CHARACTERIZATION OF TAPPING PANEL DRYNESS (TPD) OF *HEVEA BRASILIENSIS* IN CLONE RRII 105

Thesis submitted to

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Under the Faculty of Science

By

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Under the supervision and guidance of

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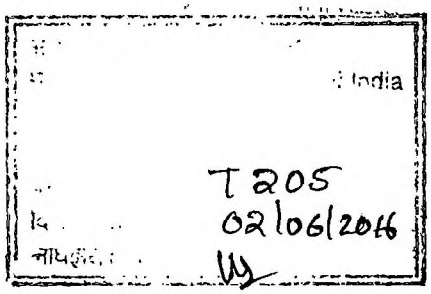
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DECLARATION

I hereby declare that the thesis entitled **"Characterization of tapping panel dryness (TPD) of *Hevea brasiliensis* in clone RRII 105"** is an authentic record of original research carried out by me under the supervision and guidance of Dr. C. Kuruvilla Jacob (Director, Rubber Training Institute, Rubber Board, Kottayam, Kerala) in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY of the Mahatma Gandhi University and no part of this work has been presented for any degree, diploma or any other similar titles of any university.

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CERTIFICATE

This is to certify that the thesis entitled "**Characterization of tapping panel dryness (TPD) of *Hevea brasiliensis* in clone RRII 105**" is an authentic record of original research carried out by Mr. Thomson Abraham under my supervision and guidance and no part of this work has been presented for any degree, diploma or any other similar titles of any university.

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ABSTRACT

Tapping panel dryness (TPD) syndrome is a serious malady of rubber (*Hevea brasiliensis*) occurring in all rubber growing areas resulting in partial to complete drying of tapping panel. A detailed study was conducted to understand different symptoms of TPD and its prevalence in the rubber clone RRII 105. The possibility of association of viroid with TPD syndrome was also investigated.

It was observed that TPD is initiated in two ways. In the first case, extreme fluidity of latex with abnormally low DRC and the latex flow only from the inner most layers of latex vessels was observed. In the other, very viscous latex with abnormally high DRC and partial dryness of varying degrees and vessels devoid of latex even in the inner layer was seen. When dryness was observed in the virgin (BO 1) panel, the cracks were found to be extended to the opposite panel. Cracks and necrosis started from the tapping panel in some trees while in others it originated near the bud union and later extended towards the tapping cut. The present study revealed that cracking and bulging of bark increased with the age of trees and with progression in period of tapping. Nearly 40% reduction in total latex volume was observed for trees in the category of less than 50% TPD and nearly 90% reduction was observed when the trees were in the category of more than 75% TPD when tapped in BO 1 panel. Contrary to total latex volume, an increase in DRC was noted as the intensity of TPD increased. Dryness of root system was observed along with necrosis, cracking and bulging (as observed on the trunk) in TPD affected trees. The roots corresponding to the dry portion of scion showed dryness although those roots originated from the root stock.

It was observed that the number TPD affected trees increased as the year of tapping progressed at all the locations under observation both in small holdings and in large estates. Out of the 18900 trees observed at Adoor region 3508 trees (18.56%) showed more than 50% TPD, while it was 2425 out of 13700 (17.70%) at Nedumangad, 2153 out of 12700 (16.95%) at Taliparampa, 2821 out of 17300 (16.30%) at Mannarkad, 3106 out of 20200 (15.37%) at Pala and 2273 out of 15100 (15.05%) at Kanjirappally. The percentage of trees in the category of very high TPD intensity (>75%) showed a clear trend of increase from the first year to the last year of tapping at all the locations. The number of TPD trees in the other categories (low, medium and high) did not show such a remarkable trend of increase from BO 1 to BI 1 (renewed) panels. The scale of increase in TPD was more in older trees than in trees at the initial stages of tapping.

Percentage of trees showing TPD symptoms was less when the panel was changed but it increased even to higher values a year after such change. Reversion of TPD symptoms was observed only at a young age. Evidence for natural transmission of TPD from one tree to the other was observed in the present field studies as the number of TPD trees in clusters showed a significant increase with progressive tapping. Only 23.8% of the small holders adopted tapping rest when TPD was observed. When the TPD affected trees were continued to tap in the upward system, more than 50 per cent of the trees showed dryness after four months.

An infectious LMW RNA was detected from different samples drawn from varying ages of trees from different locations. The consistency in the presence of LMW RNA over three years of observation in TPD affected trees and its absence in the healthy ones points to the association of the LMW RNA with TPD. About 30 per cent of the apparently healthy trees which may be symptomless carriers also showed presence of LMW RNA. Such trees later showed TPD symptoms. Presence of bands in apparently healthy samples indicates that the biotic agent can be detected in the tree much before the TPD symptoms are visible. The PCR of the cDNA using viroid specific primers consistently amplified products in the range of viroids in R-PAGE positive TPD affected samples but was absent in R-PAGE –ve apparently healthy samples. The amplified LMW RNA showed 98% sequence homology to Potato Spindle Tuber Viroid (PSTVd) on BLAST analysis.

The R-PAGE test of bud grafted plants under transmission studies showed that all the plants tested from the group in which both stock and scion were viroid +ve, maintained the viroid bands. Viroid was observed to be transmitted from viroid +ve stock to viroid –ve scion. Test tapping showed TPD in both group of plants, namely plants budded with scion from TPD affected trees as well as plants with scion from healthy trees. This shows that root stock also plays a role in the development of TPD. Epinasty symptom development on tomato plants inoculated with total RNA isolated from TPD affected trees showed that the viroid present in rubber can be transmitted to the indicator host indirectly satisfying the Koch's postulates. The viroid was reisolated and it showed LMW RNA band in R-PAGE. The sequence homology of the RT-PCR product obtained from the inoculated tomato with that of Potato Spindle Tuber Viroid proved its viroid relationship.

Key words: *Hevea*, RRII 105, TPD, symptoms, prevalence, viroid, transmission

PREFACE

Natural rubber (NR) has become an indispensable commodity in our day-to-day life. Rubber tree (*Hevea brasiliensis*) is the primary source of natural rubber. One of the most important constrains for the production of NR is the occurrence of tapping panel dryness (TPD) since, it renders the trees non-productive. As rubber is a perennial tree crop with a production span of nearly 25 years, the loss due to TPD is enormous especially if a tree is affected early in its economic life. Tapping panel dryness syndrome of rubber has been reported from the plantations of different rubber clones from the very early stages of rubber cultivation. There have been several efforts to investigate the cause of the TPD syndrome, but so far, no clear picture has emerged.

TPD has become more important now as almost all the new high yielding clones of rubber are highly susceptible to TPD. Clone RRII 105, developed by Rubber Research Institute of India (RRII) is not an exception. This clone is widely cultivated in India covering more than 85% of the area, both in small holdings and in large estates. This single clone is responsible for the country in achieving the highest productivity of rubber worldwide. Although reports are available on the symptoms of TPD in clone RRII 105 a systematic study from different locations and at various stages of rubber cultivation is lacking. Identification of initial symptoms of TPD on clone RRII 105 in untapped as well as trees at various stages of tapping is necessary for adopting management practices to minimize losses.

Detailed study on external and internal symptoms may throw light on the development stages of TPD syndrome. Some of the management practices adopted by farmers need critical analysis for their effectiveness. Also farm practices that may trigger development/spread of the disorder require close scrutiny. Although earlier investigations could not indicate biotic etiology for TPD there is scope for fresh investigation in this line due to availability of new molecular biological tools which can highlight involvement of sub microscopic molecular pathogens if any.

This thesis includes six main chapters. The first chapter 'General introduction' is intended to give a general idea of the topic of research, its relevance as well as economic importance and objectives of the present study. The second chapter 'Symptoms of TPD' is a detailed account on the various symptoms of TPD in clone RRII 105. It describes TPD symptoms on different plant parts both external as well as internal symptoms at different stages of the tree.

The third chapter 'Incidence of TPD' describes the occurrence of TPD in different years of tapping at various locations both from the small holdings as well as estates. It also gives an account of various management practices adopted and its feasibility and success. The pattern of occurrence of TPD in plantations is also described in this chapter. The fourth chapter 'Molecular studies on the biotic etiology of TPD' deals with the investigation on the association of LMW RNA in different tissues of TPD trees and also the characterization of the LMW RNA. The fifth chapter entitled 'Transmission studies' gives an account of investigations on the infectious nature of the LMW RNA isolated from TPD affected rubber trees. Major findings of the work and future prospective are included in the sixth chapter.

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ABBREVIATIONS

BLAST	–	Basic Local Alignment Search Tool
DNA	–	Deoxyribonucleic acid
cDNA	–	Complementary DNA
DEPC	–	Diethyl pyro-carbonate
dNTP	–	deoxyribonucleotide triphosphate
DRC	–	Dry rubber content
E. coli	–	*Escherichia coli*
EDTA	–	Ethylene Diamine Tetra Acetic acid
Gl	–	Glenshiel, Malaysia
H. brasiliensis	–	*Hevea brasiliensis*
IPTG	–	Isopropyl β-D-1-thiogalactopyranoside
Kb	–	Kilobase
LMW RNA	–	Low molecular weight RNA
NaCl	–	Sodium Chloride
NCBI	–	National Center for Biotechnology Information
NR	–	Natural Rubber
PAGE	–	Polyacrylamide Gel Electrophoresis
PCR	–	Polymerase Chain Reaction
PDA	–	Potato Dextrose Agar
ppm	–	Parts per million
PSTVd	–	Potato spindle tuber viroid
RNA	–	Ribonucleic acid
rDNA	–	Ribosomal DNA
ROS	–	Reactive Oxygen Species
R-PAGE	–	Return Polyacrylamide Gel Electrophoresis
rpm	–	Revolutions per minute
RRII	–	Rubber Research Institute of India
RRIM	–	Rubber Research Institute of Malaysia
rRNA	–	Ribosomal ribonucleic acid
RT-PCR	–	Reverse Transcription PCR
TBE	–	Tris/Borate/EDTA
TE	–	Tris/EDTA
TEMED	–	Tetramethylethylenediamine
Tjir	–	Tjirandji, Indonesia
TNA	–	Total nucleic acid
TPD	–	Tapping Panel Dryness
X-GAL	–	5-Bromo-4-chloro-3-indolyl-β-D- Galactopyranoside

Chapter 1

GENERAL INTRODUCTION

1.1 *Hevea brasiliensis* - The Para rubber tree

Hevea brasiliensis (Willd. ex A. Juss.) Muell.Arg., originally a forest tree indigenous to the tropical rain forests of Central and South America and the only major commercial source of natural rubber (NR), is one of the most recently domesticated crop species in the world. The genus *Hevea*, belonging to the family Euphorbiaceae, grows wild in the Amazon River basin and in the surrounding regions. The genus was introduced into parts of Asia and Africa where large plantations were established (Fig. 1.1). Although NR has been found in the latex of other plants belonging to 311 genera of 79 families, 99 per cent of the global natural rubber production is from *H. brasiliensis*, the Para rubber tree. Latex containing vessels are present in all parts of the tree except wood.

