level in T_4 medium might have interfer with the normal development of the embryos raised in that medium. Embryos deriv from treatments T_2 and T_3, exhibited continous growth leading to successful pla regeneration (Table.5.1). Hence these lines can be considered as tolerant lines wi regenerative ability.

<table>
<tr><th>Treatments</th><th>Toxin enriched embryo induction medium (6 weeks)</th><th>Embryo induction medium without toxin (3 weeks)</th><th>Toxin enriched embryo induction medium (6 weeks)</th><th>Embryo induction medium without toxin (3 weeks)</th><th>Embryo maturation medium</th><th>Embryo germination medium</th><th>Plant regeneration medium</th></tr>
<tr><td>T_1 (0.1%)</td><td>Callus proliferation without any growth inhibition</td><td colspan="6">Since there was no selection the cultures from this treatment were not continued</td></tr>
<tr><td>T_2 (0.2%)</td><td rowspan="3">50-70% inhibition of callus growth. Surviving calli transferred to non toxin medium</td><td rowspan="3">Proliferation of the selected surviving calli</td><td rowspan="3">Death of 20-30% of the proliferated calli whereas rest of the calli continued proliferation</td><td rowspan="3">Embryo induction with a frequency of 50% leading to the development of embryos at different stages of embryogenesis</td><td rowspan="3">Development of mature cotyledonary embryos</td><td rowspan="2">Bipolar differentiation of induced embryos</td><td rowspan="2">Regeneration of normal germinating embryos into complete plantlets</td></tr>
<tr><td>T_3 (0.5%)</td></tr>
<tr><td>T_4 (0.8%)</td><td>Abnormal embryo development</td><td></td></tr>
<tr><td>T_5 (1.0%)</td><td>Complete inhibition of callus growth</td><td colspan="6">No further development</td></tr>
</table>

Table 5.1. Effect of CCF on callus growth and on different developmental stages of embryos

During the hardening process of regenerated plants, an initial survival rate of 20% was obtained in the earthenware pots (Fig. 5.1d). Survival rate of these hardened plants upon transfer to big polybags (Fig. 5.1e) was 80%. Thus around 20% of the regenerated plants could be hardened and were taken for field planting (Fig. 5.1f). All these plants established well under field conditions with no mortality rate.

5.3.2. Laboratory level confirmation of tolerance of the regenerated plants towards CLFD

The reliable plant bioassays performed in the laboratory to confirm the CLFD tolerance of the *in vitro* selected variants showed positive and encouraging results. In leaf wilt bioassay, the two group of plants- control (P_0) and experimental (P_1, P_2, P_3 and P_4) reacted differently towards the exposure to CCF as evidenced in Fig. 5.2. In the control plant (P_0), leaves subjected to CCF treatment showed wilting symptoms, within 24 hrs of treatment, characterized by browning and curling up of leaves, initially at the tip which then proceeded gradually towards the leaf base and complete wilting was noticed by about 48 hrs (Fig. 5.2a). In the experimental group, leaves of the plants P_2, P_3 and P_4 (derived from line T_3) showed no signs of wilting and

remained almost intact until 48 hrs of treatment [Fig. 5.2(c-e)]. In contrast, leaves of the plant P_1 (derived from line T_2) showed slight wilting symptoms, within 24 hrs of treatment, characterized by browning and curling up of leaves, initially at the tip which then proceeded gradually towards the leaf base and increased wilting was noticed by about 48 hrs (Fig.5.2b). On the contrary, in both the groups, the control set of leaves immersed in sterile distilled water remained intact without any wilting signs until 48 hrs.

In the vacuum infiltration experiment, the same trend was observed in terms of the damage occurred due to the toxic effect of the CCF (Fig. 5.3). In the control group, leaf segments of plant (P_0) vacuum infiltrated with CCF started showing necrotic symptoms by 24 hrs of treatment. Intensity of necrosis increased considerably with increase of exposure time, showing dark brown lesions followed by partial and complete collapse of tissue by 48 hrs of treatment. In the experimental group, leaf segments of the plants P_2, P_3 and P_4 remained green without any necrosis even after 48 hrs of CCF treatment, indicating tolerance of these plants towards the CCF. Leaf segments of plant (P_1) showed slight necrosis after 36 hrs of CCF treatment which got intensified by about 48 hrs. On the contrary, leaf segments from both the groups, vacuum infiltrated with distilled water remained the same without any sign of necrosis. Moreover the colour difference between necrotic and non necrotic tissue was made clear using eye dropper tool.

The control plant P_0 and plant P_1 in the experimental group exhibited enhanced electrolyte leakage whereas the plants P_2, P_3 and P_4 did not show much electrolyte leakage. All plants in both groups showed lower EL% values at 24 and 72 hrs of incubation, while the same plants showed maximum EL% values at 48 hrs of incubation (Fig. 5.4). The control plant P_0 showed the maximum electrolyte leakage (64.79 %) at 48 hrs incubation. The experimental plant P_1 also exhibited an enhanced electrolyte leakage of 50.03%. Other *in vitro* raised plants P_2, P_3 and P_4 exhibited a lower EL% of 22.19%, 24.13% and 24.47% respectively. In the variance analysis (ANOVA) applied to the data set, the effects due to the *Hevea* plants x treatment interaction were highly significant ($p=0.00018$). The significance of *Hevea* plants x treatment effects was compared separately for each plant at different treatment durations using Duncan Multiple Range Test (DMRT) calculated on the basis of mean

EL per cent values. Analysis of variance on the DMRT data showed that plant belonging to the control group (P_0) and experimental plants differed significantly with regard to electrolyte leakage in response to CCF treatment. The plants regenerated were thus ranked based on their mean response to the treatment, from the more sensitive (P_0, P_1) to the less sensitive (P_2, P_3 and P_4).

Comparison of chitinase activity before and after treatment (Fig. 5.5) revealed that treatment with the CCF significantly increased the enzyme activity in both the group of plants. The increase was found to be significantly higher in plant P_2 (0.07 U) followed by plants P_3 (0.06 U) and P_4 (0.06 U). The increase was found to be much lower in both P_1 (0.02 U) and P_0 (0.015 U). This difference in the rise in intrinsic chitinase activity following CCF treatment clearly indicates the differences in tolerance of the respective plants towards CLFD. Thus, the significant increase in chitinase activity of P_2, P_3, and P_4 plants following CCF treatment compared to P_0 and P_1 plants indicated their enhanced CLFD tolerance.

ROS was produced in the leaf discs under the stressful conditions of CCF treatment. The treated leaf discs of control plant (P_0) and experimental plant P_1 showed very high levels of formazan precipitate formation after NBT staining, indicating higher amount of ROS production in these plants (Fig. 5.6). However, in the case of experimental plants P_2, P_3 and P_4, no colour formation was observed after NBT staining indicating the absence of ROS production. These findings indicate that the cells of the experimental plants P_2, P_3 and P_4 were less damaged by CCF treatment, whereas cells of P_0 and P_1 became infected with crude fungal exudates and suffered extensive cell damage during treatment.

The leaf samples of the plants under study had significantly different catalase and peroxidase activity profiles before and after CCF treatment. Compared to the respective controls, increase in CAT activity was shown by all treated plants belonging to both the groups. Of these, plants P_0 and P_1 showed the highest CAT activity. The increased catalase formation is intended to remove the ROS (H_2O_2) produced in these clones as a result of infection. After treatment, the mean values of CAT activity progressively increased over the controls. Differences between mean values of treated samples and untreated samples were considered significant at $p<0.05$

(Fig. 5.7). The P_0 and P_1 have shown the highest statistically significant increase of 160% and 140% respectively over their controls. The other plants P_2, P_3, and P_4 showed only a slight increase in CAT activity by 33.33%, 50% and 33.33% respectively and that this increase was found to be statistically insignificant.

Upon CCF treatment, all plants under study showed increased POX activity compared to their respective controls. Of these, plants P_0 and P_1 showed the highest POX activity. The increased peroxidase formation is intended to remove the ROS produced in these clones as a result of infection. After treatment, the mean values of POX activity progressively increased over the controls. Differences between mean values of treated samples and untreated samples were considered significant at $p<0.05$ (Fig. 5.8). The plants P_0 and P_1 have shown the highest statistically significant increase of 140% and 83.33% respectively over their controls. The other plants P_2, P_3, and P_4 showed only a slight increase in POX activity by 33.33%, 66.66% and 66.66% respectively and that this increase was found to be statistically insignificant.

5.3.3. Multiplication and field evaluation of *in vitro* raised plants

It was noted that 20% buddding success was obtained [Fig. 5.9 (a-e)]. Once the multiplied plants reached two whorl stage they can be taken to CLFD hot spots for field screening towards CLFD tolerance.

Fig.5.1. ***In vitro*** **selection of tolerant callus and plant regeneration. a- Difference in the sensitivity of calli of** ***Hevea*** **clone RRII 105 on exposure to different levels of CCF T_1-T_5 (0.1- 1.0 % v/v), b- Somatic embryos induced from the proliferated surviving callus, c- Embryo germination and plant regeneration, d- Plants undergoing initial phase of hardening in earthenware pots, e- Plants hardened in big polybags, f- Hardened plantlets established under the field conditions**

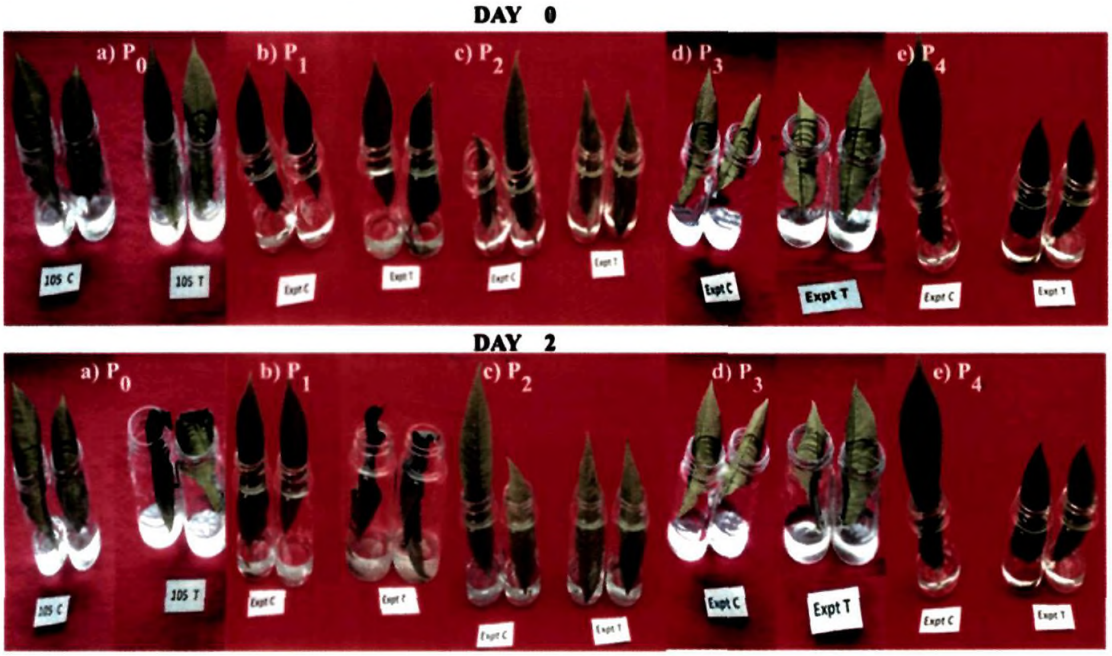


Fig.5.2. Leaf wilt bioassays performed on control plant (P_0) and experimental plants (P_1, P_2, P_3 and P_4). Day 0- Immediately after immersing the cut end of leaves in CCF (T) / distilled water (C), Day 2- Observed for disease symptoms after incubation for 48 hrs

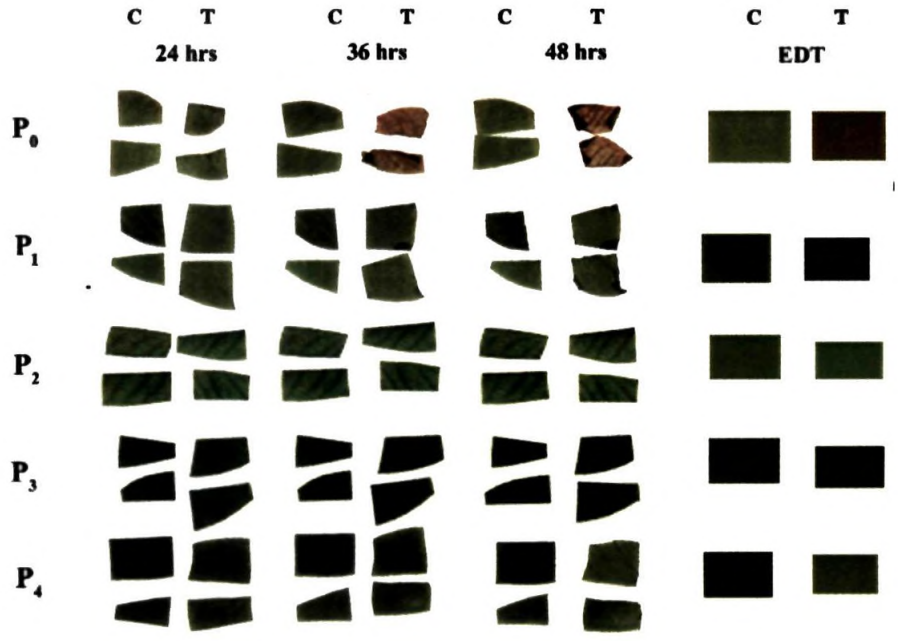
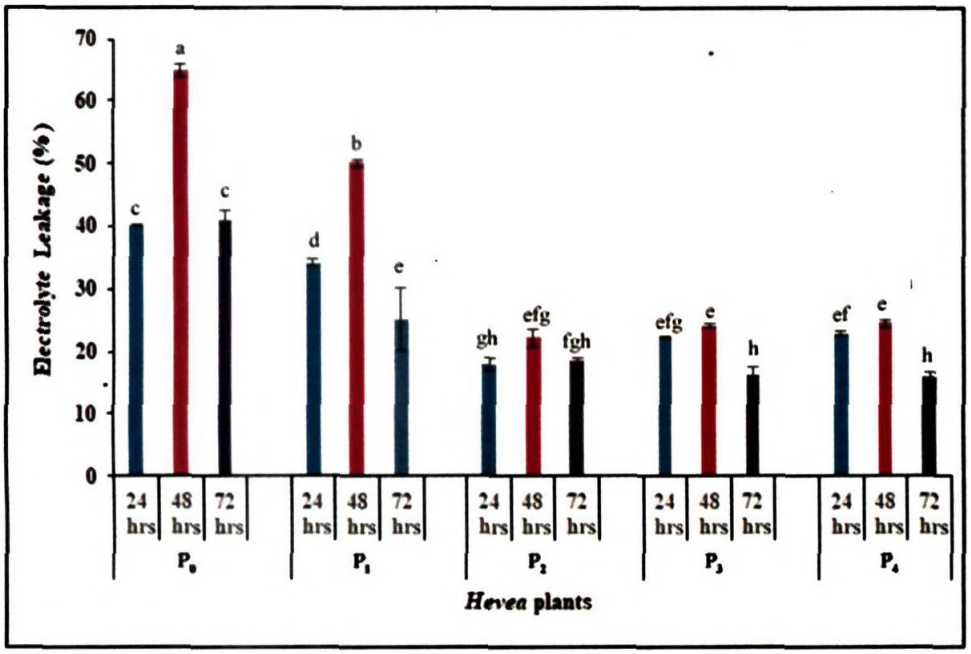
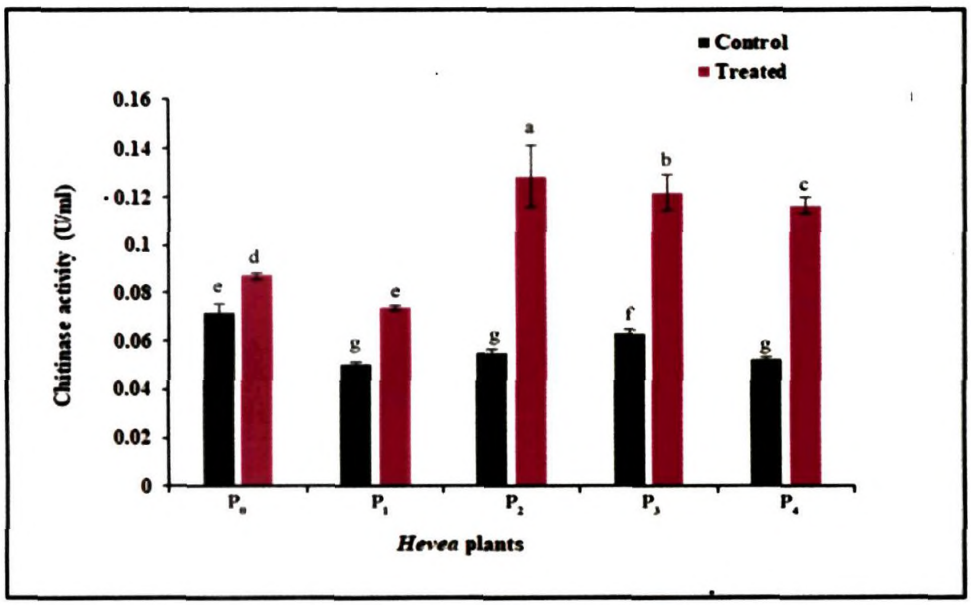