1.2 Rubber

Rubber is a constituent of latex, a milky substance produced in the laticiferous tissues. Even though latex is present in almost all parts of the plant, the laticifers exploited commercially are from the bark. The latex vessels are developed by the activity of vascular cambium (Premakumari and Saraswathyamma, 2000). Latex is obtained by controlled wounding of bark of rubber tree, termed as tapping (Fig. 1.2). Trained workers do the tapping, using special knives, by controlled wounding during which a thin layer of bark consisting of the hard bast and major part of the soft bast is removed.

Fig. 1.1 Rubber plantation

Fig. 1.2 Rubber tapping

1.3 History of commercial NR cultivation

The modern age of natural rubber started during the 1870s when the British successfully transported *Hevea* seeds from Brazil for planting in the British India (Markham, 1876; Petch, 1914). The original genetic material of the Para rubber tree, referred to the 'Wickham gene pool' was introduced to South East India by Sir Henry Wickham in 1876.

1.4 Rubber plantation in India

The cultivation of rubber in India began in 1878 from the rooted cuttings imported from Royal Botanic Gardens, Heneratgoda, Ceylon (RBGK, 1898; Petch, 1914). The growth of the Indian rubber plantation industry has been mainly through the expansion of rubber cultivation in Kerala. The British planters initiated rubber cultivation on a plantation scale and the state administration encouraged them by providing land, labour, capital and trade facilities.

Rubber cultivation on a plantation scale was initiated in India only during 1902. Within a period of 100 years the country has emerged as the fourth largest producer of natural rubber in the world (Table 1.1).

Table 1.1 Natural rubber: Area and production in major producing countries (2012).

Country	Area ('000 ha)	Production ('000 tonnes)
Thailand	2785	3512
Indonesia	3484	3015
Malaysia	1041	925
India	759	919
Vietnam	911	864
China	1110	795
Sri Lanka	131	152
Philippines	179	111

Source: 'Rubber Statistical Bulletin'- Rubber Board

1.5 Tapping panel dryness (TPD)

The disorder derives its name from the obvious symptom of the tapping panel going dry which can be partial or total. TPD, a commonly known disorder of *Hevea* trees continued to remain unresolved despite extensive research by many workers over several decades. TPD is characterized by the gradual or sudden drying up of the latex vessels near the tapping panel resulting in abnormally low yield or complete stoppage of latex production. The disease was reported for the first time in Brazil in 1887 (Rutgers and Dammermann, 1914). This was reported in plantations in Asia since the beginning of the 20th century (Rands, 1921). Panel dryness is of economic importance as it renders the trees non-productive.

1.6 Economic impact of TPD

As rubber is a perennial tree crop with a production span of nearly 25 years, the loss due to TPD is enormous especially if a tree is affected early in its economic life. TPD syndrome of rubber has resulted in considerable losses to the rubber plantation industry in all the rubber producing countries. It is estimated that the global annual loss due to this disorder is US $ 900 million (Sethuraj, 1998). TPD incidence is on the increase, particularly in areas where high yielding clones such as RRII 105 are being used for commercial plantations. An annual crop loss of 10-15 per cent due to this disorder is estimated (Sethuraj, 1998). However there are reports of variations in the intensity of TPD in relation to the age of the plant, stages of tapping and systems of tapping.

At an estimated incidence of 15 per cent, the crop loss due to TPD in India is 320 kg/ha/year resulting in a loss of Rs. 24.2 billion per year (Table 1.2).

Table 1.2 Estimated revenue loss due to TPD in India

Total rubber area under tapping (ha)	504000
Total production of natural rubber (Tonnes)	913700
Productivity (yield) (kg/ha)	1813
Yield potential (in the absence of TPD) (kg/ha)	2133
Yield loss due to TPD@15% (kg/ha)	320
Total yield loss (Tonnes)	161250
Price of natural rubber RSS 4 (Rs/kg) (approximate)	150
Loss to India per year (Billion, Rs)	24.2

1.7 Clone RRII 105

Clone RRII 105 is the first indigenously evolved high yielding hybrid clone of India. This clone is widely cultivated across the country in more than 85% of the area under small holdings and estates. Net revenue added per annum by cultivation of this clone is Rs.2856 crores (US$ 571 million). More than 80 per cent of the rubber plantations in India are under smallholdings sector. The incidence of TPD in this clone adversely affects the economic sustainability of the small farmers.

1.8 Objectives of the study

Tapping panel dryness syndrome of rubber has been reported from the plantations from the very early stages of rubber cultivation in different rubber clones. Although several reports are available on the symptoms of TPD in clone RRII 105 a systematic study from different locations and at various stages of rubber cultivation is lacking. Hence, one of the objectives of the study was to understand different symptoms in untapped as well as trees from the first to the last year of tapping in clone RRII 105. Identification of symptoms on different plant parts like roots which were not studied in detail earlier and symptoms on trees tapped on different tapping panels like virgin/renewed and controlled upward tapping (CUT) panels were attempted. Different easily noticeable external symptoms like cracks and bulges and internal symptoms which were difficult to identify such as bark necrosis on trunk and root required detailed study. Variations in total latex volume and DRC at different intensities of TPD were analysed.

There are reports of variations in the incidence of TPD with regard to clone, age of the plant, stages of tapping, system of tapping etc. Hence, another objective of the study was to assess the actual extent of TPD in clone RRII 105 in different years of tapping at various locations both from the small holdings as well as large estates. An attempt was also made to study various management practices adopted, their feasibility and success in containing TPD. The spread of TPD from the affected to the nearby trees was also investigated.

Various factors which were assumed to be the cause of TPD could not conclusively prove its etiology. Occurrence of TPD in adjacent trees in the same line, consistent increase in the incidence with age, similarity in symptoms and anatomical abnormalities to some known diseases, inability to revive the affected trees by resting and the presence of dry rubber trees from the start of exploitation prompted studies on biotic etiology for TPD. Investigations on the association of LMW RNA in different tissues of TPD trees and characterization of the LMW RNA utilizing the molecular biological tools now available formed the third objective.

The fourth objective of the present study was to investigate the pathogenicity of the LMW RNA isolated from TPD affected rubber trees, thus verifying biotic nature of causal agent.

Chapter 2

SYMPTOMS OF TPD

2.1 INTRODUCTION

Tapping panel dryness (TPD) of rubber (*Hevea brasiliensis* Muell. Arg.) is a serious disorder with unknown etiology occurring in all rubber growing regions resulting in severe loss of latex yield. TPD, earlier called as 'brown bast' can be defined as a process of drying of the tapping panel resulting in abnormally low yield or complete cessation of latex production (Sethuraj, 1992) and interestingly, without the loss of the tree. This drying up of the tapping cut is generally accompanied by various anomalies or symptoms. However, attention was first drawn by brown discoloration of bark cortex and the phenomenon was first called the "Brown Bast disease".

2.1.1 First reports

Brown bast syndrome of rubber has been reported from the plantations from the very early stages of rubber cultivation. This was noticed in Brazil as early as in the 1880's in wild rubber in the Amazon forest (Rutgers and Dammerman, 1914) suggesting that TPD has been there right from the beginning of the commercial cultivation of natural rubber. Its existence in the Asian plantations was first reported by Rutgers and Dammerman (1914) followed by Belgrave (1917), Belgrave and South (1918), Bobilioff (1919), Petch, (1921), Rands (1921a,b), and Keuchenius (1924). In another early report from Malaysia, TPD was observed to affect mostly high yielding clones of rubber (Sharples, 1936).

2.1.2 Scenario in India

In India, TPD is known to affect rubber plantations since the beginning of commercial cultivation. Rubber cultivation on a plantation scale was initiated in India only during 1902 (INRO, 1999; Thomas and Panikkar, 2000; Anon, 1911). However, it became more important with the introduction of the high yielding clones and the loss incurred due to TPD in rubber production over the years has been on the increase. The high yielding clone RRII 105 planted in more than 85% of rubber planted area in India is susceptible to TPD. As rubber is a perennial tree crop with a production span of nearly 25 years, the loss due to TPD is enormous. At an estimated average incidence of 15 per cent and based on the price of NR (Rs.150/kg), the country is loosing over Rs. 24.2 billion every year on account of TPD.

2.1.3 Symptoms reported

The most important symptom of TPD syndrome are reduced latex yield, eventually leading to total drying of tapping panel which gives the name to the

syndrome, tapping panel dryness. Some trees may also suddenly stop producing latex. More frequently, only part of the tapping cut dries up. This phenomenon of bark dryness has long been recognized as the first obvious symptom of TPD (Petch, 1921; Sanderson and Sutcliffe, 1921). Occurrence of TPD even in untapped trees has also been reported.

Different types of TPD have been described (Jacob and Prevot, 1989) with different symptoms and possibly having same or different causes, but all eventually have the common symptom of panel dryness. These are TPD with or without any symptoms other than panel dryness, reversible or irreversible panel dryness, partial or complete, inner or outer bark affected, panel dryness preceded or not preceded with the late dripping, dryness with or without necrosis and chronic or necrotic TPD (Sethuraj, 1992).

Some reports suggest two types of TPD - necrotic TPD and over-exploitation induced TPD. The former occurring randomly in a plantation and then spreading, often along the lanes of trees, and latter being a physiological fatigue feature (Lacrotte *et al.*, 1997). Rhodes (1930) described panel dryness and brown bast as different entities. According to him brown bast was not present in the case of panel dryness but panel dryness was noticed in the case of brown bast. However, in both cases panel dryness is the eventuality. Thus, TPD became a common terminology although there can be different developmental etiologies behind the observed dryness.

Intense stimulation enhanced the development of TPD (De Fay, 1988). Stimulation resulted in larger dry zones in the tapping panel. Comparative histology of bark dryness following intensive stimulation and bark affected by brown bast revealed different symptomatology. It was suggested that TPD due to over exploitation is characterized by reduced sucrose content in the latex in the early stages and can be reversible or permanent depending on the degree of fatigue. This may or may not evolve into necrotic TPD which is apparently similar to the bark necrosis induced TPD described by Nandris *et al.*, (1991a, b). The transient reversible phenomenon of partial dryness was not accompanied by any histological anomalies (De Fay, 1988). If the dryness suddenly worsens to about half of the tapping cut or more with or without the other accompanying symptoms it may be an indication of brown bast and the tree becoming irreversibly nonproductive (De Fay, 1988).

In TPD affected trees one or more of the following symptoms can be noticed along with panel dryness. Very often only a part of the tapping cut dries up which may

or may not lead to full TPD. Late dripping was reported as the initial indication of TPD (Steinnmann, 1925). Trees may be partially or completely dry. Brown discoloration of the cortical tissue is a common observation, but not always. Thickening and splitting of the bark below the tapping panel are noticed in majority of the affected trees. Sometimes these symptoms appear even on bark above the tapping panel. Partial emptiness of the latex vessels and coagulation of latex inside the vessels are also observed (Gomez *et al.*, 1990). Excessive meristamatic activity and unusual cell division leading to nodule and burr formation and distortion, cracking and sloughing off of the bark are also noticed (Petch, 1921; Rhodes, 1930; Gomez and Gandhimathi 1990; Sanderson and Sutcliffe, 1921). Histological symptoms such as formation of cross walls, invasion of latex vessels (e.g. tyloses), flocculation of latex, partial or complete coagulation of latex within the vessels and damaged lutoids and other membrane structures are also common features of TPD (Paranjothy *et al.*, 1975; De Fay, 1981; Hao and Wu, 1994).

Besides reduction and cessation of latex flow terminal symptoms like formation of woody burrs and cracking of the bark has been reported (Rands, 1921). The symptoms range from partial dryness with no browning of the tapping cut, browning and thickening of the bark and cracking and deformation of the bark in some cases (Pakianathan *et al.*, 1992; Gomez *et al.*, 1990b). The disease zone occurring on the first tapping panel can spread rapidly onto the second panel (Murong *et al.*, 1994).

According to de Fay and Jacob (1989), external symptoms of natural bark dryness is the drying of the tapping cut. Observation of the bark during tapping and a thorough study of tapping panel, show that drying up of bark can reach various degrees, simple tendency to dryness; typical dryness, more or less extensive and complete dryness. Simple tendency to dryness is the starting stage of dryness in rubber. Here the drops of latex emerge irregularly and swell slowly in the fresh cut (Rands, 1921; de Fay, 1981). Two other types of flow have often been described, either extreme fluidity of the latex, which flows from the cut for a long time (Petch, 1921; Rands, 1921), or in contrast, very viscous latex leading to premature coagulation on the cut. These features are often interpreted as forecast of the typical dryness caused by tapping panel dryness (Petch, 1921; Rands, 1921; Peries *et al.*, 1964).

In second stage of dryness i.e., in typical dryness the latex does not flow at all unless the tapping cut is made deeper. The dry part of the tapping cut is distributed in one or two zones of whole tapping cut and later it becomes completely dry (de Fay and

Jacob, 1989). It is generally considered that dryness begins at the tapping cut and then extends sideways and downwards very rapidly (Rands, 1921).

In the last stage of dryness drops of latex do not appear whatever be the depth of tapping, known as complete dryness. In complete dryness 100 per cent dryness of tapping panel is observed (de Fay and Jacob, 1989). Other anomalies observed in the dry zones of bark are browning, a tendency to thicken, cracking and peeling, deformation and abnormal growth of trunk (de Fay and Jacob, 1989; Gomez and Gandhimati, 1990).

Browning of dry bark and abnormal coloration were reported by many workers (Rands, 1921; Petch, 1921; Sanderson and Sutcliffe, 1921). Cracking, usually vertical, may occur in the outer part of the bark. It is generally observed below the tapping cut where it runs towards the base of the trunk (Petch, 1921; Rands, 1921) and enables the old, diseased bark to flake off. It dries and separates off as a thick scale that is hard, brittle and cracked. (de Fay, 1981). Another important symptom is the formation of abnormal bulges on the lower part of the tree trunk. (Rands, 1921; Sanderson and Sutcliffe, 1921). It has been observed that though the occurrence of TPD is at random in plantations, there is some level of clustering of infected plants (Taysum, 1960; de Fay, 1981).

A detailed study on the TPD symptoms on clone RRII 105 is essential since it is a high yielding clone planted in more than 85% of rubber planted area in India but highly susceptible to TPD. Hence, detailed systematic study on the symptoms of TPD in untapped as well as trees from the first to the last year of tapping in clone RRII 105 was carried out with the aim of understanding different symptoms of TPD. Symptoms on different plant parts like roots which were not studied in detail earlier and symptoms on trees at different ages and tapped on different tapping panels like virgin/renewed and controlled upward tapping (CUT) panels were studied in detail. Different easily noticeable external symptoms like cracks and bulges were studied in detail. Internal symptoms which were difficult to identify such as bark necrosis on trunk and root, visible only on scrapping the external bark, was also studied in detail. Variations in total latex volume and DRC at different intensities of TPD were also recorded.

2.2 MATERIALS AND METHODS

Healthy and TPD trees of clone RRII 105 at different years of tapping were closely observed to study the external symptoms. Trees were selected from the first year to the final year of tapping from different locations in Kerala and Kanyakumari district of Tamil Nadu. External symptoms on trunk and root were closely observed on each tree. External symptoms on tapping cuts were observed during and immediately after tapping for the nature and colour of the bark, wet and dry areas in bark and flow of latex. Trees were also observed before opening for tapping to identify symptoms of TPD in untapped trees.

2.2.1 Design of experiment

2.2.1.1 Location

Since the plantation management practices differ between small holdings and large estates, observations were made from both (Table 2.1). Observations in small holdings were carried out at six locations under different regions spread all over the Kerala state namely Nedumangadu, Adoor, Kanjirappally, Pala, Mannarkadu and Thaliparampa. Locations were selected to represent different types of rubber growers in Kerala ranging from Pala and Kanjirappally with highest productivity and having very good management practices to locations like Nedumangadu and Adoor with lesser productivity and inferior management practices. In each location there is slight variation in the systems of tapping adopted.

Observations on trees in estates were recorded from Kulasekharam area of Kanyakumari district in Tamil Nadu. The estates included were New Ambady Estate, Maruthi Estate, Kottukulam Gardens, Vrindavan Estate, Nataraja Estate, Vaikundam Agrotech, Vaikundam Plantations, Bethany Estate, Babu Gardens, Ranipuram Estate, Kamadhenu Estate and Sivalokam Estate. In each estate slightly different management practices are adopted.

2.2.1.2 Age of the plants

Trees of clone RRII 105 were selected from the first year of tapping in BO 1 panel to the last year of tapping in BI 2 panel. Usually each of the four panels is tapped for six years under normal system of tapping and hence a total 24 years of tapping was studied. The consecutive panels are termed BO 1, BO 2, BI 1 and BI 2 respectively, BO indicating original and BI indicating renewed bark. In small holdings 1000 trees each from all four panels were studied from each location. From the large estates 100 trees in

each tapping year was selected and observed every month continuously for one year to study the symptom development.

2.2.1.3 Observations

Detailed study on the symptoms of TPD in untapped as well as trees in all stages of tapping in clone RRII 105 was performed to understand different developmental stages of TPD. Symptoms on different plant parts like roots and trunk at different ages and in different tapping panels like virgin/renewed panels and CUT panels were studied in detail. Different easily noticeable external symptoms like cracks and bulges were observed closely. Internal symptoms such as bark necrosis on trunk and root which is visible only on scrapping the external bark was also studied in detail. Variations in total latex volume and DRC at different intensities of TPD were also recorded.

Table 2.1 Experiment details

Parameters	Small holding	Estates
Location	1. Nedumangadu 2. Adoor 3. Kanjirappally 4. Pala 5. Mannarkadu 6. Thaliparampa	Kulasekharam (Kanyakumari district Tamil Nadu)
No. of panels	4	3
Years of tapping in each panel	6	6
No. of blocks in each year of tapping	10	1
No. of trees in each block	100	100
Total no of trees observed	144000	1800
Frequency of observation	Single observation	Monthly

2.2.1.4 Categorization of TPD trees

TPD trees were categorised based on the intensity of TPD. The length of the panel showing dryness, which directly influences latex production, was considered as the indicator of intensity. For estimating the intensity of TPD, trees were tapped and the dry portions were marked below the cut with a chalk immediately after tapping, before the latex flows. Total length of the panel and the length of the dry portion were measured. The intensity of TPD in terms of per cent dryness was calculated based on the formula,

$$\text{Intensity of TPD (\%)} = \frac{\text{Length of the cut affected by TPD x 100}}{\text{Total length of the cut}}$$

In order to express the degree of intensity of the disorder, the following score was used (Fig. 2.1).

(1) 0% - Healthy (2) up to 25% - Low (3) 25-50% - Medium (4) 50-75% - High (5) > 75% - Very high

(a) (b) (c)

(d) (e)

Fig. 2.1 Categorization of TPD trees (a) Healthy, (b) Low, (c) Medium, (d) High (e) Very high

2.3 RESULTS

Tapping panel dryness (TPD) derives its name from the effect it produces on the tree, characterised by drying up of the latex vessels resulting in partial or complete cessation of latex production. The external symptoms other than panel dryness varied from tree to tree. It included malformations such as bulging, cracking, necrosis on the bark of the tree trunk. Some trees did not show any external symptoms other than bark dryness. TPD trees showed such symptoms on root also. Leaves did not show any characteristic symptoms in TPD trees.

2.3.1 Symptoms on tapping cut

2.3.1.1 Primary symptoms and appearance of TPD

Tapping panel dryness was noticed on a rubber tree when latex vessels upon tapping produces abnormally low or no latex. According to Sanderson and Sutcliffe (1921), a tree can be considered to be affected when the tree is unable to yield latex at the usual depth of tapping. It was observed that development of panel dryness varied from tree to tree. Close observations of the tapping cut and panel showed different types of dryness. Mainly two types of panel dryness were observed.

2.3.1.1.1 *Dryness in the entire stretch of tapping panel*

In some trees, dryness was noticed in the complete stretch of the tapping panel all at once and only the inner most layer of latex vessels produced latex throughout the length of the tapping panel (Fig. 2.2). In such cases complete stretch of tapping panel dries all at once followed by bark cracking symptoms. TPD symptoms develop immediately whenever a fresh panel is opened either on the same side or on the opposite side in such trees. In most cases, dryness spreads to the adjacent virgin panel, affecting the entire circumference. Soon the inner layer also turn dry and the tapping panel turns to fully dry within one to two months' of tapping. This kind of development of TPD symptoms were mostly observed in the initial years of opening of a tapping

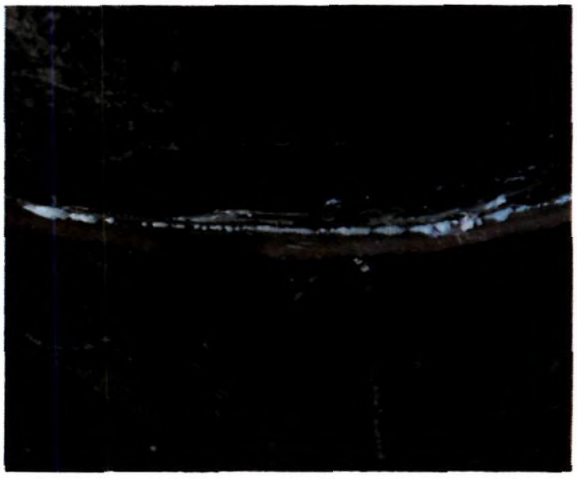


Fig.2.2 Latex flowing only from the inner bark throughout the length of the panel

panel, either virgin or renewed (Fig. 2.3 a&b). Such trees often show prolonged flow of watery latex (late dripping) with drastic reduction in DRC.

(a) (b)

Fig. 2.3 a. Latex flow only from inner bark throughout the length of the panel on virgin bark
b. Dryness appearing simultaneously in the entire length of panel on renewed bark

2.3.1.1.2 ***Intermittent dry and wet zones on the tapping panel***

In most of the TPD trees, partial dryness of varying degrees was noticed. Such trees had one or more dry patch on the tapping panel with no latex flow even from the inner latex vessels (Fig. 2.4). The dry part of the cut is distributed in one or two or even more zones (Fig. 2.5 a & b). As the tapping progress on such trees, the dry area may continue to remain as dry patches while the remaining area produced latex or in some cases the dry areas coalesce leading to complete drying of the tapping panel (Fig. 2.6). In such trees excessive pre-coagulation on the tapping cut with abnormally high DRC was observed which later lead to the development of dryness (Fig. 2.7a). Such trees also showed necrotic symptoms with brownish discolouration on the tapping panel when the outer bark was scrapped (Fig. 2.7b). The terminal symptoms like bark cracking, flaking and development of burr and nodules were also observed. But

Fig. 2.4 Partial dryness - intermittent dry and wet zones with brownish bark

importantly this kind of trees did not show prolonged flow of watery latex (late dripping) or reduction in DRC.