Fig.5.3. Vacuum infiltration showing difference in the sensitivty of leaf segments towards the culture filtrate treatment. P_0- control plant; P_1, P_2, P_3 and P_4- experimental plants P_1- experimental plant C- water treated control, T- toxin treated, EDT- eye dropper tool at 48 hrs



CD (treatment x time) =4.51, p≤ 0.05

Fig.5.4. Electrolyte leakage in leaf discs of control plant (P_0) and experimental plants (P_1, P_2, P_3 and P_4) at different treatment durations. Top letters on each bar indicate the significance of the difference between treatments



CD (clone x treatment) = 0.034 with p ≤ 0.05

Fig.5.5. Comparison of chitinase activity in leaves of control plant (P_0) and experimental plants (P_1, P_2, P_3 and P_4) before and after CCF treatment

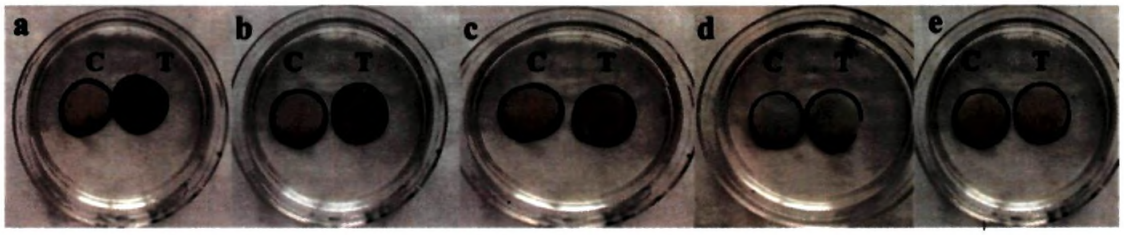
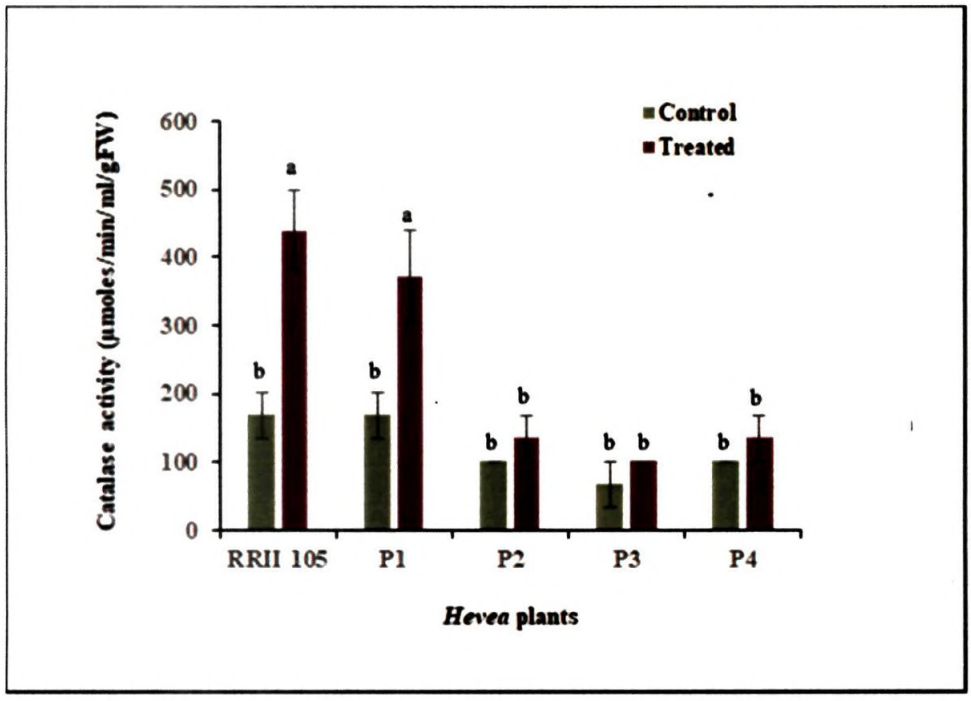
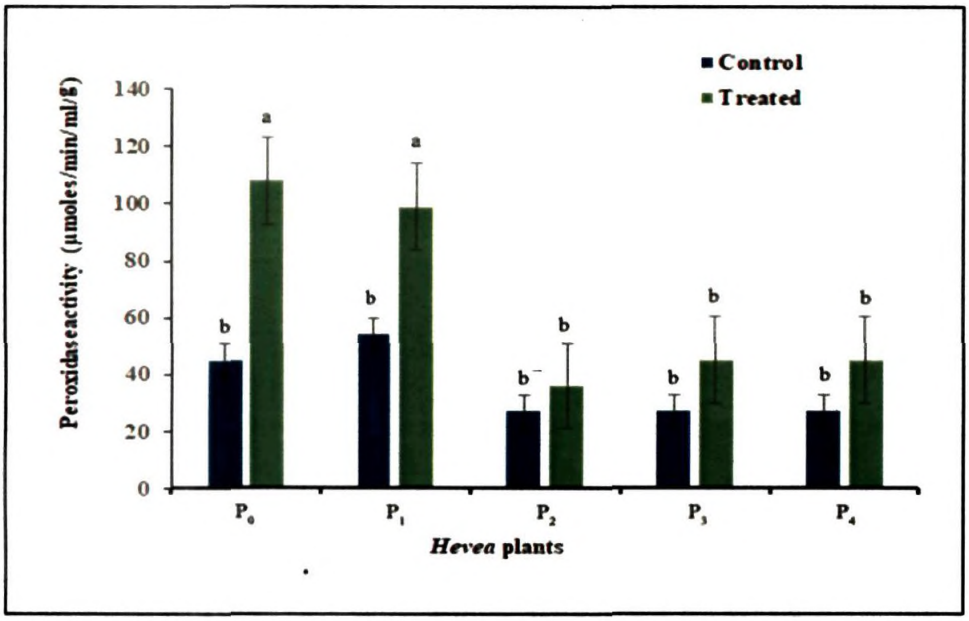


Fig. 5.6. Detection of ROS by NBT staining in the leaf discs of control plant (P_0) and experimental plants (P_1, P_2, P_3 and P_4). ROS production is visualized as dark blue coloration whereas absence of dark blue-colored formazan deposit indicates no ROS production. a- Control plant P_0, b-plant P_1, c-plant P_2, d-plant P_3 and e-plant P_4. C- Control, T-Treated.



CD (clone x treatment) = 0.001 with $p \leq 0.05$

Fig 5.7. Comparison of catalase activity in leaves of control plant (P_0) and experimental plants (P_1,P_2, P_3 and P_4) before and after CCF treatment



CD (clone x treatment) = 0.001 with $p \leq 0.05$

Fig 5.8. Comparison of peroxidase activity in leaves of control plant (P_0) and experimental plants (P_1, P_2, P_3 and P_4) before and after CCF treatment

Fig 5.9. Multiplication of *in vitro* regenerated plants. a- Stock plants, b-Bud grafted plant, c- plant with sprouted bud, d and e- cut back plant along with the sprouted bud

5.4. Discussion

Present study focused on developing CLFD tolerant lines from callus cultures of a highly susceptible clone RRII 105 through *in vitro* toxin challenge. The initial findings of this study demonstrate the applicability of crude culture filtrate in the development of CLFD tolerant plants through *in vitro* selection. The use of relatively small explants, suspension cells, or protoplasts can reduce the risk of escapees and chimaeras. Accordingly, embryogenic calli of appropriate size was used as the explant in our study. The small size of this explant, along with its friable nature, explains the uniformity with which cells of the *Hevea* calli were exposed to the CCF contained in the growth medium. Since *C.cassiicola* has been reported to produce a host selective toxin called cassiicolin (Breton *et al.*, 1997), it is very likely that this cassiicolin toxin present in the CCF may act as a primary determinant of pathogenesis. CCF's ability to reproduce Corynespora infection met the condition put forward by Aducci *et al.*, (1997), that for a culture filtrate to be assigned the role of a disease factor, it must be able to cause at least a portion of the disease when placed in healthy plant cells.

It has been observed that a high concentration of CCF in the growth medium considerably affected the *in vitro* growth of embryogenic callus cultures thereby resulting in browning and necrosis of those cells whereas growth of calli on medium containing low concentration of CCF was little affected. This correlation between *Hevea* callus susceptibility and CCF toxicity suggests that CCF could be used for *in vitro* screening of Corynespora disease resistance. The same phenomenon of brown discolouration of callus in response to treatment with different culture filtrates has been reported in many crops such as bean (Fernandez *et al.*, 2000); sugarcane (Sengar *et al.*, 2009); garlic (Zhang *et al.*, 2012) and pineapple (Borras-Hidalgo and Bermudez, (2010). The brown discoloration of the calli accompanying necrosis may be due to the presence of phenolic compounds and their oxidation products. According to Hidalgo *et al.*, (1998), callus browning is a good indicator of callus sensitivity to culture filtrate.

All of these findings confirm the assumption that CCF contains either *C.cassiicola* toxin or toxic metabolites. Several previous studies observed that the toxin present in the culture filtrate could limit cell growth and that the cells of the host

plants were more sensitive towards the toxin than those of non-host plants Selvapandiyan *et al.*, (1988); Hartman *et al.*, (1984); Malepszy and El Kazzaz, (1990). A considerable decrease in the growth and proliferation rate of the *Hevea* callus was observed when the CCF concentration in the medium was increased. The same trend of decreased or inhibited callus growth was observed when calli of garlic cultivars were exposed to increased concentration of *Sclerotium cepivorium* culture filtrate (Zhang *et al.*, 2012). In our study, two cycles of selection against CCF followed by proliferation resulted in the production of calli that are tolerant to the CCF, because such a selection cycle reduces chimerism and allows only resistant cells to proliferate on the selection medium. This selection cycle was in consistence with those reported by El-Kazzaz and Abdel-Kader, (1998); El-Kazzaz *et al.*, (1999) and El-Kazzaz and Ashour, (2004).

In the current experiment, calli cultured over different concentrations of CCF responded differently, with the callus growth rate being inversely proportional to the concentration of CCF in the medium. The growth and proliferation of *Hevea* calli on T_1 medium containing low concentration (0.1%) of CCF remained unaffected, indicating that this concentration was insufficient to inhibit callus growth. On the contrary, growth of *Hevea* calli was completely inhibited on T_5 medium containing a high concentration (1.0%) of CCF. The high concentration of toxin in this medium could have destroyed the viability and regenerative capacity of the callus thereby leading to tissue death. Even though embryo induction was obtained from selected surviving calli retrieved from media supplemented with intermediate levels of toxin (T_2, T_3 & T_4) germination and plant regeneration could be obtained only from the embryos of T_2 & T_3 whereas the embryos developed in T_4 showed several abnormalities whereby further growth was suspended and finally those embryos got dried up. The comparatively higher toxin level (0.8%) in T_4 medium might have interfered with the normal development of the embryos raised from that medium. The inhibitory effect of CCF may be due to the formation of some growth regulators and/or some antimetabolic inhibitors secreted by the fungus which suppress the expression of genes responsible for embryo regeneration as explained by El-Kazzaz *et al.*, (1999). Successful plant regeneration was achieved only from T_2 (0.2%) and T_3 (0.5%) lines. One possible explanation for this may be that some of the cells within

the calli derived from T_2 and T_3 lines might have acquired CLFD tolerance so as to survive in the presence of toxin. Such cells might have proliferated, underwent differentiation and gave rise to embryogenic callus, embryos and finally plantlets. The regenerated plants from T_2 and T_3 lines showed no phenotypic variation and were similar to the control plants raised from calli unchallenged with CCF.

A number of successful methods have been developed to select for pathogen resistance in plants by using culture filtrate or purified toxins in *in vitro* selection experiments. Potato plants resistant to culture filtrate of *Phytophthora infestans* (Behnke, 1979), peach plants insensitive to culture filtrate of *Xanthomonas campestris pv.pruni* (Hammerschlag, 1988), soyabean plants resistant to culture filtrate of *Septoria glycines* (Song *et al.*, 1994), groundnut plants resistant to culture filtrate of *Cercosporidium personatum* (Venkatachalam *et al.*, 1998), carnation plants resistant to culture filtrate of *Fusarium oxysporum f.sp.dianthi* (Thakur *et al.*, 2002), turmeric plants tolerant to culture filtrate of *Pythium graminicollum* (Gayatri and Kavyashree, 2005), sunflower plants tolerant to culture filtrate of *Alternaria helianthi* (Rao and Ramgoapl, 2010), citrus plants tolerant to culture filtrate of *Phytophthora parasitica* (Savita *et al.*, 2011) and garlic plants resistant to culture filtrate of *Sclerotium cepivorum* (Zhang *et al.*, 2012) are some successful studies.