(a) (b)

Fig. 2.5 Distribution of dry zones (a) single dry zone (b) multiple dry zones

Fig. 2.6 Complete dryness

(a) (b)

Fig. 2.7 (a) Pre-coagulation on the tapping cut (b) necrotic symptoms

2.3.1.2 Endurance of TPD

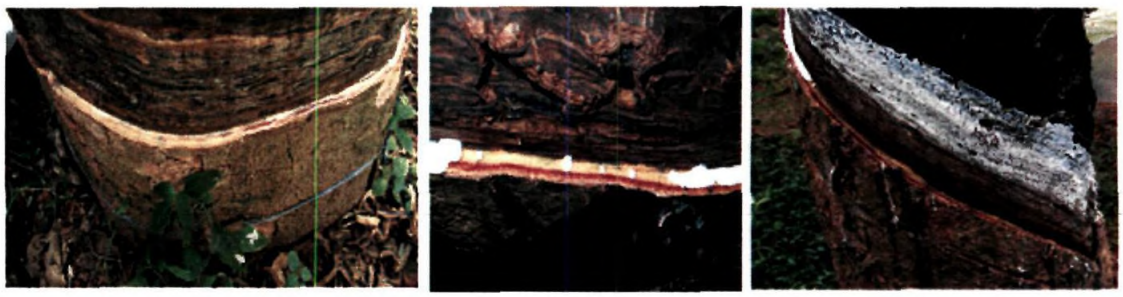
When regular tapping was continued on the partially dry panels, changes in the severity of TPD occurred. Conversion from low to medium TPD intensity and vice-versa or back to '0' level (healthy) was commonly observed. In some cases both low and medium intensity groups turned to fully dry. But in almost all cases the trees that showed high TPD intensity turned to very high intensity and never recovered. When a panel was fully dry, the opposite panel (virgin or renewed) also got affected (Fig. 2.8 a, b & c). The renewed bark formed in TPD trees after scraping off and application of a wound dressing compound (Rubber kote) also could not yield latex continuously. However, the upper panel of fully affected trees were found to yield latex for a short period. But here also with continued tapping, the panel went dry after a short period of tapping (Fig. 2.8 d & e). However, satisfactory yield was obtained when CUT method was adopted.

Fig. 2.8 (a) Cracking on the opposite side of tapping panel on virgin bark
(b & c) Cracking below the tapping panel on the renewed bark.
(d & e) Panel dryness in upward tapping

2.3.2 Symptoms on the bark

2.3.2.1 Abnormal colouration of the bark

Abnormal colouration of dry bark was observed in TPD affected trees of clone RRII 105. The natural colour of the bark changed to yellowish to brown yellow or dark brown to grey (Fig. 2.9). The brown patches generally appeared and developed along with the dryness.



(a) (b) (c)

Fig. 2.9 Panel dryness with abnormal colouration observed on (a) virgin bark (b) & (c) renewed bark

2.3.2.2 Bark thickening

The bark of the dry trees was observed to thicken in some cases in clone RRII 105. This was very clear in partially dry trees when dry zones in panel were thicker than the healthy zones (Fig. 2.10).

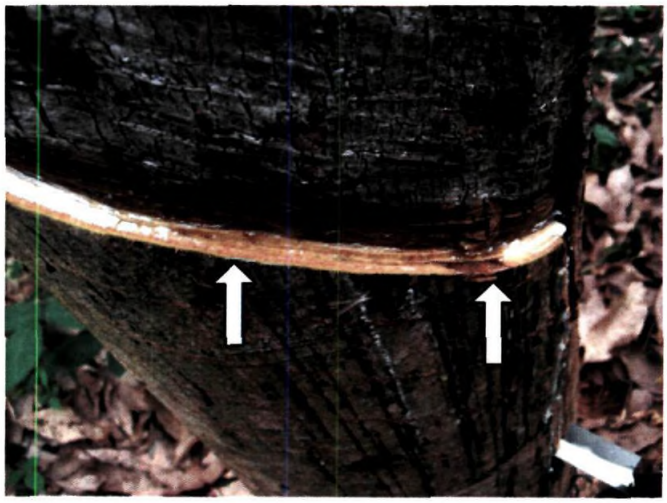


Fig. 2.10 Bark thickening in TPD trees (Arrows indicates the thicker dry zone)

2.3.2.3 Cracking and flaking

Cracks were observed in the vertical direction all over the bark in most of the TPD trees of clone RRII 105. It was generally observed below the tapping cut where it runs towards the base of the trunk (Fig. 2.11 a & b). When dryness was observed in the

BO 1 panel, the cracks were seen extended to the opposite panel, yet to be opened for tapping (Fig. 2.11 c).

(a) (b) (c)

Fig. 2.11 (a) & (b) Cracking below the tapping panel up to the base of the trunk on virgin bark
(c) Cracking on the opposite side of tapping panel on virgin bark

Cracks also extended to the upper side of the panels in rare cases (Fig. 2.12). With age, cracked bark in TPD affected plants was found to flake or peel off (Fig. 2.13).

Fig. 2.12 Crack extending to the upper side of the panel on the virgin bark

Fig.2.13 Cracked bark peeling off as new tissues develop inside affected bark

2.3.2.4 Necrosis

In many cases, along with the cracks, necrotic patches were also seen. In some TPD trees cracks and necrosis started from the tapping panel (Fig. 2.14). It is generally considered that dryness begins at the tapping cut and then extends sideways and downwards very rapidly (Rands, 1921). But, there were instances where the cracks were seen only at the bottom of the panel, near the bud union, which later extended towards the tapping cut (Fig. 2.15). On scrapping the outer bark, necrotic patches were often seen in the inner bark (Fig. 2.16). Pin head size droplets of latex oozed out from the inner bark (Fig. 2.17), but did not flow down the channel as in normal trees.

Fig. 2.14 Tree with cracking and necrosis below the tapping panel

Fig. 2.15 Tree with cracking and necrosis at the bottom

Fig. 2.16 Necrotic patches on the inner bark exposed on shaving of the outer bark

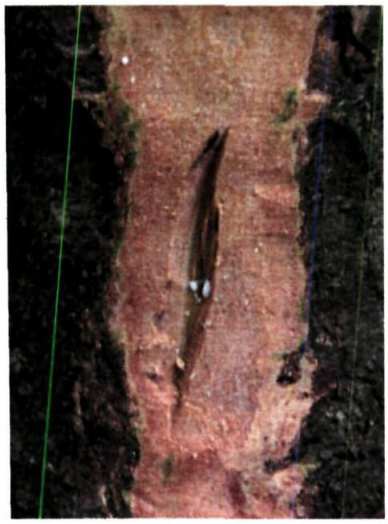


Fig. 2.17 Pin head size droplets of latex oozing out from the inner bark of TPD tree

2.3.2.5 Burr formation

Besides reduction and cessation of latex flow, symptoms like formation of woody burrs was also observed in clone RRII 105. The tapping panels of certain rubber trees were deformed by large vertically elongated swellings of the bark. Such woody burrs of varying size and shape developed in the bark was common in TPD trees of clone RRII 105 (Fig. 2.18 a, b & c). These abnormal growths were also seen along with cracking and flaking (Fig. 2.18 b & c).

(a) (b) (c)

Fig. 2.18 The woody burrs and bulges of variable size and shape along with cracks developed on the bark

2.3.2.6 Symptomless bark

On some TPD affected trees the bark was observed to dry up without any external symptoms other than panel dryness (Fig. 2.19 a & c). The inner bark showed necrotic patches spreading to all sides extending from the bottom to the tapping cut or in the opposite direction or in certain cases above the cut also (Fig. 2.19 b &d). Necrotic patches were observed in the renewed bark also (Fig. 2.19e).

(a) (b) (c) (d)

(e)

Fig. 2.19 (a) & (c) TPD tree with panel dryness symptom only
(b) & (d) Necrotic patches exposed on scraping
(e) Renewed bark in TPD affected tree showing bark dryness and necrotic patches exposed on scraping

2.3.2.7 Symptoms on untapped trees

The dryness of the panel was generally noticed only when the tree was opened for tapping. In most cases only after a few tapping, tapping panel dryness could be identified. But there were instances of untapped trees showing external symptoms of bark cracking and bulging (Fig 2.20). These trees were found to be dry and no latex oozed out even when deep pricking was attempted. Many trees of clone RRII 105 were observed to go dry immediately after opening, within 3 to 10 tapping (Fig. 2.21 & 2.22) without any external symptoms. Close examination of such trees showed the typical symptoms of panel dryness with discolouration of the bark (Fig. 2.22b).

Fig. 2.20 Untapped tree showing bark cracks

Fig. 2.21 TPD identified after a few tappings

(a) (b)

Fig. 2.22 (a) Tree showing dryness immediately after opening
(b) Panel dryness with discolouration of the bark

2.3.2.8 TPD symptoms on trees tapped in different tapping panels

Different external symptoms of TPD on various tapping panels were compared in trees of clone RRII 105 which were having more than 50% TPD in small holdings (Fig. 2.23). Panel dryness alone was observed in 66, 63, 56, and 44% and cracking was observed along with panel dryness in 20, 20, 27, and 29% in panel BO 1, BO 2, BI 1 and BI 2 respectively in clone RRII 105. Bulging was observed along with panel dryness in 7-14% and both cracking and bulging were observed along with panel dryness in 7-13% in the different panels observed.

It was interesting to note that in BO 1 panel among the TPD plants with more than 50% dryness, 66 per cent had only drying of panel without any other external symptoms like cracking and bulging. On the same panel, 20 per cent TPD trees showed cracking symptoms, 7 per cent showed bulging symptoms and another 7 per cent showed both cracking and bulging symptoms along with panel dryness.

There is an increasing trend of cracking symptom with increase in age of the trees. In BO 1 panel only 27 per cent trees showed cracking symptoms while in BI 2 panel it increased up to 42 per cent. However, there was a decreasing trend in the number of trees with symptom of drying alone as the tapping progressed. In the BO 1 panel it was 66 per cent and in BI 2 panel it was only 44 per cent. Among the various symptoms noted, cracking was predominant in the TPD plants as the tapping proceeded.

The trend was similar in estates also (Fig. 2.24) where panel dryness alone was observed in 69, 66, and 51% in panel BO 1, BO 2 and BI 1 respectively. An increasing trend of bulging and cracking with the age of the trees was noticed in estates also although the tapping was more systematic than in small holdings.