Previous workers have attributed different reasons for the acquired tolerance of toxin exposed cells. Some researchers believed that by applying selection pressure of various selection agents [natural pathogen isolates, modified fungal cultures, phytotoxins and pathotoxins, bacterial phytotoxins, fungal host-selective toxins (HSTs), fungal cell wall components (elicitors)] to susceptible cells, resistance could be induced. One explanation is that the reason behind a few cells acquiring disease tolerance might be due to the loss or alteration of the toxin's target or through detoxification (Hammond-Kosack and Jones, 1997) indicating that the selected tissues might get altered to produce toxin suppressors or detoxifying enzymes which either suppress or detoxify the crude toxin (Savita *et al.*, 2011). Another possible explanation for acquired tolerance of regenerated plants, as claimed by El-Kazzaz and Ashour, (2004) when selecting genetically resistant cucumber plants to wilt pathogen, is that the genetic background of these plants may contain heterogeneous gene structures for CCF/toxin resistance. Furthermore, El-Kazzaz *et al.*, (1999) suggested

that resistance of regenerated plants could be attributed to the induction of somaclonal variations within selected host callus cells. The acquired tolerance may be due to the ability of cells to neutralize the toxin or because the toxin is recognised by specific receptors (Breton and d'Auzac, 1999; Umoh and Fashoranti *et al.*, 2018) and Jayasinghe, 2000). In his comprehensive review paper titled "tissue culture and the selection of pathogen resistance to pathogens," Daub, (1986) explained that a successful selection mechanism needs that the selecting agent either kills the cells or significantly inhibits their growth, allowing the resistant cells to outgrow the susceptible cells and thus be identified. Similarly, in our study as well, culture filtrate in the medium might have acted as a selection agent thereby killing or inhibiting the infected cells and thus helping in the identification of cells resistant to the culture filtrate.

The same bioassays that were used in preliminary experiments to test the CLFD sensitivity of susceptible and tolerant *Hevea* clones were used here also to confirm the Corynespora tolerance of the experimental plants of clone RRII 105 regenerated through *in vitro* selection. Based on the result of leaf wilt bioassay, three plants derived from line T_3 were found to be CCF tolerant, while the plant derived from line T_2 turned out to be susceptible towards CCF. The results obtained by vacuum infiltrating CCF into the leaf segments of regenerated plants were also in accordance with the leaf wilt bioassay.

In addition, measurement of electrolyte leakage can be considered as another effective bioassay to confirm CLFD tolerance of CCF selected *in vitro* regenerated plants. Significantly lower electrolyte leakage was observed in the experimental plants P_2, P_3 and P_4 as evidenced by the electrolytic conductivity values whereas the control plant P_0 and plant P_1 showed significantly higher electrolyte leakage values indicating high electrolyte leakage. The lower electrolyte leakage values of plants P_2, P_3 and P_4 indicate that when the leaf discs of these plants were infiltrated with CCF no alterations occurred in their membrane permeability. Hence these plants can be considered as tolerant. In the case of plants P_1 and P_0, higher electrolyte leakage values indicate that membrane permeability got altered upon infiltration with CCF and these plants are termed susceptible.

Biochemical assays were carried out for the enzymes chitinase, catalase and peroxidase in the plants belonging to both control (P_0) and experimental (P_1, P_2, P_3 and P_4) groups before and after CCF treatment. The experimental plants P_2, P_3 and P_4 exhibited enhanced chitinase activity upon CCF treatment whereas the corresponding increase in plants P_0 and P_1 was found to be much less indicating that the former set of plants are more CLFD tolerant than the latter group. According to previous reports, *chitinase* gene activation has been widely used to improve disease tolerance in plants (Khan and Shih, 2004; Ganesan and Jayabalan, 2006). Disease resistance was induced in mango (Jayasankar and Litz, 1998), berries (Robert *et al.*, 2002), cotton (Ganesan and Jayabalan, 2006), (Shimizu *et al.*, 2013), peanut (Abd El-Hai and Ali, 2019) and other plants by the expression of chitinase enzymes through fungal culture filtrate. The inoculation of *Colletotrichum fragariae* spores on healthy tomato leaves also increased chitinase activity (Fiume and Fiume, 2003). All of these reports, as well as our findings, suggest that one reason for the induction of CLFD tolerance in *in vitro* raised Corynespora susceptible *Hevea* clone RRII 105 plants could be due to the activation of PR protein chitinase in response to CCF selection pressure.

After CCF treatment, cells of the P_0 and P_1 plants showed high levels of blue formazan residue formation in response to NBT staining, indicating increased levels of $O2^{\bullet-}$ generated in these cells whereas cells of the plants P_2, P_3 and P_4 showed no coloration in response to NBT staining indicating the absence of $O2^{\bullet-}$ generation in these cells. To put it in another way, P_0 and P_1 plants were susceptible to CLFD, and as a result, their cells became infected with crude fungal exudates and suffered extensive cell damage, whereas plants P_2, P_3 and P_4 were tolerant to CLFD, and that their cells were less damaged by CCF treatment. According to Vijaya kumar *et al.*, (2008) *in vitro* screening methods using culture filtrate induces oxidative stress which can often lead to elevated intracellular levels of reactive oxygen species (ROS).

Antioxidant enzymes are thought to play an important role in the defense mechanism by scavenging ROS produced in response to CCF-induced oxidative stress. In the present study, both CAT and POX activity increased in CCF-treated leaves of P_0 and P_1 plants compared to their untreated controls, indicating that these clones produced more H_2O_2 to defend themselves from CCF stress. On the other hand, the increase in CAT and POX activity in response to CCF treatment was

significantly lower in experimental plants P_2, P_3 and P_4 indicating a lower amount of H_2O_2 production which is sufficient to defend the minor damage caused by CCF stress. Increased levels of ROS coupled with increased activity of the antioxidant enzymes CAT and POX in the case of plants P_0 and P_1 in response to CCF treatment indicate that these plants are susceptible to CLFD. Similarly, lower levels of ROS as well the enzyme activities of CAT and POX in the plants P_2, P_3 and P_4 upon CCF treatment revealed that these plants are more tolerant towards CLFD.

In the light of the observations from the bioassays as well as the biochemical assays conducted using plants regenerated from CCF challenged calli of clone RRII 105 along with control plant of RRII 105 raised from unchallenged calli, it can be concluded that the experimental plants raised from the treatment line T_3 proved to be more tolerant towards CLFD compared to the control plant as well as a plant originated from the treatment line T_2. In other words, it is possible to develop Corynespora tolerant plants through *in vitro* challenge with CCF. Acclimatisation followed by field establishment of these *in vitro* raised plants, both experimental and control plants could be achieved successfully. Eventhough the plants P_2, P_3 and P_4 proved to be tolerant towards CLFD in the laboratory level studies their disease tolerance in the field need to be yet confirmed. For the same purpose these tolerant plants have been multiplied by bud grafting and are at different stages of development. These plants after acquiring the required maturity need to be planted in CLFD hot spots for field level confirmation of tolerance. Once confirmed the tolerant plants can be included in future breeding programmes. Moreover, field trials may be performed with these tolerant plants to assess yield and other secondary parameters. If any such tolerant plant turns out to be a superior one endowed with high yield and other promising secondary characters, it can be released as a new variety after necessary confirmatory trials. In such trials, if a tolerant candidate with high yield and other promising secondary parameters could be identified it can be further tested and be released as a new variety.

Despite the apparent popularity and practicality of this *in vitro* selection method for developing resistant species, only a few of these studies have been carried out beyond the laboratory. Field trials of alfalfa selected for tolerance to *Fusarium oxysporum* f. sp. *medicaginis* culture filtrates have yielded promising results (Hartman

and Knous, 1984). Wenzel, (1985) conducted field testing of selected potato lines resistant to culture filtrate of *Phytophthora infestans*, but due to damage caused by other diseases it was impossible to accurately determine their resistance to late blight. According to Daub, (1986), Sacristan conducted field evaluations of four spring rape lines selected for resistance to *Phoma lingam* culture filtrates and found that these lines were more resistant than the susceptible parent, but not as resistant as some currently available varieties.

Chapter 6

Cloning and characterization of *chitinase* gene for the construction of a chitinase gene expression cassette

6.1. Introduction

Plants possess both preformed (structural and biochemical) and inducible defense systems to fight against the invasion of pathogens. The production of pathogenesis related (PR) proteins are one of the most common and well-documented inducible defense responses in plants. Among the various PR proteins, chitinase and β -1, 3 glucanase are the two most important hydrolytic enzymes induced in many plant species in response to various fungal infections. Both these proteins are antifungal enzymes which are capable of degrading the cell wall components of invading pathogenic fungus. Generally, PR protein production serves as an alternative defense strategy in plants when the first line of defense is compromised by certain fungal infections. However, most fungi rapidly develop resistance to these PR proteins, resulting in the breakdown of plant defense mechanisms. In order to combat this, larger levels of PR protein expression in the host would be preferable. This can be done by overexpressing PR genes in host plant by means of transformation. The two most prioritised PR proteins used in genetic engineering procedures for the production of fungal disease-resistant plants are chitinases and glucanases. The reason for this priority is that inducing these enzymes causes rapid breakdown of chitin and glucans, which are the major components of most fungal cell walls, preventing those pathogens from entering leaf tissue.

Chitinases are hydrolytic enzymes responsible for the degradation of chitin, a high molecular weight linear polymer of N-acetyl-D-glucosamine units (Wang and Yang, 2007). They can be isolated from several sources like bacteria, fungi and plants. In numerous studies, transgenic plants expressing the *chitinase* gene demonstrated increased resistance to fungal diseases. The *Rhizopus oligosporus* fungal *chitinase* gene (*chil*) conferred antifungal activity to transgenic tobacco plants

against the discomycete pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Terakawa *et al.*, 1997). Transgenic cucumber [*Dendranthema grandiflorum* (Ramat.) Kitamura] plants harboring the rice chitinase cDNA (*RCC2*) exhibited enhanced resistance to gray mold (*Botrytis cinerea*) (Tabei *et al.*, 1998). Chrysanthemum (Takatsu *et al.*, 1999), grapewine (Yamamoto *et al.*, 2000) and trifoliate orange (Mitani *et al.*, 2006) are examples of some other transgenic plants with the same rice chitinase cDNA (*RCC2*). Transgenic soybean plants expressing the two antifungal protein genes, bean chitinase (*Chi*) and barley ribosome-inactivating protein (rip) genes were developed by Li *et al.*, (2004). Tohidfar *et al.*, (2005) developed transgenic cotton plants that expressed the bean chitinase (*Chi*) gene, which increased resistance to the verticilliosis-causing *V. dahlia*. Transgenic potato plants harboring *chitinase*, (*ChiC*) isolated from *Streptomyces griseus* strain HUT 6037 showed enhanced resistance to fungal infections and herbicide applications (Khan *et al.*, 2008). Fungal-resistant finger millet was developed by Antony and Ignacimuthu in 2012 by inserting rice *chitinase* gene (*chi11*) into its genome. Thus genes coding for chitinase enzymes are widely used in genetic engineering processes to produce fungal disease resistant transgenic crop plants.

Some of the most popular and widely cultivated clones of *Hevea brasiliensis* are susceptible to CLFD and the matter is of great concern and needs to be addressed due to its devastating effects. Eventhough *Hevea* possesses various defense mechanisms, the severity of various fungal infections led to the breakdown of its inherent defense mechanisms. Hence, it is imperative to develop alternative strategies for the development of durable and broad-spectrum resistance to fungal diseases. Therefore, as discussed above, genetic engineering can be considered as a good alternative tool to increase the tolerance of various *Hevea* clones to this fungal disease of *Hevea brasiliensis*. Chitinase, one of the major PR proteins, can be selected for serving this purpose. *Hevea brasiliensis* contains many classes of *chitinase* genes (Neuhaus, 1999), the expression of which is increased in response to different infections. According to reports, the *chitinase* gene family in rubber consists of 39 members from the glycoside hydrolase (GH) classes. Based on their sequence and structural features, there are classified as 7 Class I [GH 19], 1 Class II [GH 19], 16 Class III [GH18], 5 Class IV [GH19], and 10 Class V [GH18] sequences.

Furthermore, Class I chitinases have a similar sequence to plant lectins. Notably, lysozyme-like domains in Class III chitinases, also known as hevamines, provide specialised machinery for pathogen resistance. EST and transcriptome analyses show that Class IV and V chitinases are only expressed in leaves, whereas others are expressed in both leaves and latex (Misra, 2015). In light of the previous facts and the importance of engineering plants for improving disease tolerance, goal of this study was to attempt genetic transformation for introducing *chitinase* gene in *Hevea brasiliensis*. For serving this purpose, a *chitinase* gene expression cassette has to be constructed in the binary vector pCAMBIA 1301 so that this gene construct could be used to transform callus cultures of CLFD susceptible *Hevea* clones. As mentioned earlier, since different types of chitinase families have already been reported in *Hevea*, *chitinase* gene isolation could be done from *Hevea* itself so that this transformation process is considered as an attempt to over-express the *chitinase* gene in *Hevea* so as to confer CLFD tolerance.

6.2. Materials and Methods

6.2.1. Bacterial strains and nutrient medium

The bacterial strain *Escherichia coli* DH5α engineered by American biologist Hanahan was used in this study. The medium used for *E. coli* was Luria-Bertani (Tryptone-10 g, Yeast extract- 5 g, NaCl-10 g, Bacteriological agar-15 g, Distilled water-1000 ml, pH 7.4). This medium was supplemented with antibiotics such as ampicillin or kanamycin when used to grow transformed *E. coli* cells.

6.2.2. Plasmids

1) Bacterial cloning vector pGEM-T Easy:

They are bacterial cloning vectors with high-copy-number and are used in the cloning studies. The pGEM-T Easy pre-linearized Vector contains 3′-T overhangs at the insertion site to provide a compatible overhang for purified PCR products. A multiple cloning site within the α-peptide coding region of the enzyme β-galactosidase is flanked by T7 and SP6 RNA polymerase promoters. Recombinant clones can be identified directly by blue/white screening on indicator plates owing to insertional inactivation of the α-peptide.

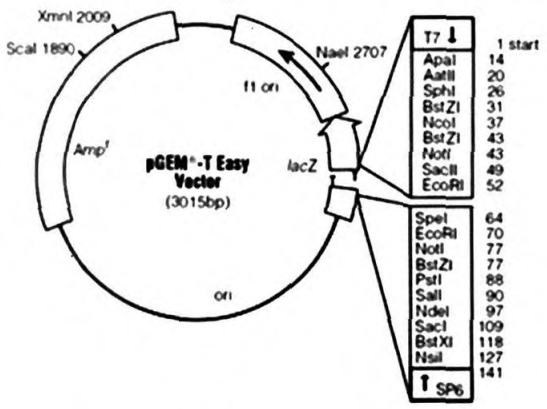


Fig.6.1a. Bacterial cloning vector pGEM-T Easy (Promega, USA).

2) Plant expression vector pRT 101:

It is a novel plant expression vector that permit expression of any gene of interest under the control of strong constitutive 35S promoter from Caulifower Mosaic Virus (CaMV) and its corresponding polyadenylation (poly (A)) signal (Topfer *et al.*, 1993). It allows the insertion of foreign genes, either as transcriptional fusions behind plant specific promoters, and the pRT plasmids are typical (Walden *et al.*, 1990). It also carries multiple cloning sites between the promoter and terminator regions as well as an ampicillin resistance gene that can be used as a selectable marker for bacterial selection.