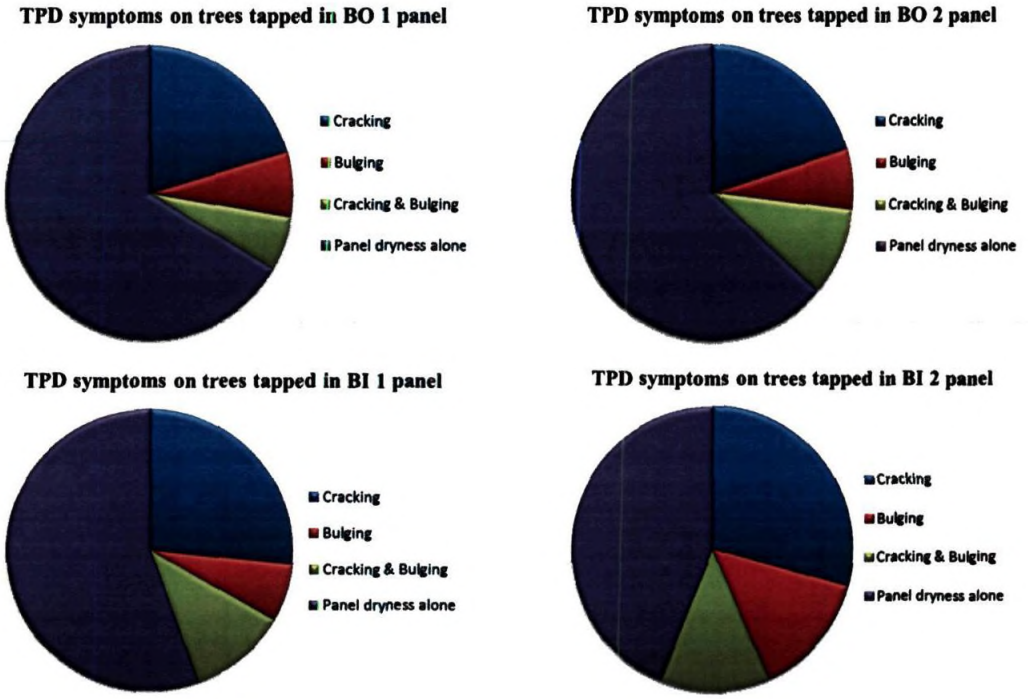


Fig. 2.23 TPD symptoms on trees of clone RRII 105 tapped in different tapping panels – small holdings

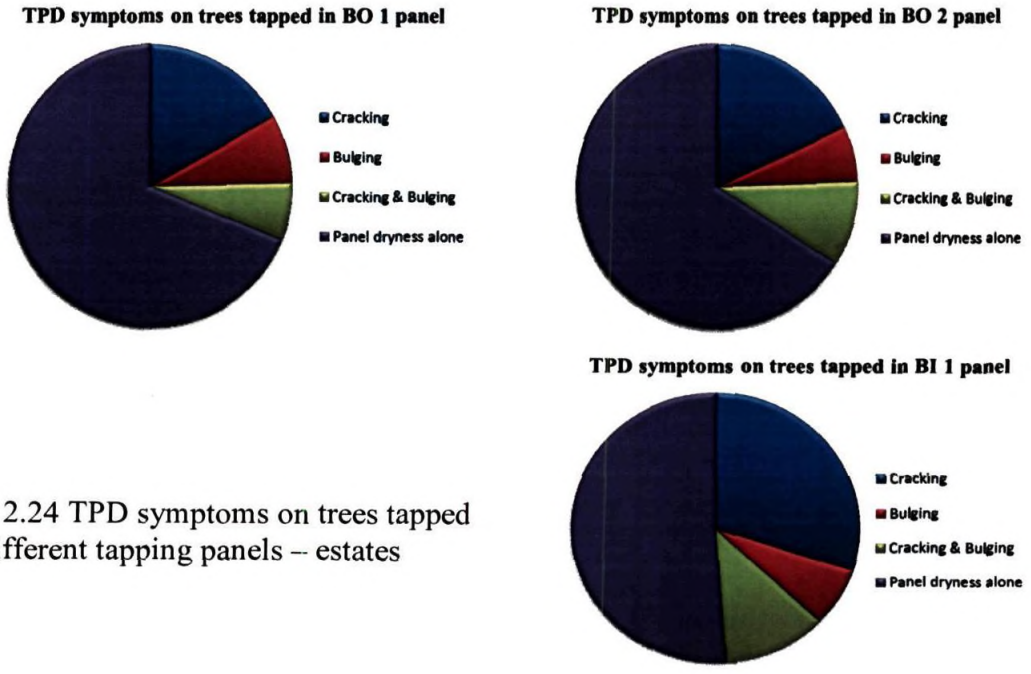


Fig. 2.24 TPD symptoms on trees tapped in different tapping panels – estates

2.3.3 TPD and latex volume

Total latex volume was higher at Pala (Fig. 2.27) and Kanjirappally (Fig. 2.28), whereas it was lower at Adoor (Fig. 2.26) and Nedumangadu (Fig. 2.25). Interestingly at Nedumangadu region, where management practices are poor, the total latex volume in BO 1 panel was high compared to the subsequent panels (Fig. 2.25). This contradicts with observations from other regions. This may be due to the unscientific method of tapping (over exploitation) practices followed in that region.

It was noted that as the TPD intensity increases, a considerable reduction in total latex volume was observed (Fig. 2.25 to 2.30). This phenomenon was observed in all the locations irrespective of the age of the tree or tapping stage. When the reduction in total latex volume due to TPD at all the locations were analysed, nearly 40% reduction in total latex volume was observed when the tree falls in the category of less than 50% TPD and nearly 90% reduction was observed when the trees are in the category of more than 75% TPD in panel BO 1.

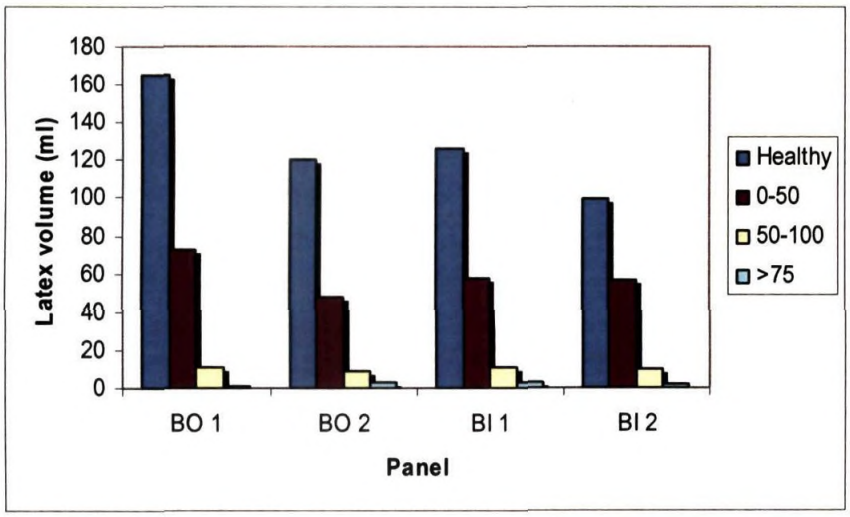


Fig. 2.25 TPD and latex volume (ml/tree/tap) (Location-Nedumangad)

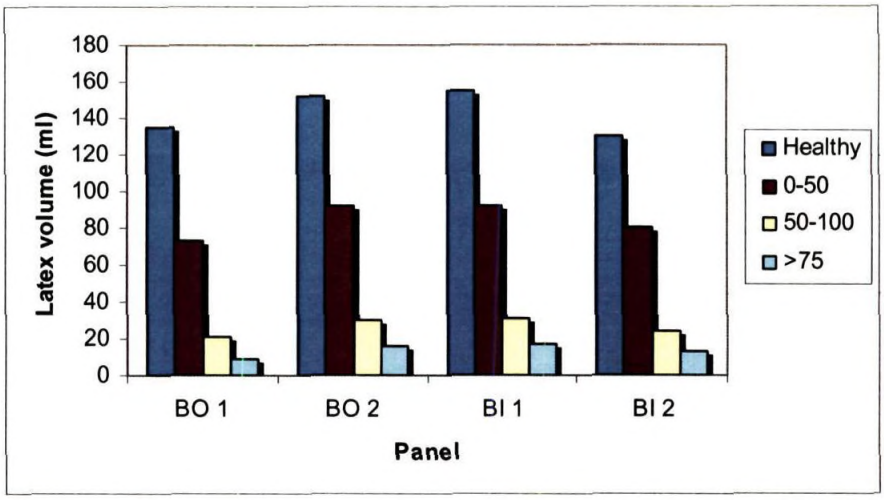


Fig. 2.26 TPD and latex volume (ml/tree/tap) (Location- Adoor)

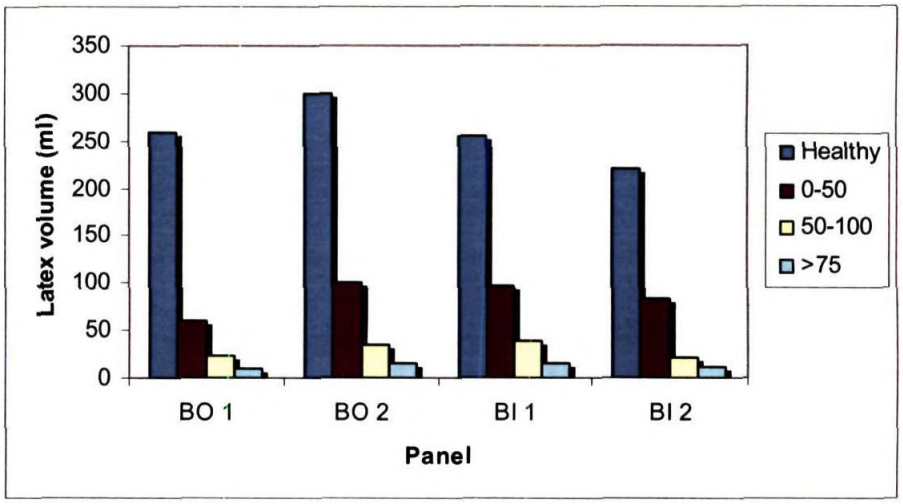


Fig. 2.27 TPD and latex volume (ml/tree/tap) (Location- Kanjirappally)

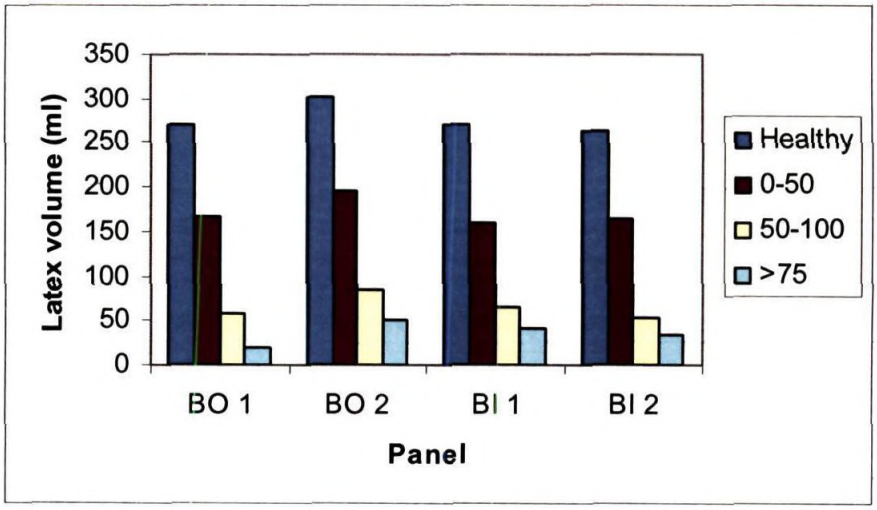


Fig. 2.28 TPD and latex volume (ml/tree/tap) (Location- Pala)

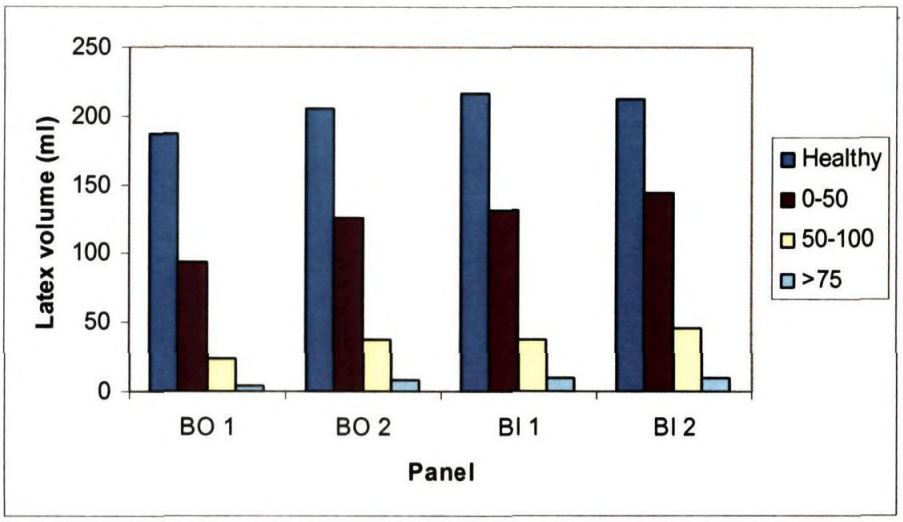


Fig. 2.29 TPD and latex volume (ml/tree/tap) (Location- Mannarkad)

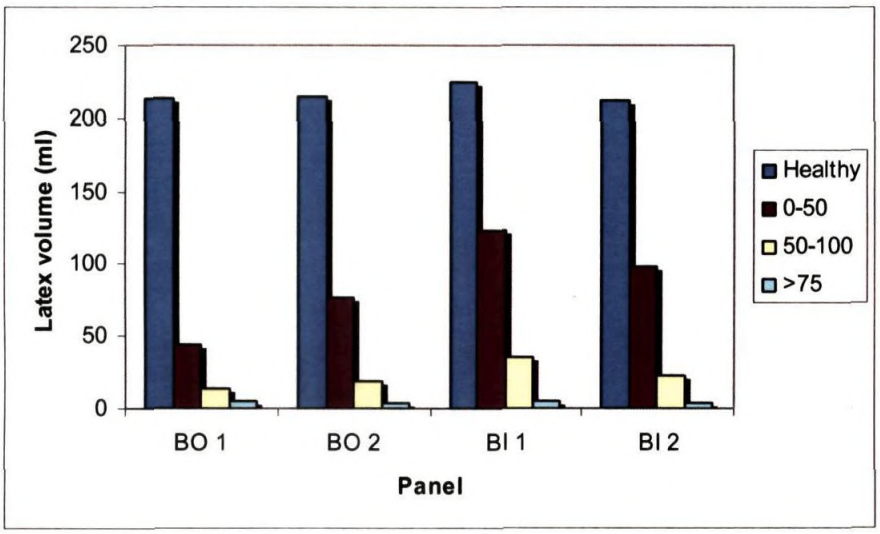


Fig. 2.30 TPD and latex volume (ml/tree/tap) (Location- Thaliparampa)

2.3.4 TPD and Dry Rubber Content (DRC)

Generally, Dry Rubber Content (DRC) of latex was found to be increasing with age at all the locations. Contrary to total latex volume, an increase in DRC was noted as the TPD intensity increases (Fig. 2.31 to 2.36). Late dripping was reported as the initial indication of TPD (Steinnmann, 1925). But, once the tree succumbs to dryness, even if it is partial dryness only, the DRC was found to be increasing with per cent dryness. The same trend was observed in all the locations irrespective of the tapping panel. The highest DRC was recorded by trees with highest intensity of TPD, if the tree is still yielding latex.

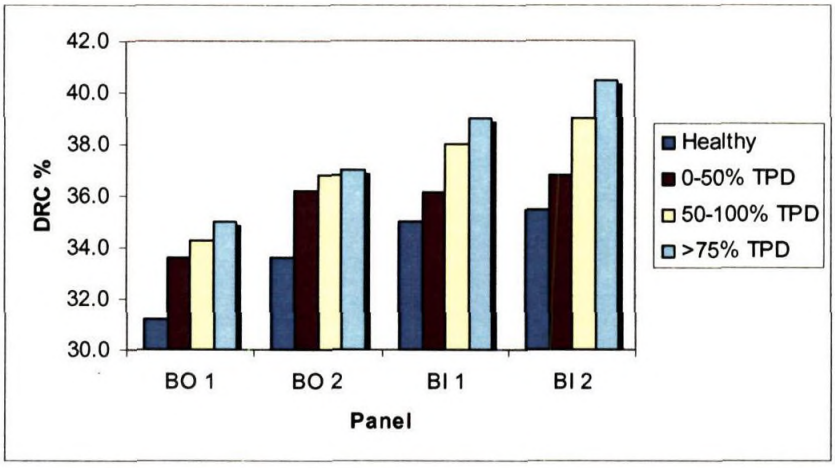


Fig. 2.31 TPD and DRC (Location – Nedumangad)

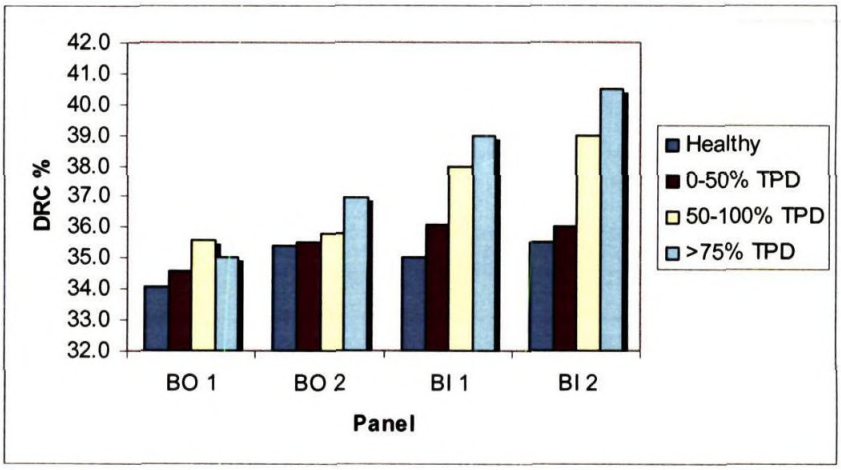


Fig. 2.32 TPD and DRC (Location – Adoor)

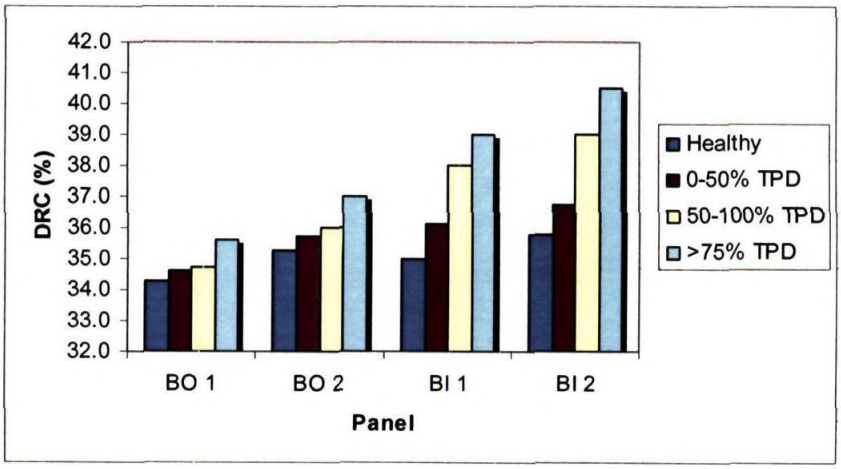


Fig. 2.33 TPD and DRC (Location – Kanjirappally)

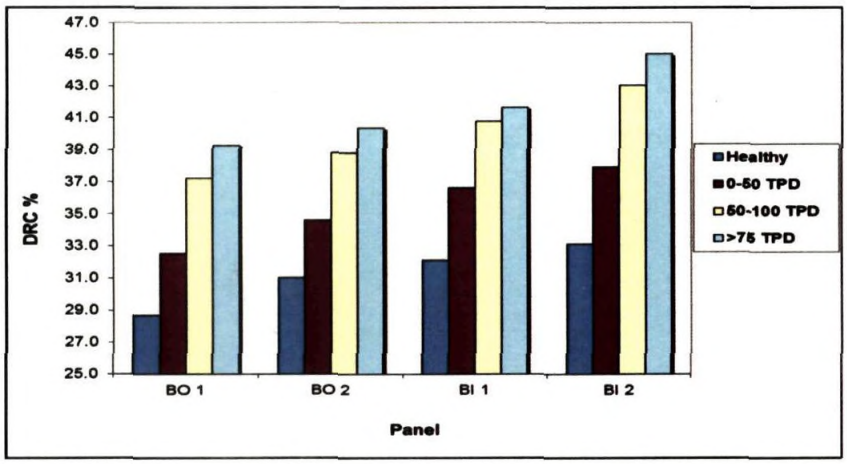


Fig. 2.34 TPD and DRC (Location – Pala)

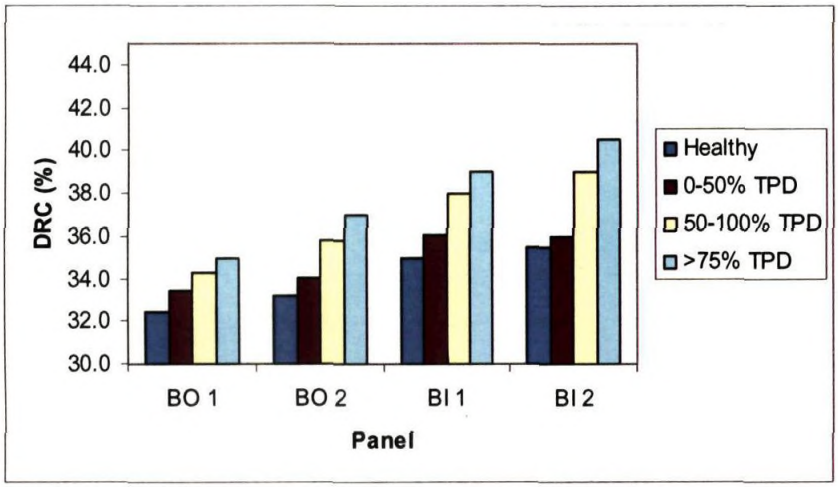


Fig. 2.35 TPD and DRC (Location – Mannarkad)