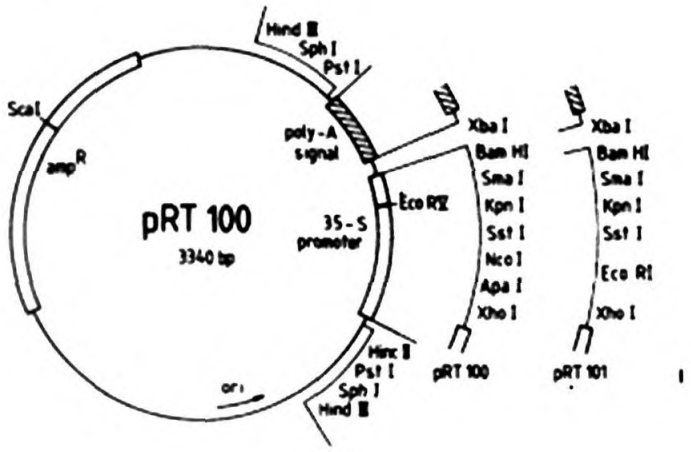


Fig.6.1b. Plant expression vector pRT 101

3) Binary vector pCAMBIA 1301:

The binary vector pCAMBIA 1301 is widely used in transformation experiments. *Escherichia coli* strain DH5∞ was used for the maintenance and

multiplication of these plasmids. It contains kanamycin and hygromycin resistance genes as the selectable markers for bacterial and plant selection, respectively, as well as GUS (β-glucuronidase) reporter genes, all of which are driven by the strong, nominally constitutive CaMV 35S promoter from Caulifower Mosaic Virus (Kathiresan *et al.*, 2009).

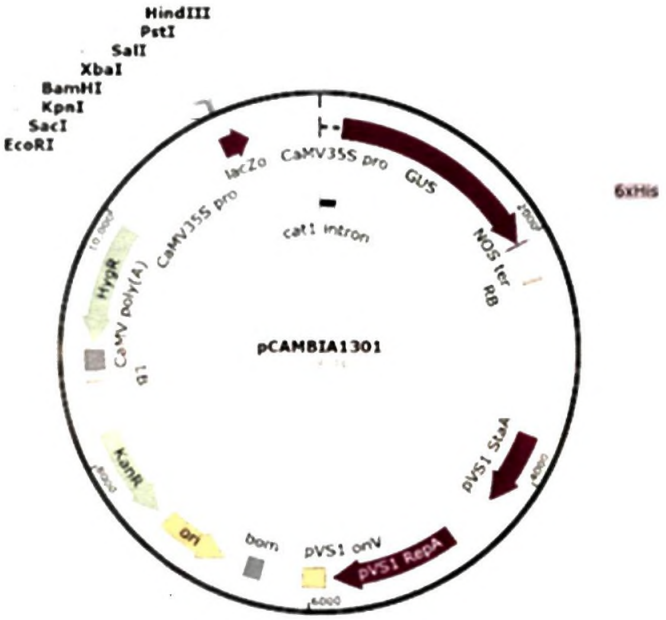


Fig.6.1c. Map of binary vector pCAMBIA1301. Binary vector pCAMBIA1301 harbours hygromycin resistance (HygR) as antibiotic marker gene and GUS as reporter gene both under the control of CaMV 35S promoter

6.2.3. Enzymes and reaction kits

Restriction enzymes *Bam H1* (New England Biolabs, USA), *Sac I* (Promega, USA) and *Hind III* (Invitrogen, USA) were used for DNA digestion. Taq DNA polymerase from Sigma-Aldrich, USA was used for routine PCR experiments.

Following kits were used for different purposes in this study:

- pGEMT-Easy vector cloning kit (Promega, USA)
- QIAquick gel extraction kit (Qiagen, Germany)
- GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich, USA)
- TURBO DNA-free Kit (Ambion, USA)

6.2.4. Buffers and Solutions

The majority of buffers and solutions were prepared in the manner as described by Russell and Sambrook, (2001). Stock solutions of antibiotics were prepared in sterilized Milli-Q water [Ampicillin (100 mg/ml) or Kanamycin (50 mg/ml)] and sterilized by filtration (0.22 μm) before adding it to the cooled medium (50°C).

6.2.5. Designing and reconstitution of primers used in the study

To design the primers for the *Hevea brasiliensis chitinase* gene, available mRNA sequences encoding *Hevea* chitinase reported from gene sequences of previously known chitinases submitted to the Genbank database of NCBI site were downloaded, and multiple sequence alignment was performed using the CLUSTAL W software (Thompson *et al.*, 1994). Based on the consensus sequences obtained from the multiple sequence alignment, *chitinase* gene specific primers were designed using the primer BLAST tool. The two primer sets designed and used for *chitinase* gene amplification were either flanked with (No.1) or without (No.2) restriction sites (***Sac I*** in the forward primer and ***Bam H I*** in the reverse primer).

Sl. No	Primer Sequence		Primers
1	Chit RT F	5′CTGCGAGCTCTTTACTTGCAATAATGGCCA 3′	Forward
	Chit RT R	5′ TCGAGGATCCATTTCTCCCAATTCAAAGTAC 3′	Reverse
2	Chit F	5′CTTGCAATAATGGCCAAAAG 3′	Forward
	Chit F	5′GGGTGCACCGAATAATTTCT 3′	Reverse
3	T7	5′ TAATACGACTCACTATAGGG 3′	Forward
	SP6	5′ ATTTAGGTGACACTATAGA A 3′	Reverse
4	pRT F	5'AACATGGTGGACCACGACACT 3'	Forward
	pRT R	5'CAGGTCACTGGATTTTGGTT 3'	Reverse
5	pCAM F	5′GAATGCTAGAGCAGCTTGAGCT 3′	Forward
	pCAM R	5' TAATTGCGTTGCGCTCACT 3'	Reverse

Table.6.1. List of primers used in the study

Designed primers were purchased from Eurofins Genomics India Pvt, Ltd (Bangalore, India). The volume of diluent (in microliters) required to reconstitute a 100 µM stock of a purchased primer is specified on its Certificate of Analysis. The most commonly used diluent is TE buffer (10:0.1).The working stock (10 µM) can be prepared by diluting 10 µl of the stock solution with 90 µl of sterilized Milli-Q water.

6.2.6. Cloning and sequence characterization of *chitinase* gene

As we learned more about the significance of various defense related genes in *Hevea brasiliensis* and their applications, it was realised that the *chitinase* gene was the most suitable and effective gene for the genetic engineering studies for developing fungal disease-resistant *Hevea* plants. Our research also revealed that the chitinase enzyme plays an important role in inducing defense responses in CLFD susceptible *Hevea* clones in response to CCF infection. The inherent chitinase activities have been found to be much higher in tolerant clones compared to the susceptible clones. In view of this, the same gene was chosen for the construction of the *chitinase* gene expression cassette in the binary vector pCAMBIA 1301. The advancements in genetic engineering technology along with the development of *Agrobacterium*-mediated transformation have enabled the incorporation of foreign genes into the desired crop plants for the development of disease-resistant varieties.

Gene cloning and its sequence characterization studies are preliminary works that need to be done prior to the construction of a gene expression cassette. These preliminary works were done to understand more about the interested gene sequence as well as to create multiple identical copies of that gene: However, in order to conduct these preliminary studies, some basic experiments must be performed first to prepare the necessary materials needed for the study. These basic experiments include gene identification, isolation, designing of chitinase gene specific primers and PCR (Polymerase Chain Reaction) amplification of *chitinase* gene by these designed primers.

6.2.6.1. Isolation of *chitinase* gene from *Hevea brasiliensis* clone RRII 105

Genomic DNA isolation

Isolation of genomic DNA from the young leaves of *Hevea* clone RRII 105 was done by CTAB extraction method. The steps involved in this extraction method

are as follows. 500 mg of leaf sample was ground to a fine powder in liquid nitrogen. The powder was then transferred to a fresh 15ml Falcon tube. After that, 5ml of CTAB extraction buffer (2 % CTAB, 20 mM EDTA·Na_2·$2H_2O$, 1.4 M NaCl and 100 mM Tris, pH 8) preheated at 60°C was added to the tube and vortexed well. The samples were then incubated at 60°C in a water bath and mixed well by inversion in every 10 min for 45 min-1 hr. After cooling, an equal volume of chloroform: isoamyl alcohol (24:1) was immediately added to the solution and mixed thoroughly by inversion. The sample was again incubated for 10 min at room temperature. After incubation, the sample was centrifuged for 10 min at 10,000 rpm at 22°C. The supernatant was then decanted into a new 15 mL Falcon tube without disturbing the interphase. 25 µl of RNase A (10 mg/ml) was added to the supernatant and incubated at 37°C for 1-1.5 hrs. Following this, extraction with an equal volume of chloroform: isoamyl alcohol (24:1) was performed. After incubation, the sample was centrifuged for 10 min at 7,000 rpm at 22°C. For precipitation of DNA, the upper aqueous phase was pipetted out into a new tube, and 0.6 volumes of isopropanol was added and gently mixed by inversion. The precipitated DNA was then pooled out and transferred to a 1.5 ml eppendorf tube containing 1 ml of ice cold 75% ethanol. The sample was then centrifuged for 10 min at 10,000 rpm at 4°C. Thereafter, the supernatant was decanted without disturbing the pellet. The pellet was subsequently washed with 1 ml of ice cold 75% ethanol. Ethanol was carefully decanted and DNA pellet was air-dried at room temperature. The pellet was dried long enough to remove alcohol, but without completely drying the DNA. Once dried, DNA was suspended in 200 µL of 10: 0.1 TE buffer (10 mM Tris, pH 8, 1 mM EDTA). The quality and concentration of the extracted DNA was analysed using a NanoDrop UV/Vis spectrophotometer. After this the extracted DNA was run on 0.7% (w/v) agarose gel.

6.2.6.2. Polymerase Chain Reaction for amplification of *chitinase* gene

PCR amplification of *chitinase* gene from the isolated genomic DNA was done using the second set of chitinase primers. The reaction was carried out in a total volume of 20 µl containing 200 µM dNTP's, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase and 50 ng template DNA, in a thermal cycler. The PCR profile was as follows: 95°C for 5 min followed by 40 cycles of amplification at 94°C for 30 sec, 55°C for 30 sec, 72° C for 1 min and with a final extension at 72°C for 10

min. The PCR products were then electrophoresed on 1.0 % agarose gel along with λ-DNA *EcoR1* and *Hind III* double digest as DNA marker, at 70V for 2 hrs.

Agarose gel electrophoresis, the most commonly performed laboratory technique plays an important role in the purification and quality control of DNA fragments used in gene cloning. The following are the steps involved in casting and running an agarose gel. 0.45 g agarose was melted in 45 ml TAE buffer solution in a conical flask or bottle. After cooling the solution to around 50°C, 3.2 µl of ethidium bromide stock (10mg/ml) was added to the molten agarose solution. The melted agarose was then poured into a casting tray. When the gel solution solidifies, a comb was laid across the end of the casting tray to make wells. After the gel had solidified, it was placed in an electrophoresis chamber filled with 1X TBE buffer. Samples of PCR products were prepared for electrophoresis by mixing them with loading dyes. These samples were loaded to the sample wells with a clean micropipette and the gel was run at 80V for 30 min to 1hr. After removing the gel from the running chamber, it was analysed and photographed by placing it on a Stratagene Eagle Eye Gel Imaging system to visualize the DNA. The gel slice containing the amplified gene fragment was excised from the gel with a sterile scalpel and was kept in a clean eppendorf tube. This amplified gene fragment present in gel slice must be separated and purified from agarose gel before being used for cloning and sequencing experiments.

Extraction and purification of PCR product was done by using the QIAquick PCR Purification Kit. The manufacturer's protocol for this extraction process is as follows. The gel slice that had been excised was weighed. To 1 volume of gel (100 mg gel~100 l), three volumes of buffer QG were added. The sample was then incubated for 10 min at 50°C. To dissolve the gel, the tube was vortexed every 2–3 min. After that, 1 gel volume of isopropanol was added to the sample and mixed thoroughly. A QIAquick spin column was placed in a 2 ml collection tube and the sample was applied to this column. The column along with tube was then centrifuged for 1 minute. After centrifugation, the flow-through was drained and the QIAquick column was put back into the same tube. For washing the sample, 750 µl of buffer PE was added to the QIAquick column and centrifuged for 1 minute. The QIAquick column was replaced into a clean 1.5 ml microcentrifuge tube. To elute DNA, 10- 20 µl of sterilized Milli-Q water was added to the centre of the QIAquick membrane and

the column along with tube was centrifuged for 1 minute. The quality and concentration of the eluted DNA was analysed using a NanoDrop UV/Vis spectrophotometer.

6.2.6.3. Sequence characterization of *chitinase* gene

The sequence structure of *chitinase* gene was initially studied by analyzing the available genomic database of *Hevea*. Accordingly, the available mRNA sequences of chitinase (AJ010397.1, DQ873889.2 and AJ007701.1) were blasted with the *Hevea* whole genome in the public database. In order to further confirm the identity and sequence structure of *chitinase* gene, it is necesssary to determine the nucleic acid sequence of *chitinase* gene. A series of experiments must be carried out to determine this. Experiments in the series include cloning the PCR amplified *chitinase* gene fragment (extracted and purified from the agarose gel) into the pGEM-T easy vector, transforming *E. coli* DH5∞ competent cells with the recombinant pGEM-T easy vector, isolating recombinant plasmid DNA from transformed colonies, and sequencing this isolated recombinant plasmid with vector specific primers. In order to avoid repetitive boredom, the methodology of each of the experiments mentioned here is well explained while describing about the construction of the *chitinase* gene expression cassette, which indeed was the next focus of our study.

6.2.7. Construction of *chitinase* gene expression cassette

Before cloning a particular gene, it is necessary to learn more about the open reading frame (ORF) of that gene. ORF's are coding regions of gene which begins with an initiation codon and ends with a termination codon. So to learn more about the ORF of *chitinase* gene which is intended to be cloned, total RNA was isolated from the leaves of *Hevea brasiliensis* clone RRII 105 and first strand of cDNA was synthesised using this isolated RNA as the template. Second strand of cDNA was synthesised by PCR amplification using *chitinase* gene specific primers. This amplified *chitinase* gene was extracted from the gel and cloned into the pGEM-T easy cloning vector. The recombinant plasmids obtained after cloning were then used to transform *E.coli* cells for plasmid multiplication. Multiplied plasmids were isolated and sequenced to obtain the ORF of the concerned *chitinase* gene.

6.2.7.1. Isolation of RNA

All reagents required for performing this experiment were prepared in 0.1% DEPC-treated water to inactivate the RNases. Total RNA isolation from the infected leaves of *Hevea* clone RRII 105 was done by pine tree method as follows.

DAY 1:

Frozen tissue sample (1.5 g of leaf) was ground to a fine powder in liquid nitrogen using prechilled mortar and pestle. Powdered sample was then quickly transferred into a RNase-free falcon tube containing 10 ml of RNA extraction buffer [2% CTAB (hexadecyltrimethylammonium bromide), 2% PVP (polyvinylpyrrolidone K 30), 100 mM Tris-HCl - pH 8.0, 25 mM EDTA, 2.0 M NaCl and 0.5 g/l spermidine are mixed and autoclaved. 2% β-mercaptoethanol was added just before use] prewarmed at 65°C. The contents were then mixed thoroughly by inverting the tube for 3-4 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the homogenate and mixed thoroughly by vortexing. The mixture was centrifuged at 7000 rpm for 5 min at room temperature to separate the phases. The top aqueous phase was then transferred to another centrifuge tube, and the chloroform: isoamyl alcohol extraction was repeated. The mixture was centrifuged again with the same conditions as before. The top phase was recovered again and 0.3 volumes of 8 M LiCl were added. The contents were mixed thoroughly by inverting the tube for 3-4 min. It was then stored at 4°C, to allow overnight precipitation.