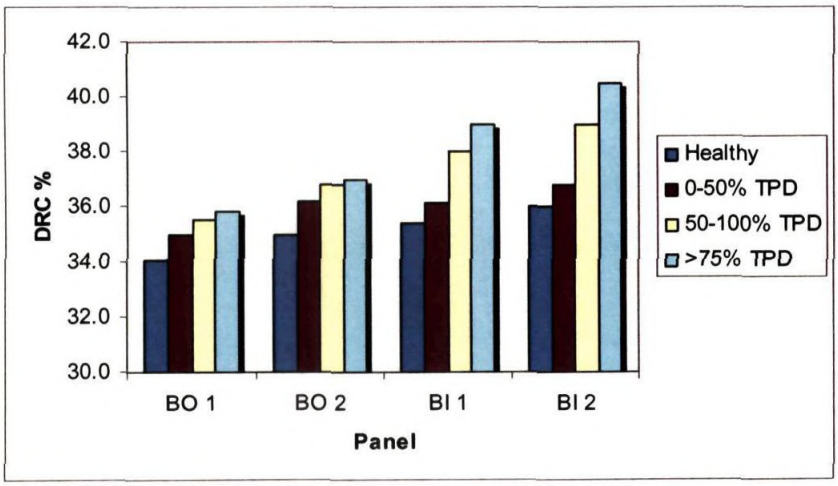


Fig. 2.36 TPD and DRC (Location – Thaliparampa)

2.3.5 Symptoms on the root system

On close observation of the root system (which belongs to the stock part of the budded tree) of TPD affected trees as well as healthy trees after excavation, it was interesting to observe that the root system of TPD trees had necrosis, cracking and bulging (Fig. 2.38) as seen on the trunk (which belongs to the scion part of the tree) of TPD affected trees. In many cases, the root and the collar region close to the bud union showed the typical cracking and peeling symptoms (Fig. 2.37). Necrotic patches were seen on scraping the outer bark of such roots (Fig. 2.38b). Typical brown colour was observed in the bark of the root also (Fig. 2.39).

Fig. 2.37 Cracking symptoms observed on root and collar region

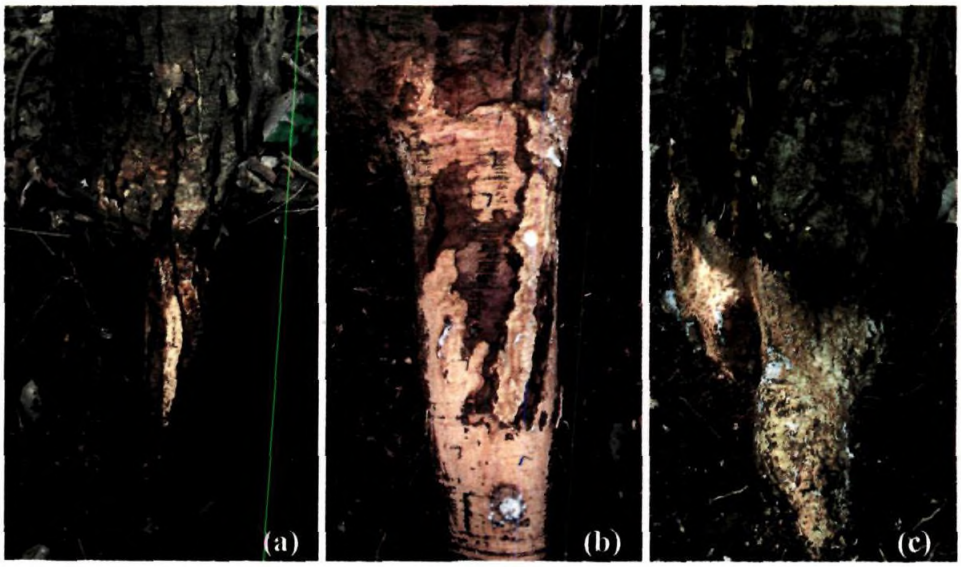


Fig. 2.38 Symptoms noticed on the root stalk (a) Cracking, (b) Necrosis (c) Bulging

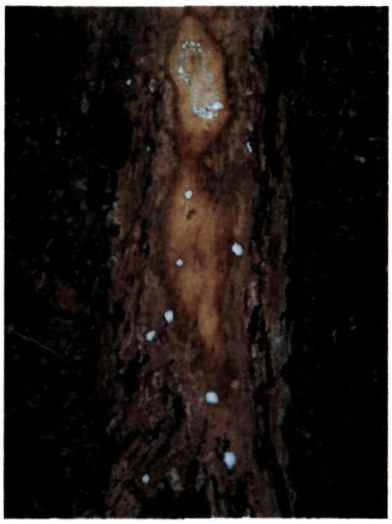


Fig. 2.39 Brownish colour on roots of TPD affected trees

In TPD trees, both the root system and the trunk portion were affected. It was interesting to note that in partially dry trees, the root system corresponding to the dry area in the trunk portion was only affected (Fig. 2.40 & 2.41).

(a) (b)

Fig. 2.40 (a) Root below the dry area on the trunk showing no latex flow
(b) Root with normal latex flow on opposite side (healthy side) of the partially

Fig. 2.41 Partially dried tree showing dryness in root below the dried portion in the scion and normal flow of latex below the wet bark

The dryness symptom was observed also on the collar and root. Very small quantity of latex exuded when the root system of the affected plants were pricked, compared to healthy roots (Fig. 2.42). When 100 trees each were studied from trees in which tapping panel was healthy, partially dry and fully dry, the rootstocks of 85% healthy trees, 25% partially dry trees and only 13% fully dry trees showed normal flow of latex (Fig. 2.43). Rootstock was showing reduced latex flow in 15% healthy trees as well as in 54% partially dry and 28% fully dry trees. Those 15% healthy trees showing reduced latex flow from the root may turn to TPD in future. It was interesting to note that none of the healthy trees showed fully dry root system and only 13% of the fully TPD trees showed normal latex flow from the root. In 59% fully dry trees and 21% partially dry trees the rootstock was also fully dry.

The observation of dryness and associated symptoms such as necrosis, cracking and bulging on the root system (which belongs to the stock part of the tree) of TPD trees as seen on the trunk (which belongs to the scion part of the tree, usually a high yielding clone) is a first report for rubber.

(a) (b) (c)

Fig. 2.42 (a) Normal flow of latex on root of a healthy tree
(b) Root of TPD affected tree with reduced latex flow
(c) Root of TPD affected tree with no latex flow showing necrosis

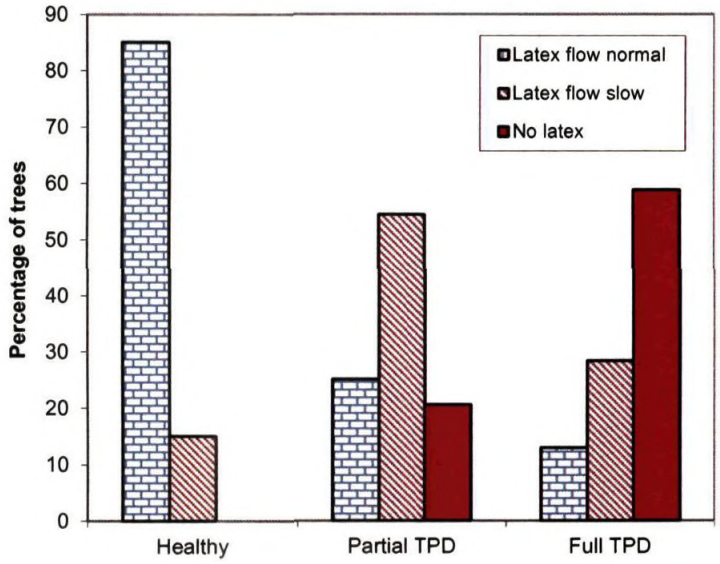


Fig. 2.43 Latex flow from the root

2.4 DISCUSSION

Tapping panel dryness syndrome of rubber has been reported from the plantations from the very early stages of rubber cultivation. The most important symptom of TPD syndrome are reduced latex yield, eventually leading to total drying of tapping panel which gives the name to the syndrome, tapping panel dryness. The detailed study on the TPD symptoms on clone RRII 105 was essential as it is a high yielding clone planted in more than 85% of rubber cultivated area in India but highly susceptible to TPD. Hence, detailed systematic study was performed with the aim of understanding different symptoms of TPD in clone RRII 105. Symptoms on different plant parts like roots which were not studied in detail earlier and symptoms on trees at different ages and tapped in different tapping panels were studied in detail.

Close observations of the tapping cut and panel on the trees of clone RRII 105 showed different types of dryness. Mainly two types of panel dryness were observed. In some trees, dryness was noticed in the complete stretch of the tapping panel all at once and only the inner most layer of latex vessels produced latex throughout the length of the tapping panel. This symptom was mostly observed in the initial years of opening of a tapping panel, either virgin or renewed. Such trees often show prolonged flow of watery latex (late dripping) with drastic reduction in DRC. Late dripping was reported as the initial indication of TPD (Steinnmann, 1925). De Fay and Jacob (1989) observed that partial or intermittent drying of panel leads to such TPD, the intermittent zones coalesce and finally the whole panel dries. In such cases when complete stretch of tapping panel is dry, the inner bark also will be completely dry. In most of the other TPD trees, partial dryness of varying degrees was noticed with one or more dry patch on the tapping panel devoid of latex even from the inner latex vessels. As the tapping progressed on such trees, the dry area continued to remain as dry patches and the remaining area produced latex or in some cases the dry areas coalesced leading to complete drying of the tapping panel. If the dryness suddenly worsens to about half of the tapping cut or more with or without the other accompanying symptoms it may be an indication of brown bast and the tree becoming irreversibly non-productive as observed by De Fay (1988). In such trees excessive pre-coagulation of latex on the tapping cut with abnormally high DRC was observed.

Earlier workers observed that drops of latex emerge irregularly and swell slowly on the tapping cut in some trees on tapping (de Fay, 1981; Rands, 1921). This is due to the irregular drying of the bark in the tapping cut (Rands, 1921).

In this study it was observed that TPD is initiated mainly by two ways either with extreme fluidity of the latex in which the latex flows from the cut for a long time as observed by Petch (1921) and Rands (1921) or with very viscous latex leading to its premature coagulation on the cut as observed by Compagnon *et al.*, (1953) and Petch (1921).

Abnormal colouration of dry bark observed in TPD affected trees of clone RRII 105 was similar to those reported in other clones (Petch, 1921; Rands, 1921; Sanderson and Sutcliffe, 1921). Thickening of the bark of the dry trees was similar to what Rands (1921) observed in other clones. Pakianathan *et al.*, (1992) also reported browning and thickening of the bark in other clones. However bark can be dry and thick, if regenerating bark has been deeply wounded or in trees which are not very vigorous (de Fay and Jacob, 1981).

The phenomenon of bark cracking was observed by earlier workers in other clones (Petch, 1921; Rands, 1921). But, interestingly in the clone RRII 105 when dryness was observed in the BO 1 panel, the cracks were seen extended to the opposite panel which is yet to be opened for tapping which is not so common in other clones.

It is generally considered that dryness begins at the tapping cut and then extends sideways and downwards very rapidly (Rands, 1921). In some TPD trees cracks and necrosis started from the tapping panel. But, there were instances where the cracks were seen only at the bottom of the panel, near the bud union, which later extended towards the tapping cut.