DAY 2:

Prior to start of the experiment the SSTE buffer was prewarmed at 65°C in a water bath. The tube containing LiCl-precipitated sample was centrifuged at 7,000 rpm for 20 min at 4°C and the supernatant was discarded. The pellet was washed again with 2 ml of 2 M LiCl and then centrifuged at 7,000 rpm for 20 min at 4°C. The supernatant was discarded and the pelleted RNA was dissolved in 500 µl of prewarmed SSTE solution [1 M NaCl, 0.5% SDS, 10 mM Tris HCL (pH 8.0), 1 mM EDTA (pH 8.0)] by vigorous vortexing for about 45 min to ensure complete resuspension. An equal volume (500 µl) of chloroform: isoamyl alcohol was added to the tube and mixed well. The contents were then transferred to a new 1.5 ml RNAse-free eppendorf tube. After that, an equal volume (500 µl) of chloroform: isoamyl

alcohol was added to it and mixed well. The eppendorf tube was then centrifuged at 12,000 rpm for 10 min. The upper aqueous phase was transferred to a new 1.5 ml RNAse-free microfuge tube. To this, double the volume of ethanol was added and the contents were mixed by inversion. RNA was allowed to precipitate by keeping the sample at -80°C for 1 hr. The sample was then centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant (ethanol) was carefully aspirated off. The RNA pellet was resuspended in 50µl DEPC-treated water and mixed well by vortexing. The tubes were then incubated at 65°C for 2 min before placing them on ice. RNA concentration and purity was determined using a NanoDrop UV/Vis spectrophotometer and then the extracted RNA was run on 1% agarose gel. To remove contaminating genomic DNA, 1µg of RNA was incubated with 1µl of RNase free DNase (10U/µl) for 15 min at 37^0C. The quantity of RNA obtained was again determined by spectrophotometer.

cDNA synthesis

First strand of cDNA was synthesised using the isolated RNA as the template and second strand of cDNA was synthesised by PCR amplification using *chitinase* gene specific primers flanked by the restriction sites (***Sac I*** in the forward primer and ***Bam HI*** in the reverse primer).

Forward primer – 5′CTGCGAGCTCTTTACTTGCAATAATGGCCA 3′

Reverse primer- 5′ TCGAGGATCCATTTCTCCCAATTCAAAGTAC 3′

a) First strand synthesis:

4.5 µl of RNA sample (1 µg) and 1µl of oligo (dT) (0.5 µg) were mixed together and heated at 70°C for 10 min. Soon after incubation the mixture was chilled on ice for 2 min. To this mixture, 10X RT-PCR buffer (4 µl), RNase inhibitor (0.3 µl), dNTP mix (1.2 µl), reverse transcriptase from Murine leukemia virus (1 µl) and DEPC-treated water (2.7 µl) were added sequentially and mixed well. The resulting mixture was then incubated at 42°C for 1 hr, and the reaction was terminated by heating at 92°C for 10 min. This mixture was then stored at -80°C before using it as template for PCR amplification.

b) Second strand synthesis

PCR amplification of *chitinase* gene in synthesized cDNA was carried out using the set of primers tagged with the restriction sites *Sac 1* and *Bam H1*. The PCR reaction was carried out in a total volume of 20 µl containing 200 µM dNTP's, 0.2 µM of each primer, 2mM $MgCl_2$, 0.5 U Taq DNA polymerase and 50 ng template DNA, in a thermal cycler. The PCR profile was as follows: 95°C for 5 min followed by 40 cycles of amplification at 94°C for 30 sec, 55°C for 30 sec, 72° C for 1 min with an extension for 10 min at 72°C. PCR product was then electrophoresed on agarose gel along with λ-DNA *EcoR1* and *Hind III* double digest as DNA marker, at 70V for 2 hrs. The desired band was excised and eluted from the gel by QIAquick Gel Extraction Kit following the manufacturer's protocol.

6.2.7.2. TA Cloning for integration of *chitinase* gene in pGEM-T easy vector

The eluted PCR amplified product was then cloned into pGEM-T easy vector (Fig. 6.1a) by means of ligation. For successful ligation reaction, ratio of plasmid and insert DNA is crucial and should be adjusted to 1:3. Ligation of the PCR product into pGEM-T easy vector was done as follows: 0.5 µl of pGEM-T easy vector, 1.5 µl of insert (eluted DNA), 2.5 µl of 2X ligation buffer and 0.5 µl of T_4 DNA ligase were mixed well and incubated overnight at 16°C in a cooling water bath. After ligation, heat inactivation of ligase enzyme was done by incubating the ligation mix at 65°C for 10 min. This cloned vector was named pGEM-T-Chitinase. The products of ligation reaction were finally introduced into chemically competent DH5α *E.coli* cells.

Transformation of DH5α competent cells

The goal of this transformation is to replicate the *chitinase* gene in amounts suitable for further analysis and manipulation studies.The competent cells of the *E. coli* DH5α strain were prepared using a modified version of the protocol described by Russell and Sambrook, (2001). High efficiency competent cells prepared according to this protocol were stored frozen at -80°C in small aliquots. Just before the start of transformation experiments, these frozen competent cells were taken out from the freezer and placed in an ice bath for about 5 min until completely thawed. The contents were gently mixed by twirling the tube. Transformation of *E.coli* DH5α competent cells with the recombinant pGEM-T easy vector was done as follows. 1.5

µl of ligation mixture was added to 50 µl of competent cells taken in a chilled eppendorf tube and mixed by gentle tapping. The mixture was then incubated on ice for 20 min and heat shock was given by immediate shifting of the chilled mixture to a water bath (preheated at 42°C) for exactly 1 min. The tube was then immediately returned to ice for 5 min. To this, 500 µl of nutrient medium (LB broth containing 25% sucrose) was added and the tube was incubated at 37°C for 1.5 hrs with shaking at 220 rpm. The cell suspension was centrifuged at 3000 rpm for 3 min and 400 µl of supernatant was discarded and the pellet was resuspended in the remaining medium. 50-100 µl of transformed *E.coli* cells were spread onto LB agar plates containing ampicillin (20 µl of 100 mg/ml stock), X-gal (20 µl of 20 mg/ml stock) and IPTG (40 µl of 100 mM stock) using a sterile spreader and the plates were incubated overnight at 37°C.

Screening transformants for inserts

Selection of the transformed host cells and identification of the clone containing the desired gene fragment is made possible with the help of selectable markers (genes conferring resistance to ampicillin) and reporter or scorable marker genes (which provide blue/white screening *via* α factor complementation on X-gal medium) present in the pGEM-T easy vector. The selectable markers also select cells that have been transformed by plasmids, though they may not always be the cells with the recombinant plasmids. As a result, these selection steps do not absolutely guarantee the presence of DNA insert in the selected recombinant cells. So additional tests are needed to be done to confirm that cloning was successful. This may be accomplished by means of various techniques such as colony PCR, restriction fragment analysis and/or DNA sequencing. Moreover, these techniques also provide an insight into the size of gene, coding regions (ORFs) of the gene, prediction of amino acid sequence of protein coded by the gene, gene sequence homology etc which help us to understand more about the *chitinase* gene. Positive colonies (white) growing on ampicillin containing plate were checked for insertion of *chitinase* gene by colony PCR using gene specific primers. For this, a small amount of some randomly chosen transformed bacterial colonies (white) were removed seperately from the ampicillin containing transformation plates and dispensed into individual PCR tubes each containing 1.5 µl of 10X PCR buffer, 0.3 µl of 25 mM $MgCl_2$, 0.3 µl of 10 mM dNTP

mix, 0.3 µl of 10 µM forward primer, 0.3 µl of 10 µM reverse primer, 0.12 µl of 5 U/µl heat stable Taq DNA polymerase and 12.18 µl sterile distilled water as master mix. Separate sterile toothpicks were used for picking each colony from the plate and dispensing it into individual PCR tubes. PCR was carried out using conditions appropriate for the gene insert as follows: 94°C for 7 min followed by 40 cycles of amplification at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1:15 sec with an extension at 72°C for 7 min. Colony PCR was performed using gene specific primers to check for the insertion of *chitinase* gene in the selected recombinant colonies. The PCR products were electrophoresed on 1% agarose gel along with λ-DNA *EcoR1* and *Hind III* double digest as DNA marker, at 70V for 2 hrs. The colonies, whose colony PCR tests turned out to be positive, were identified and cultured individually in LB broth (10 ml) containing ampicillin at 37°C with overnight shaking at 220 rpm.

Plasmid isolation, DNA sequencing and restriction digestion

Plasmid DNA was isolated from the cultured colonies using the GenElut HP Plasmid Miniprep Kit following the manufacturer's protocol. Sequence analysis of these colonies were performed using T7 and SP6 primers, thereby checking the complete ORF of the *chitinase* gene in the resulting recombinant plasmid pGEM-T-Chitinase. The vector sequences were trimmed from the selected good quality sequences and considered for sequence homology search using the BLASTn program at the National Centre for Biotechnology Information. Isolated plasmids were further checked for the incorporation of restriction sites *Sac1*and *Bam H1* flanked on both ends of amplified *chitinase* gene insert by digesting them with those restriction enzymes. The reaction mixture for restriction digestion was as follows: 5 µl of plasmid DNA, 1.5 µl of *Sac 1*, 1.5 µl of *Bam H1*, 0.3 µl of BSA, 3 µl of 10X buffer and 18.7 µl of water were mixed well and incubated overnight at 37°C. The restriction enzymes were heat inactivated the next day by incubating the restricted samples at 65°C for 25 min. Digested products were analysed on 1.5 % agarose gel. The obtained gel bands were checked to determine whether the results in the gel match the predicted sizes and the released fragment of *chitinase* gene flanked by the restriction sites was eluted from the agarose gel using QIAquick Gel Extraction Kit.

6.2.7.3. Subcloning of the *chitinase* gene fragment flanked with restriction sites into the intermediate vector pRT 101

Intermediate vector pRT 101 (Fig. 6.1b) contains a strong constitutive 35S promoter from Caulifower Mosaic Virus (CaMV) and its corresponding polyadenylation signal. For subcloning, pRT 101 vector was first linearized by digestion with two restriction enzymes *Sac 1* and *Bam H1*, which make their ends sticky. The sticky restriction fragment (cDNA encoding amplified *chitinase* gene flanked with restriction sites) released by digestion of pGEM-T- Chitinase construct with restriction enzymes *Sac1* and *Bam H1* was then subcloned into *Sac1* and *Bam H1* restricted pRT 101 vector. The procedure was carried out by ligation reaction as follows: 2.0 µl of linearized pRT 101vector, 5 µl of released gene fragment, 1.5 µl of 10X ligation buffer, 1.0 µl of T_4 DNA ligase and 5.5 µl of sterile distilled water were mixed well and incubated overnight at 16°C in a cooling waterbath. After ligation, heat inactivation of ligase enzyme was done by incubating the ligation mix at 65°C for 10 min. This ligated product (pRT101-Chitinase) was then used to transform chemically competent DH5α *E.coli* cells and the transformed colonies were selected on antibiotic (ampicillin) plates. Colonies were selected at random, and colony PCR was performed using gene specific primers to check for the integration of *chitinase* gene in the selected recombinant colonies. The colonies that turned positive for colony PCR were then processed for plasmid isolation. The gene insert in the extracted plasmids was again verified by DNA sequencing and restriction digestion. Sequence analysis of these colonies were performed using vector specific primers, thereby checking the sequence of *chitinase* gene and its flanked restriction sites in the resulting pRT101- Chitinase construct. The vector sequences were trimmed from the selected good quality sequences and considered for sequence homology search using the BLASTn program at the National Centre for Biotechnology Information. Isolated plasmids were further checked for the incorporation of *chitinase* gene fragment flanked with restriction sites *Sac 1*and *Bam H1* between the *Hind III* sites of pRT 101 vector by digesting them with *Hind III* restriction enzymes. The reaction mixture for restriction digestion was as follows: 5 µl of plasmid DNA, 2 µl of *Hind III*, 0.3 µl of BSA, 0.25 µl of 10X buffer and 14.75 µl of water were mixed and incubated at 37°C overnight. Heat inactivation of the restriction enzymes was done by incubating the

restricted samples at 65°C for 25 min. Digested products were analysed on 1.5 % agarose gel. The obtained gel bands were checked to determine whether the results in the gel match the predicted sizes, and the released gene fragment of the *chitinase* gene flanked by the restriction sites along with the strong 35S promoter from CaMV was eluted from the agarose gel using QIAquick Gel Extraction Kit.

6.2.7.4. Construction of *chitinase* gene expression cassette in binary vector pCAMBIA 1301

Binary vector pCAMBIA 1301 (Fig. 6.1c) carries *GUS* as reporter gene and hygromycin phosphotransferase gene (*hpt*) as antibiotic marker gene, both under the control of CaMV 35S promoter. pCAMBIA 1301 vector was first linearized by digestion with *Hind III* restriction enzyme. For the construction of *chitinase* gene expression cassette, the restriction fragment produced as a result of restriction digestion of pRT101-Chitinase construct comprising of restriction site tagged *chitinase* gene along with the strong constitutive 35S promoter from CaMV was cloned at *Hind III* restriction sites in binary vector pCAMBIA 1301 resulting in the construct pCAMBIA-Chitinase. Joining of the excised gene fragment from the pRT101- Chitinase construct with the linearized pCAMBIA 1301 vector was done by ligation reaction as follows: 2.0 µl of linearized pCAMBIA 1301 vector, 5 µl of released gene fragment with the complete cassette, 1.0 µl of 10X ligation buffer, 1.0 µl of T_4 DNA ligase and 1.0 µl of sterile distilled water were mixed well and incubated overnight at 16°C in a cooling water bath. After ligation, heat inactivation of ligase enzyme was done by incubating the ligation mix at 65°C for 10 min. This ligated product was then used to transform competent DH5α *E.coli* cells and the transformed colonies were selected on antibiotic (kanamycin- 50µg/ml) plates. Colonies were selected at random, and colony PCR was performed using gene specific primers tagged with restriction sites to check for the integration of *chitinase* gene in the selected recombinant colonies. The colonies that turned positive for colony PCR were then processed for plasmid isolation. The gene insert in the extracted plasmids was again verified by DNA sequencing. Sequence analysis of these colonies were performed using vector specific primers, thereby checking the sequence of *chitinase* gene and its flanked restriction sites along with the strong constitutive 35S promoter from CaMV in the resulting construct pCAMBIA-

Chitinase. The vector sequences were trimmed from the selected good quality sequences and considered for sequence homology search using the BLASTn program at the National Centre for Biotechnology Information.

6.3. Results

6.3.1. Isolation and sequence characterization studies of *chitinase* gene from *Hevea brasiliensis* clone RRII 105

Blast results of the available mRNA sequences of chitinase (AJ010397.1, DQ873889.2, AJ007701.1) with the *Hevea* whole genome in the public database revealed that the *chitinase* gene in these forms are intronless (Fig. 6.2.a). *Chitinase* gene fragment of size 936 bp was obtained by PCR amplification of the genomic DNA of *Hevea* clone RRII 105 using chitinase gene-specific primers (Fig. 6.2b). The purified PCR product when cloned into the pGEM-T Easy vector yielded the plasmid pGEM-T- Chitinase, which was used to transform competent DH5∞ *E. coli* cells. White recombinant colonies containing the *chitinase* gene insert were selected based on ∞ complementation. Colony PCR analysis of these recombinant plasmids with chitinase gene-specific primers also gave the desired gene band size of 936 bp (Fig. 6.2c). Colonies that turned positive for colony PCR were chosen, regrown, plasmid isolated and sequenced. BLASTn analysis of the recombinant plasmid (P1) sequence revealed 99.59% homology with *Hevea brasiliensis* chitinase mRNA (DQ873889.2) deposited in GenBank of NCBI (Fig. 6.2d). BLASTn analysis of the recombinant plasmid (P2) sequence revealed 100% homology with *Hevea brasiliensis* universal stress protein PHOS32-like mRNA (XM 021815095.1) deposited in GenBank of NCBI.

6.3.2. Preparation of *chitinase* gene expression cassette

Double-stranded cDNA was synthesized from the isolated RNA sample (Fig. 6.3a.). PCR amplification of cDNA with restriction site tagged chitinase gene specific primers yielded an amplified product of 973 bp (Fig. 6.3b), which is 37 bp larger than the size of the amplified chitinase gene fragment obtained by PCRamplific ation of the genomic DNA with chitinase gene specific primers. The incorporation of restriction site *Sac I* on 5' end and *Bam HI* on 3' end of *chitinase* gene fragment is the reason for this increase in size of the gene fragment. When restriction enzymes are present at the ends of the amplified *chitinase* gene fragment, it is easy to make

their ends sticky by cutting them with the appropriate restriction enzymes, allowing these sticky ended genes to be easily ligated to the cloning vector linearised with the same restriction enzyme. This restriction enzyme tagging of the gene insert also helps to simplify subsequent cloning procedures. Cloning of the restriction sites incorporated *chitinase* gene fragment (amplified chitinase cDNA fragment) into the pGEM-T Easy vector, resulted in the recombinant plasmid pGEM-T-Chitinase, which was used to transform competent DH5∞ *E. coli* cells. White recombinant colonies containing the chitinase gene insert were selected based on ∞ complementation. Colony PCR analysis of these recombinant plasmids with restriction sites tagged chitinase gene-specific primers gave an amplified gene band of size 973 bp which confirmed the integration of restriction sites tagged chitinase gene with in pGEM-T easy vector (Fig. 6.3c). Colonies that turned positive for colony PCR were chosen were chosen, regrown, plasmid isolated and sequenced. The complete ORF of chitinase gene was determined by submitting the chitinase gene sequence (Fig. 6.3d) obtained from plasmid DNA sequencing in a program named ORF Finder, which is available at the NCBI web site. In ORF Finder, the *chitinase* gene sequence got translated into six frames of nucleotide sequence and it displayed a graph showing a total of 9 ORF's, their position and length. The longest ORF was 936 bp long and contained 311 amino acids. BLASTp search for this ORF showed 99.36% homology with *Hevea brasiliensis* Hevamine- A, chitinase (P23472.2) deposited in Gen Bank of NCBI. Analysis of ORF's revealed that the integrated gene was *chitinase* itself. This was again confirmed by translation (936 bp/ 311 amino acids) and sequence homology results of obtained ORF. Consequently, it was confirmed that there will be no errors in the expression of this gene while using it in genetic engineering studies. BLAST search for cloned chitinase gene in pGEM-T-Chitinase showed 97% homology with the *Hevea brasiliensis* chitinase mRNA (DQ873889.2) deposited in Gen Bank of NCBI (Fig. 6.3e). Release of three fragments of size 2971 bp, 963 bp and 54 bp by digestion of the recombinant plasmid pGEM-T- Chitinase with *Bam HI* and *Sac I* restriction enzymes, again confirmed the incorporation of *Bam HI* and *Sac I* restriction sites on either ends of amplified chitinase gene fragment cloned in pGEM T- easy vector (Fig. 6.3f).

Subcloning of the released restriction sites tagged *chitinase* gene fragment (963 bp) into *Sac I* and *Bam HI* restricted sites of the intermediate vector pRT 101 resulted in pRT101- Chitinase construct which placed the *chitinase* gene under the control of strong CaMV 35 S promoter and nos terminator elements of the vector. Colony PCR analysis of the recombinant plasmids pRT 101-Chitinase with restriction sites tagged *chitinase* gene-specific primers gave an amplified gene band of size 973 bp which confirmed the integration of restriction site tagged *chitinase* gene at *Bam HI* and *Sac I* restriction sites in intermediary vector pRT 101 (Fig. 6.3g). The colonies that tested positive for PCR were chosen, regrown, plasmid isolated and sequenced. Analysis of the nucleotide sequences of the recombinant plasmids (Fig.6.3h) by BLAST algorithm showed 98.46% homology with the *Hevea brasiliensis* chitinase mRNA (DQ873889.2) deposited in Gen Bank of NCBI. Release of two fragments (2629 bp and 1663 bp) by digestion of the recombinant plasmid pRT 101- Chitinase with *Hind III* restriction enzyme, again confirmed the incorporation of *Bam H I* and *Sac I* tagged *chitinase* gene insert at *Bam HI* and *Sac I* restricted sites of pRT 101 vector (Fig. 6.3i).

Restriction digestion of pRT 101- Chitinase construct with enzyme *Hind III* resulted in excision of the complete *chitinase* gene cassette. This excised gene cassette when ligated to the *Hind III* site of binary vector pCAMBIA 1301 resulted in pCAMBIA 1301-Chitinase construct with the complete *chitinase* gene expression cassette. Colony PCR analysis of the recombinant pCAMBIA 1301-Chitinase with restriction site tagged *chitinase* gene-specific primers gave an amplified gene band of size 973 bp (Fig. 6.3j). The colonies that tested positive for PCR were chosen, regrown, plasmid isolated and sequenced. Analysis of the nucleotide sequences of the recombinant plasmids by BLAST algorithm showed 98.56% homology with the *Hevea brasiliensis* chitinase mRNA (DQ873889.2) deposited in Gen Bank of NCBI (Fig.6.3k & l).

Fig.6.2a. Results obtained by blasting *Hevea* whole genome with the available mRNA sequences of chitinase (AJ010397.1, DQ873889.2 and AJ007701.1) in the public database.

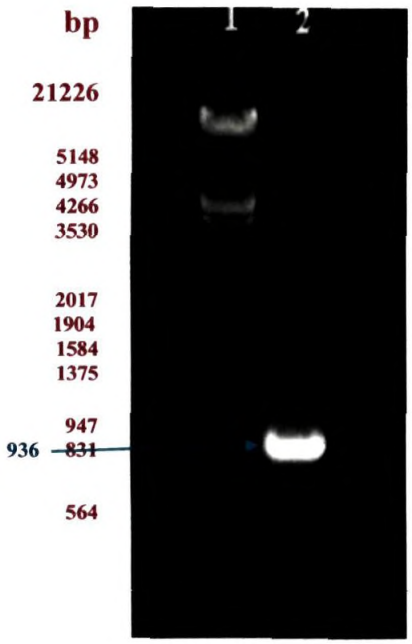


Fig.6.2b. PCR amplification of *chitinase* gene from the genomic DNA of *Hevea brasiliensis* clone RRII 105 using *chitinase* gene specific primers. Lane 1- λDNA / *EcoRI* /*Hind III* Digest (Marker), Lane 2- PCR amplified product of size 936 bp.

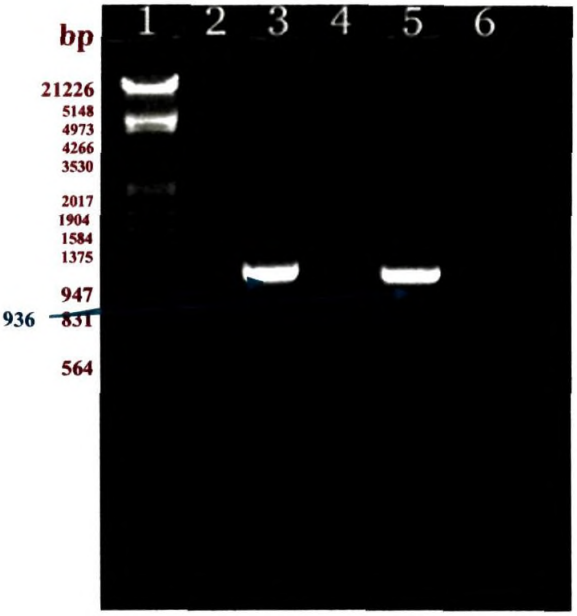


Fig. 6.2c. Colony PCR confirming the integration of *chitinase* gene in pGEM-T easy vector. Lane 1- λDNA/*EcoRI*/*Hind III* Digest (Marker), Lanes 3 and 5– colony PCR positive colonies showing amplified *chitinase* gene insert of size 936 bp, Lanes 4 and 6– negative colonies

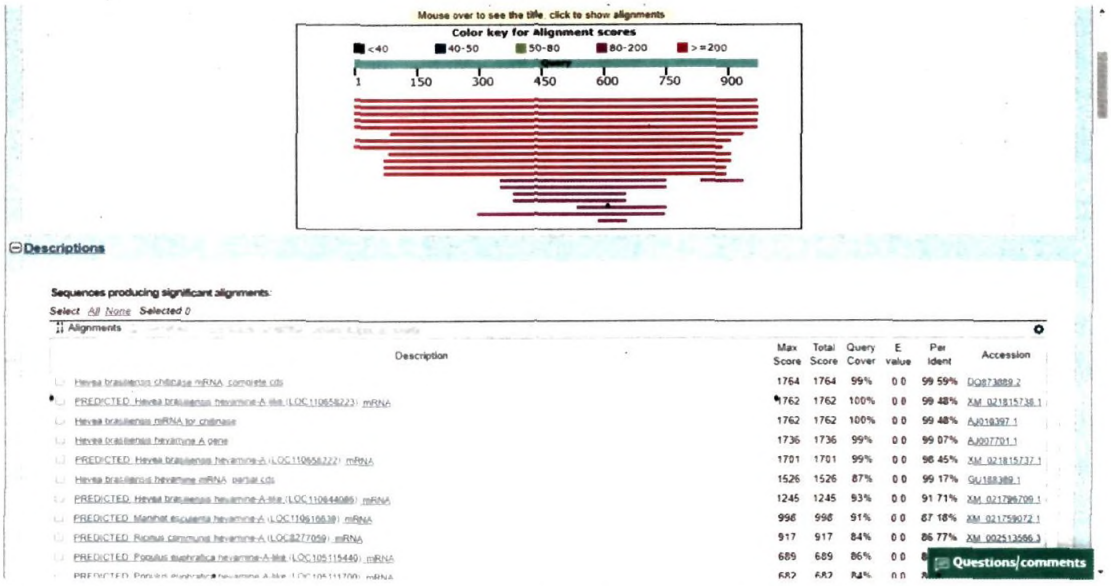


Fig. 6.2d. Results of BLASTn analysis of the nucleotide sequences of the recombinant plasmid pGEM-T easy (P1) showing 99.59% homology with *Hevea brasiliensis* chitinase mRNA (DQ873889.2) deposited in GenBank of NCBI

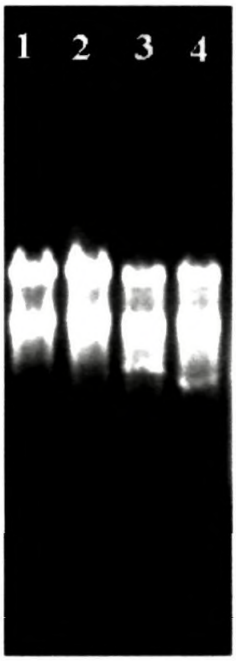


Fig.6.3a. Agarose gel electrophoresis showing RNA samples isolated from young leaves of *Hevea* clone RRII 105

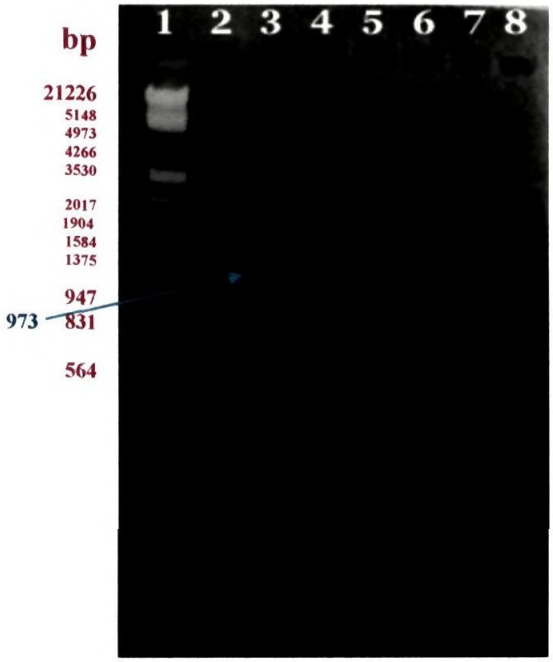


Fig. 6.3b. PCR amplification of *chitinase* gene from cDNA using first set of chitinase primers. Lane 1-λDNA / *EcoRI* / *Hind III* Digest, Lane 3- amplified chitinase cDNA product of size 973 bp

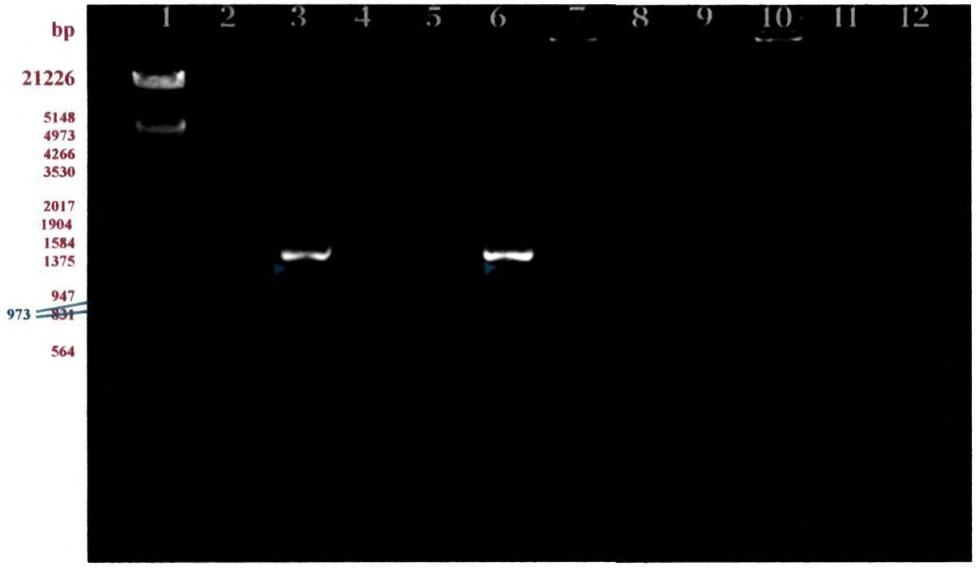


Fig.6.3c. Colony PCR confirming the integration of *chitinase* gene tagged with restriction sites in pGEM-T easy vector. Lane 1- λDNA / *EcoRI* / *Hind III* Digest (Marker), Lanes 3 and 6– colony PCR positive colonies showing amplified *chitinase* gene fragment of size 973 bp and Lanes 4, 5, 7, 8, 9, 10, 11 and 12– negative colonies

CCGCCATGGCGGCCGCGGGAATTCGATTCTGCGAGCTCTTTACTTGCAATAATGGCCAAAAGAAC
CCAAGCCATCCTTCTTCTTCTCCTAGCAATCTCACTGATTATGTCCAGCTCTCATGTTGATGGTGGTGG
CATTGCCATTTACTGGGGTCAAAATGGCAACGAAGGAACTCTAACACAAACATGCTCCACACGCAAA
TATTCTTACGTGAATATAGCCTTTCTCAATAAATTTGGCAATGGTCAAACCCCACAAATCAACCTTGC
CGGCCATTGTAACCCGGCTGCTGGAGGTTGCACCATTGTCAGCAATGGCATCAGGAGTTGCCAAATC
CAAGGAATTAAGGTGATGCTTTCTCTTGGCGGTGGGATTGGAAGCTACACCCTGGCCTCTCAAGCTG
ATGCAAAAAACGTTGCAGACTATTTGTGGAATAATTTCTTGGGTGGGAAATCTTCTTCCCGTCCCTTA
GGTGATGCTGTATTGGATGGTATTGATTTTGACATAGAGCATGGTTCAACCCTGTACTGGGACGATCT
TGCACGTTACTTATCTGCATATAGCAAGCAAGGCAAGAAGGTGTATTTAACTGCAGCTCCTCAATGT
CCATTCCCTGATAGATATTTAGGGACTGCCCTTAATACTGGTCTTTTTGACTATGTATGGGTTCAATTC
TATAACAATCCACCATGCCAGTATAGCTCAGGTAACATTAACAACATCATTAACTCGTGGAATCGAT
GGACCACATCTATAAATGCAGGGAAAATATTTTTGGGGTTGCCGGCAGCTCCTGAGGCAGCCGGAAG
CGGATATGTTCCACCGGATGTGCTGATTTCTCGGATTCTTCCTGAAATAAAGAAGTCACCTAAGTACG
GAGGTGTTATGCTTTGGTCAAAGTTCTACGATGATAAGAATGGCTATAGTTCCTCCATTCGGGACAGT
GTATTGTTCCTCCATTCTGAAAAGTGTATGACAGTACTTTGAATTGGGAGAAATGGATCCTCGAAA
TCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATA

Fig.6.3d. Nucleotide sequence of *chitinase* gene with backbone of pGEM-T easy vector. ATG- start codon, TGA- stop codon, GAGCTC- *Sac1* restriction site, GGATCC - *Bam H1* restriction site

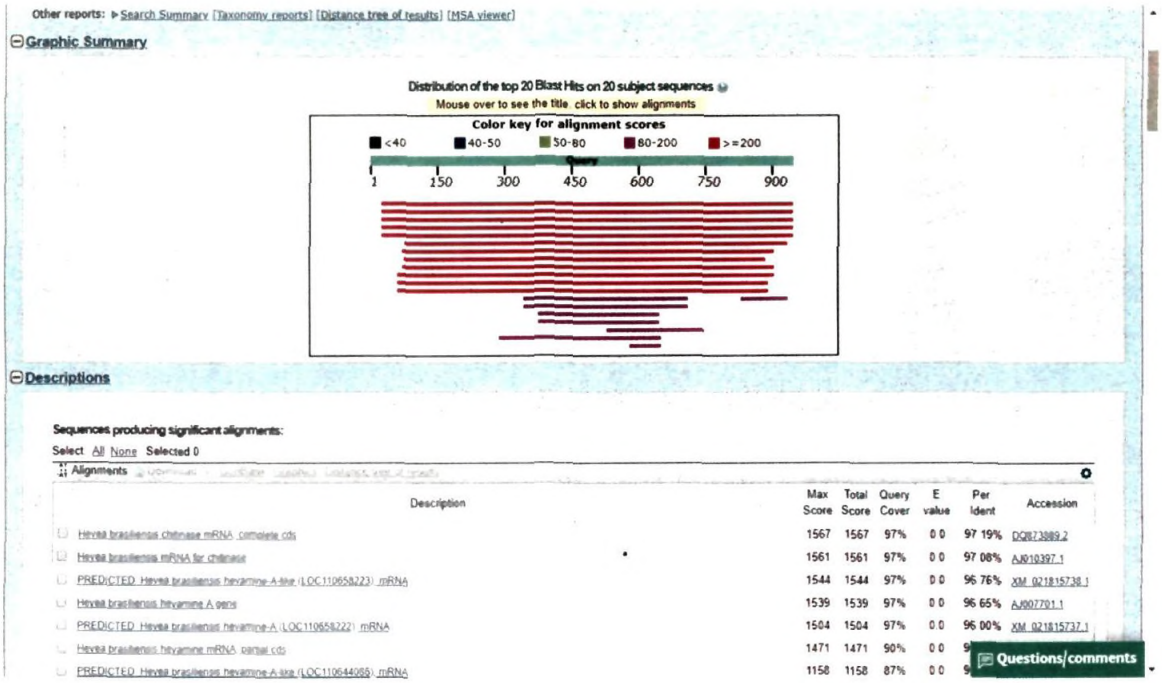


Fig.6.3e. Results of BLASTn analysis of nucleotide sequences of the recombinant plasmid pGEM-T easy showing 97% homology with the *Hevea brasiliensis* chitinase mRNA (DQ873889.2) deposited in GenBank of NCBI

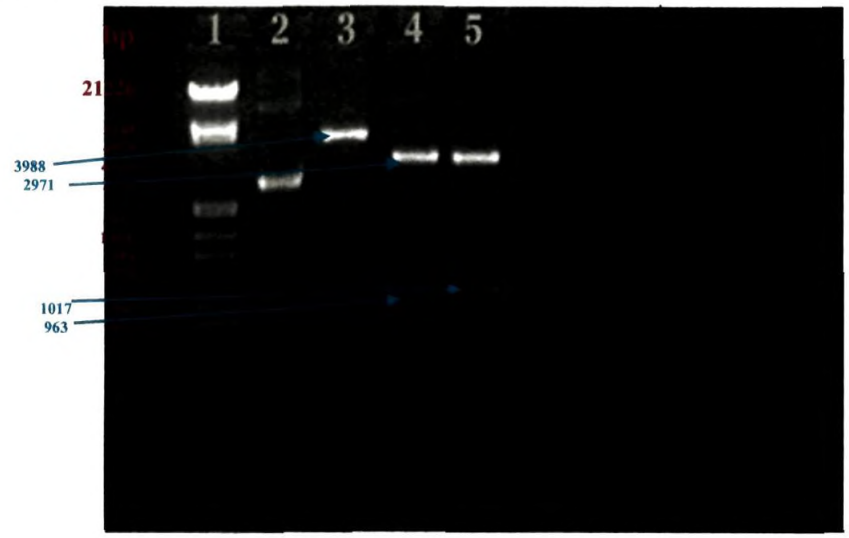


Fig.6.3f. Confirmation of the incorporation of *Bam HI* and *Sac I* restriction sites on both ends of amplified *chitinase* gene fragment cloned in pGEM-T easy vector. Lane 1- λDNA/ *EcoRI* / *Hind III* Digest (Marker), Lane 2- Uncut pGEM-T easy vector, Lane 3- *Bam HI* digested product, Lane 4- *Bam HI* and *Sac I* digested product, Lane 5- *Sac I* digested product.

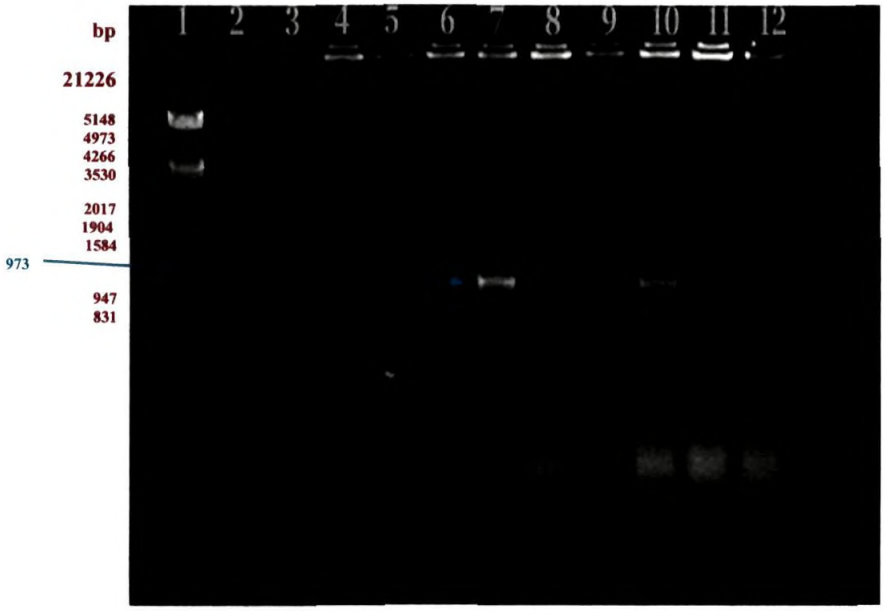


Fig. 6.3g. Colony PCR confirming the integration of *chitinase* gene with restriction sites in intermediary vector pRT 101. Lane 1- λDNA/ *EcoRI* / *Hind III* Digest (Marker), Lanes 4, 7, 10 and 12 - positive colonies showing amplified *chitinase* gene fragment of size 973 bp, Lanes 3, 5, 6, 8, 9 and 11 – negative colonies

CCAGAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAACCTCCTCGGATTCCATTG
CCCAGCTATCTGTCACTTCATCGAAAGGACAGTAGAAAAGGAAGATGGCTTCTACAAATGCCA
TCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCTACCGACAGTGGTCCCAAAGATGG
ACCCCCACCCACGAGGAACATCGTGTAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGT
GGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGA
CCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGATAGGACCTCGAGAATTCGAGCTCTTTACT
TGCAATAATGGCCAAAAGAACCCAAGCCATCCTTCTTCTTCCTAGCAATCTCACTGATTATGTCCA
GCTCTCATGTTGATGGTGGTGGCATTGCCATTTACTGGGGTCAAAATGGCAACGAAGGAACTCTAAC
ACAAACATGCTCCACACGCAAATATTCTTACGTGAATATAGCCTTTCTCAATAAATTTGGCAATGGTC
AAACCCCACAAATCAACCCTTGCCGGCCTTTGTAACCCGGCTGGTTGGAGGATGCACCATTGTTCAG
CAATGGCATCAGGAGTTTGCCAATTCCAAGGAATTAAGGTGATGCTTTCTCTTGGCGGTGGGAATGG
AAGCTACACCCTGGCCTCTCAAAGCTGATGCAAAAAACGTTGCAGACCTATTTGTGGAAATAATTTC
TTGGGGTGGGAAATCTTCTTCCCCGTCCCTTAGGTGATGCTGTATTGGATGGTATTGATTTTGACATA
GAGCATGGTTCAACCCCTGTACTGGGACGATCTTGCACGTTACTTATCTGCATATAGCAAGCAAGGC
AAGAAGGTGTATTTAACTGCAGCTCCTCAATGTCCATTCCCTGATAGATATTTAGGGACTGCCCTTAA
TACTGGTCTTTTTGACTATGTATGGGTTCAATTCTATAACAATCCACCATGCCAGTATAGCTCAGGTA
ACATTAACAACATCATTAACTCGTGGAATCGATGGACCACATCTATAAATGCAGGGAAAATATTTTT
GGGGTTGCCGGCAGCTCCTGAGGCAGCCGGAAGCGGATATGTTCCACCGGATGTGCTGATTTCTCGG
ATTCTTCCTGAAATAAAGAAGTCACCTAAGTACGGAGGTGTTATGCTTTGGTCAAAGTTCTACGATG
ATAAGAATGGCTATAGTTCCTCCATTCGGGACAGTGTATTGTTCCTCCATTCTGAAAAGTGTATGACA
GTACTTTGAATTGGGAGAAATGGATCCTCTAGAGTCCGCAAAAATCACCAGTCTCTCTCTACAAA
TCTATCTCTCTCTATTTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGT
TCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACC

Fig.6.3h. Nucleotide sequence of *chitinase* gene with backbone of pRT 101 vector. ATG- start codon, TGA- stop codon, GAGCTC- *Sac 1* restriction site, GGATCC - *Bam H1* restriction site

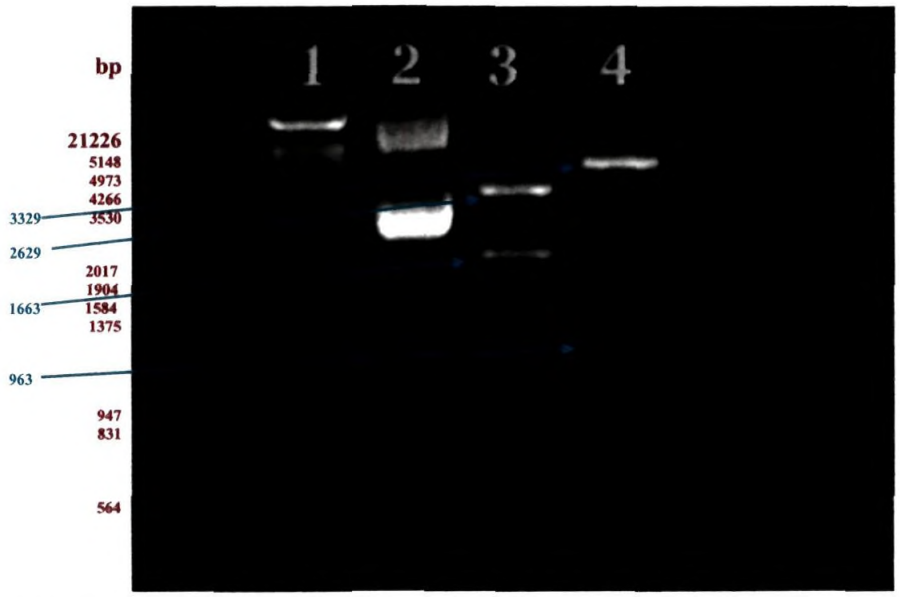


Fig.6.3i. Release of restriction sites tagged *chitinase* gene fragment along with CaMV 35 S promoter of pRT 101 vector by restriction digestion of the recombinant plasmid (pRT 101-Chitinase) with *Hind III*, *Bam HI and Sac I*. Lane 1: - λDNA / *EcoRI / Hind III* Digest (Marker), Lane 2: Uncut pRT 101 vector. Lane 3: *Hind III* digested product, Lane 4: *Bam HI and Sac I* digested product

Fig.6.3j. Colony PCR confirming the integration of restriction sites tagged *chitinase* gene fragment along with CaMV 35 S promoter in binary vector pCAMBIA 1301. Lane 1- λDNA/ *EcoRI* / *HindIII* Digest (Marker), Lanes 3, 5, 8, 9 and 10 are positive colonies showing amplified *chitinase* gene fragment of size 973 bp whereas, lanes 4, 6 and 7 are negative colonies

CAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTT
AGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTG
AGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGG
GATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGCATGCCTGCAGGTCAACATGGTGGAGCACG
ACACTCTCGTCTACTCCAAGAATATCAAAGATACAGTCTCAGAAGACCAGAGGGCTATTGAGACTTT
TCAACAAAGGGTAATATCGGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCGAA
AGGACAGTAGAAAAGGAAGATGGCTTCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTT
CAAGAATGCCTCTACCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAACATCGTGGAAAA
AGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGAT
GACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAG
GACCTCGAGGGCCCATGGGCGAGCTCTTTACTTGCAATAATGGCCAAAAGAACCCAAGCCATCCT
TCTTCTTCTCCTAGCAATCTCACTGATTATGTCCAGCTCTCATGTTGATGGTGGTGGCATTGCCATTTA
CTGGGGTCAAAATGGCAACGAAGGAACTCTAACACAAACATGCTCCACACGCAAATATTCTTACGTG
AATATAGCCTTTCTCAATAAATTTGGCAATGGTCAAACCCCACAAATCAACCCTTGCCGGCCTTTGTA
ACCCGGCTGGTTGGAGGATGCACCATTGTTCAGCAATGGCATCAGGAGTTTGCCAATTCCAAGGAAT
TAAGGTGATGCTTTCTCTTGGCGGTGGGAATGGAAGCTACACCCTGGCCTCTCAAAGCTGATGCAAA
AAACGTTGCAGACCTATTTGTGGAAATAATTTCTTGGGGTGGGAAATCTTCTTCCCCGTCCCTTAGGT
GATGCTGTATTGGATGGTATTGATTTTGACATAGAGCATGGTTCAACCCTGTACTGGGACGATCTTGC
ACGTTACTTATCTGCATATAGCAAGCAAGGCAAGAAGGTGTATTTAACTGCAGCTCCTCAATGTCCA
TTCCCTGATAGATATTTAGGGACTGCCCTTAATACTGGTCTTTTTGACTATGTATGGGTTCAATTCTAT
AACAATCCACCATGCCAGTATAGCTCAGGTAACATTAACAACATCATTAACTCGTGGAATCGATGGACC
ACATCTATAAATGCAGGGAAAATATTTTTGGGGTTGCCGGCAGCTCCTGAGGCAGCCGGAAGCGGATAT
GTTCCACCGGATGTGCTGATTTCTCGGATTCTTCCTGAAATAAAGAAGTCACCTAAGTACGGAGGTGTTA
TGCTTTGGTCAAAGTTCTACGATGATAAGAATGGCTATAGTTCCTCCATTCGGGACAGTGTATTGTTCCTC
CATTCTGAAAAGTGTATGACAGTACTTTGAATTGGGAGAAATGGATCCTCTAGAGTCCGCAAATCACC
AGTCTCTCTCTACAAATCTATCTCTCTCTATTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGA
ATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGT
AAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTGACCTGCAGGCATGCAAGCT
TGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGC
AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTT
GCGCAGCCTGAATGGCGAATGCTAGAGCAGCTTGAGCTTGGATCAGA

Fig.6.3k. Nucleotide sequence of *chitinase* gene with backbone of pRT 101 and pCAMBIA 1301. ATG- start codon, TGA- stop codon, GAGCTC- *Sac 1* restriction site, GGATCC - *Bam H1* restriction site, AAGCTT- *Hind III* restriction site

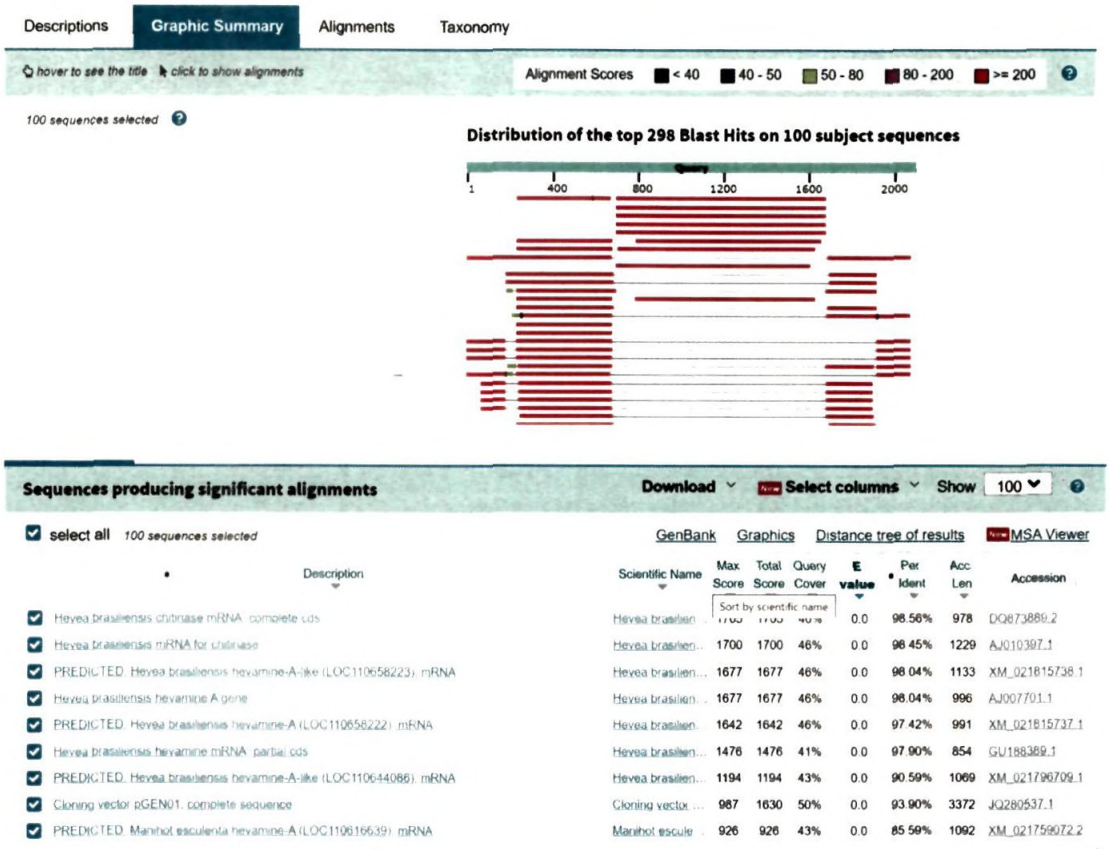


Fig.6.3l. Results of BLASTn analysis of recombinant pCAMBIA 1301 showing 98.56% homology with the *Hevea brasiliensis* chitinase mRNA (DQ873889.2) deposited in GenBank of NCBI

6.4. Discussion

Corynespora leaf fall disease (CLFD), caused by *Corynespora cassiicola*, has emerged as a major leaf disease in *Hevea* affecting cultivated clones and causing economic losses (Manju *et al.*, 2010). Management of this disease is extremely difficult, and conventional breeding for Corynespora resistance is a time-consuming and laborious task. Chemical control in plantations was also unsustainable due to different setbacks. Considering the promising results of previous studies on the transfer of defense genes into various plants, as well as our finding that the CLFD tolerance is proportional to chitinase enzyme activity, over-expression of the *chitinase* gene in *Hevea* in order to boost its CLFD resistance could be a promising alternative strategy. In the present study, the genomic DNA fragment and cDNA encoding

chitinase gene were PCR amplified, cloned, and characterized from *Hevea brasiliensis* as a first step towards the development of a *chitinase* gene expression cassette. Using various molecular biology techniques, a plant transformation vector carrying the *chitinase* gene expression cassette was constructed with the aid of three vectors: cloning vector pGEM T-easy, intermediate vector pRT 101, and binary vector pCAMBIA 1301. The restriction sites (*Sac I* and *Bam HI*) incorporated *chitinase* gene fragment (amplified chitinase cDNA fragment) was first cloned into the pGEM-T Easy vector. The restriction sites tagged *chitinase* gene fragment was then released from the recombinant pGEM T- easy vector and subcloned into the *Sac I* and *Bam HI* restricted sites of the intermediate vector pRT 101, thereby placing the *chitinase* gene under the control of the vector's strong CaMV 35 S promoter and nos terminator elements. After subcloning, the *chitinase* gene expression cassette was excised from the recombinant intermediate vector, and the excised cassette was finally ligated to the *Hind III* sites of binary vector pCAMBIA 1301, resulting in the formation of a plant transformation vector carrying *chitinase* gene expression cassette. Molecular characterization of the nucleotide sequences of cloned *chitinase* gene showed 98.56% homology with the *Hevea brasiliensis* chitinase mRNA (DQ873889.2) deposited in GenBank of NCBI. This vector construct could be used to transform the callus cultures of CLFD susceptible *Hevea* clones through *Agrobacterium*-mediated transformation method. As the isolation of *chitinase* gene was done from *Hevea* itself this transformation process is considered as an attempt to over-express the *chitinase* gene in *Hevea* so as to confer CLFD tolerance. Overexpression of a gene from the same plant species rather than a foreign transgene is advantageous in terms of public acceptance while cloning disease resistance genes (Datta *et al.*, 1999). Our institute (Rubber Research Institute of India) has successfully incorporated various transgenes such as *HbSOD*, *Tb osm* and *hmgr1* into *Hevea brasiliensis* through various protocols of *Agrobacterium* mediated transformation process which has been mentioned in the review. Genetic transformation of *Hevea* with the newly constructed vector containing the *chitinase* gene expression casette could be carried out using any of these standardized *Agrobacterium* mediated transformation protocols and the transformed *Hevea* lines are expected to show enhanced tolerance towards CLFD.

Chapter 7

Summary and Conclusions

Findings of preliminary *in vitro* screening experiments regarding the sensitivity ranking of the selected *Hevea* clones towards CLFD indicates that the results were in conformity with the field observations. On account of the similarity with the actual pathogen in inducing disease symptoms, crude culture filtrate appears to be an effective and appropriate selection agent for the *in vitro* experiments for assessing the level of CLFD sensitivity of *Hevea* clones. Moreover, the *in vitro* sensitivity tests using detached leaves and cultured calli as well as electrolyte leakage measurement, being quite simple and fast, can be employed in the early screening of clones for CLFD sensitivity. As the membrane potential and membrane permeability of leaf tissues in response to CCF can be assessed effectively with the conductivity measurements, the electrolyte leakage test seems to be a practical and sensitive approach and serves as an indicator for rapid identification of CLFD sensitivity in *Hevea* clones. Collectively, the findings of preliminary *in vitro* screening studies opens up the scope of employing *in vitro* sensitivity assays for early screening of germplasm material and breeding population including hybrid seedlings, open pollinated seedlings and pipeline clones of *Hevea brasiliensis* towards CLFD, making use of the pathotoxic culture filtrate, thus enabling early selection of tolerant clones.

The intrinsic activity of chitinase was found to be higher in tolerant clones than in the susceptible ones. The application of CCF to the leaves of *Hevea* clones caused a further increase in this intrinsic chitinase activity, with tolerant clones exhibiting higher increase than the susceptible ones. Also the application of CCF on *Hevea* leaves initiated ROS accumulation leading to cell damage. The cells of the CLFD tolerant clones were less likely to be damaged by CCF treatment, while cells of CLFD-susceptible clones suffered extensive cell damage during CCF treatment. The increase in the activity of enzymic antioxidants, catalase and peroxidase was found to be higher in the susceptible clones than tolerant ones. This increased antioxidant

activity indicated the scavenging or detoxification of excess ROS produced in response to CCF treatment.

The toxic metabolites produced by the pathogenic fungus *Corynespora cassiicola* are involved in the pathogenesis of *Hevea brasiliensis*. The presence of these compounds in the growth medium, through supplementation of CCF in the medium, significantly affected the *in vitro* growth of embryogenic callus cultures of CLFD susceptible *Hevea* clone RRII 105. Changes in calli growth were observed over time depending on the concentration of CCF in the culture medium. Medium containing the highest concentration of CCF adversely affected the growth of the embryogenic calli, resulting in browning and necrosis of those cells whereas growth of calli on medium containing the lowest concentration of CCF was little affected. Positive correlation between callus sensitivity and the concentration of culture filtrate suggested that culture filtrate can be used as an effective selection agent for *in vitro* selection for disease tolerance.

Friable embryogenic calli were found to be a suitable study material for *in vitro* screening and selection studies because of the uniformity with which cells were exposed to CCF contained in the medium. The regeneration frequency of calli selected on toxin medium was found to be lower as compared to the growth of calli under nontoxic conditions. A few CCF tolerant plantlets of *Hevea* clone RRII 105 could be developed through *in vitro* challenging of embryogenic calli with CCF, followed by selection and further development leading to plant regeneration. On laboratory level screening with CCF, most of these plants showed CLFD tolerance as evidenced from the results of leaf wilt bioassay, vacuum infiltration of leaf segments and electrolyte leakage. Biochemical assay of various parameters done on these regenerated plants after CCF treatment showed an enhanced chitinase activity, absence of ROS production and very low levels of catalase and peroxidase acitivity which are signs of better tolerance of these plants towards CLFD. These results revealed that CLFD tolerance acquired at the cellular level through CCF selection is manifested at the plant level without being lost through various stages of germination. Since CLFD tolerance could be imparted to a highly susceptible clone, RRII 105, it could be expected that *in vitro* selection followed by plant regeneration from the toxin

challenged cultures may offer plant breeders a new approach to accelerate the development of disease tolerant plants from highly susceptible parents.

The *chitinase* gene present in *Hevea brasiliensis* was cloned and characterized by DNA sequencing and restriction enzyme analysis. Using this *chitinase* gene, an expression cassette was successfully prepared in the binary vector pCAMBIA 1301 for generating transgenic *Hevea* plants overexpressing *chitinase* gene.

Highlights of the study

- An *in vitro* selection system for ameliorating the trait of CLFD tolerance has been successfully employed in a susceptible clone RRII 105, an elite and most popular *Hevea* clone with desirable traits in all other aspects
- This *in vitro* selection strategy represents an economical and expeditious way of selecting disease resistant plants from susceptible varieties, as compared to time consuming classical breeding methods
- Moreover, this technique is a promising, bio- safe method that can be used to produce non-GM (genetically modified) plants that are tolerant to various fungal diseases
- This is the first report of its kind in *Hevea brasiliensis*

Future Prospects

- *In vitro* sensitivity assays can be employed for early screening of germplasm material and breeding population including hybrid seedlings, open pollinated seedlings and pipeline clones of *Hevea brasiliensis* towards CLFD, making use of the pathotoxic culture filtrate, thus enabling early selection of tolerant clones
- *In vitro* selection followed by plant regeneration from the toxin challenged cultures, may offer plant breeders a new approach to accelerate the development of disease tolerant plants from highly susceptible and otherwise elite clones
- The plants regenerated from CCF challenged calli can be further multiplied by bud grafting and be planted in CLFD hot spots for confirmation of induced

disease tolerance. Once confirmed the tolerant plants can be included in future breeding programmes

- Moreover, field trials may be performed with these tolerant plants to assess yield and other secondary parameters. In such trials, if a tolerant candidate with high yield and other promising secondary characters could be identified it can be further tested and be released as a new variety
- Construction of the chitinase gene expression cassette in the plant transformation vector opens up another avenue for imparting CLFD tolerance to elite clones of *Hevea* by overexpression of *chitinase gene* through genetic transformation technique

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