Formation of woody burrs was also observed in clone RRII 105 as reported in other clones (Pillay, 1968; Bobilioff, 1919; Petch, 1921; Rands, 1921; Sanderson and Sutcliffe, 1921; Compagnon *et al.*, 1953). TPD affected trees without any visual symptom on the bark and untapped trees with bark cracking symptoms were also observed.

Various external symptoms such as cracking, flaking and nodule formation are reported to be associated with panel dryness (Rands, 1921; Petch, 1921; Gomez and Ghandimathi, 1990; Gomez et al., 1990, Rao, 1976; de Fay and Jacob, 1989; Sharples, 1936) in different clones although tapping panel wise progression in symptoms have not

been studied systematically. The present study revealed that cracking and bulging of bark increased with the age of trees and with progressed period of tapping.

The observation of higher total latex volume in BO 1 panel compared to the subsequent panels at Nedumangadu region where management practices are poor, may be due to the unscientific method of exploitation practices followed in that region.

As the TPD intensity increases, a considerable reduction in total latex volume was observed in all the locations irrespective of the age of the tree or tapping stage. About 40% reduction in total latex volume was observed on trees in the category of less than 50% TPD and nearly 90% reduction was observed when the trees were in the category of more than 75% TPD in panel BO 1.

DRC of latex was found to be increasing with age. Contrary to total latex volume, an increase in DRC was noted as the TPD intensity increased. Late dripping was reported as the initial indication of TPD (Steinnmann, 1925). But, once the tree succumbs to dryness, even if it is partial dryness only, the DRC was found to be increasing with per cent dryness. The highest DRC was recorded by trees with highest incidence of TPD, if the tree is still yielding latex. Gomez *et al.*, (1990) reported the partial emptiness of the latex vessels and coagulation of latex inside the vessels which indirectly supports the finding of high DRC in TPD trees.

Although different symptoms like late dripping with low DRC and partial dryness leading to complete dryness are reported as TPD symptoms, the present systematic observations could relate each initial symptom to the DRC of latex.

Dryness of root system was observed along with necrosis, cracking and bulging in TPD affected trees as seen on the trunk. The roots corresponding to the dry portion of scion showed dryness although it originated from a root stock.

2.5 CONCLUSION

The detailed study on the TPD symptoms on clone RRII 105 was essential as it is a high yielding clone planted in more than 85% of rubber cultivated area in India but highly susceptible to TPD. Symptoms on different plant parts like roots which were not studied in detail earlier and symptoms on trees at different ages and tapped in different tapping panels were studied. Mainly two types of panel dryness were observed, dryness in the complete stretch of the tapping panel all at once and only the inner most layer of latex vessels produced latex throughout the length of the tapping panel and another type with partial dryness of varying degrees devoid of latex even from the inner latex vessels, the first case with late dripping and the second without. Abnormal colouration, thickening and cracking of dry bark were also observed in TPD trees of clone RRII 105. In this study it was observed that in some TPD trees cracks and necrosis started from the tapping panel and in others cracks were seen only at the bottom of the panel, near the bud union, which later extended towards the tapping cut. TPD affected trees without any visual symptoms on the bark and untapped trees with bark cracking symptoms were also observed. The present study revealed that cracking and bulging of bark increased with the age of trees and with progressed period of tapping.

As the TPD intensity increases, a considerable reduction in total latex volume (nearly 40% reduction in less than 50% TPD and 90% in more than 75% TPD) was observed in all the locations irrespective of the age of the tree or tapping stage. It was found that once the tree succumbs to dryness, even if it is partial dryness only, the DRC was found to be increasing with per cent dryness.

The present study could closely observe and categorise TPD symptoms. Although the symptoms could be categorized on the basis of parts affected, abnormalities observed etc., the categorization on the basis of length of tapping panel affected is more desirable as it has a direct bearing on the yield. The observation of dryness of root system in TPD affected trees particularly that the roots corresponding to the dry portion of scion show dryness although it originated from a root stock is a new report which could be of significance. The finding that in many cases roots show symptoms of dryness in advance of the scion can lead to a hypothesis of spread of TPD through root system although no attempt is made here to verify such a hypothesis.

Chapter 3

INCIDENCE OF TPD

3.1 INTRODUCTION

Tapping panel dryness (TPD) of rubber trees (*Hevea brasiliensis* Muell. Arg.), also known as brown bast is a serious disorder occurring in all rubber growing countries resulting in severe loss of yield. In the early decades of the commercial cultivation of natural rubber TPD was relatively a minor issue assumed to be due to predominance of relatively low yielding clonal and bud-graft populations (Rands, 1921a). According to Bryce (1921), the incidence of TPD was very small in Ceylon where mild tapping has been the practice unlike in several other countries where the incidence was high and the tapping was severe. It is assumed that when more high yielding clones were evolved and practices including stimulation commenced, TPD became a much serious problem. Today, TPD is a major factor affecting productivity and a key stumbling block in realizing the full yield potential in plantations of newly developed high yielding clones. Although yield of affected trees can be fully lost, loss of the trees due to TPD has never been reported. Obviously, a tree getting affected during its early years of tapping will cause much more net loss to the grower than if it is affected later in its economic cycle.

Rands (1921) observed 52-85% incidence of brown bast in seedling trees in Java. Observations from China (Shaoqiong, 1989), Malaysia (Sivakumaran and Haridas, 1989) and Sri Lanka (Samaranayake and Yapa, 1989) revealed clonal differences in susceptibility (Chua, 1967; Bealing and Chua, 1972). In India, TPD has been known to affect rubber plantations since the beginning of commercial rubber cultivation. However, it became more serious with the introduction of the high yielding clones. The high yielding clone RRII 105 planted in more than 85% of rubber growing area in India is highly susceptible to TPD.

The incidence of TPD in smallholdings can be in the rage of 0-5% in the first few years of tapping which can eventually go up to as much as 5-10% or even higher (Sethuraj, 1992; Sivakumaran *et al.*,1994; Nair, 2004). There are holdings, especially in the final stages of tapping in which more than 20% trees are fully lost due to TPD. According to Chan (1996), up to 10% of the trees affected by TPD in the A panel and up to 15% in the B panel. The literature is rather confusing with respect to the extent of prevalence of TPD. Some studies have reported as much as 50% or even more TPD incidence (Lukman, 1992; Soepena, 1992).

There are reports of variations in the incidence of TPD with regard to clone, age of the plant, stages of tapping and system of tapping (Chan, 1996; Eschbach *et al.*,

1994; Lee and Hashim, 1989; Mydin *et al.*, 1999; Olapade and Ueleke, 1989; Sivakumaran *et al.*, 1986). In order to assess the actual extent of TPD in clone RRII 105 under different years of tapping, a study was made by recording observations from the field.

TPD trees in clusters of two or more have been observed in plantations (Mydin *et al.*, 1999; Taysum, 1960; de Soya, 1983). In many cases the immediate next trees to the affected tree also was affected. Even though the occurrence is at random in the initial years, immediate next trees to the affected ones in the direction of tapping are reported to be affected subsequently.

The most commonly recommended practice to manage TPD is taping rest (leaving the trees untapped) (de Silva, 1961). Tapped trees are regularly watched for incipient symptoms of early TPD. Low frequency tapping, shallow tapping, discrete use of stimulants and adopting controlled upward tapping are other management strategies (Anthony *et al.*, 1981). Application of a compound containing microelements (Wei Xiaodi *et al.*, 1997), use of petrolactum (Sethuraj, 1992) and tar has been reported (Bobilioff, 1921) for controlling TPD. Isolation of the infected bark by taking deep grooves has been found successful in China (He Ziyu *et al.*, 1983). Changing tapping from an affected A panel to a fresh B panel led to the syndrome spreading into the B panel within a matter of less than one year (Krishnakumar *et al.*, 2002). Eschbach *et al.*, (1994) reported the increase in the intensity of bark necrosis with panel change. Management of TPD by adopting upward system of tapping has been attempted by some workers. Earlier reports on management of TPD trees by adopting upward system of tapping showed that TPD trees tapped under CUT system are initially free from dryness and can be exploited although in some trees, dryness developed on upward cuts after six months of tapping (Sivakumaran *et al.*, 1986). However, results of upward tapping with stimulation were not always encouraging (Lukman, 1992). Tapping any part of the tree that is not affected was suggested as the only practical management solution for TPD trees which were impossible to recover (Commere *et al.*, 1989).

In order to assess the actual extent of TPD in clone RRII 105 from the estate sector and from small holdings in different years of tapping, a study was made by making observations from the field. The spread of TPD from the affected to the nearby trees along the direction of tapping was also investigated for getting any evidence on the biotic etiology of TPD. An attempt was also made to study various management practices adopted by the growers and its feasibility and success.

3.2 MATERIALS AND METHODS

Since, the field management practices differ between small holdings and large estates, observations were made from both. Different rubber plantations both from the estate and small holdings sectors with trees of clone RRII 105 at different years of tapping were closely observed to quantify the incidence of TPD. The experimental blocks were selected from the first year to the final year of tapping from different small holdings in Kerala and estates in Kanyakumari district of Tamil Nadu.

3.2.1 Design of experiment

3.2.1.1 Location

Observations in small holdings were carried out at six locations under different Regional Offices of Rubber Board spread all over the Kerala State namely Nedumangadu, Adoor, Kanjirappally, Pala, Mannarkadu and Thaliparampa. These locations were selected to represent different types of rubber growers ranging from those with highest productivity and traditionally considered to be adopting very good management practices (Pala and Kanjirappally) to those with lesser productivity and inferior management practices (Nedumangadu and Adoor). Field management practices differ slightly in each location.

Observations on trees in estates were recorded from Kulasekharam area of Kanyakumari district in Tamil Nadu. The estates included were New Ambady Estate, Maruthi Estate, Kottukulam Gardens, Vrindavan Estate, Nataraja Estate, Vaikundam Agrotech, Vaikundam Plantations, Bethany Estate, Babu Gardens, Ranipuram Estate, Kamadhenu Estate and Sivalokam Estate. Field management practices are slightly different in each estate.

3.2.1.2 Age of the plants

Trees of clone RRII 105 were selected from the first year of tapping in BO 1 panel to the last year of tapping in BI 2 panel (Fig. 3.1, 3.2 & Table 3.1). Usually each of the four panels is tapped for six years under ½ S d/2 system of tapping and hence a total 24 years of tapping was studied. In small holdings 1000 trees each from all four panels (BO 1, BO 2, BI 1 and BI 2) were studied from each location. From the large estates, a block of 100 trees in each tapping year was selected and observed every month continuously for 16 months to study the variations in the intensity of dryness in each tree, development of TPD in new trees and spread of TPD if any from one tree to the next tree (forward spread).

Development of dryness symptoms in TPD trees when tapped in upward direction was also studied using the same method of evaluation on the upward panel for a period of four months.

Untapped **1st year** **2nd year** **3rd year** **5th year**

Fig. 3.1 Rubber trees at different stages of tapping in BO 1 panel

(a) (b)

Fig. 3.2 (a) Tapping on BO 2 panel (7th year) (b) Tapping on renewed bark in BI 1 (13th year)

Table 3.1 Experiment details

Parameters	Small holding	Estates
Location	1. Nedumangadu 2. Adoor 3. Kanjirappally 4. Pala 5. Mannarkadu 6. Thaliparampa	Kulasekharam
No. of panels	4	3
Years of tapping in each panel	6	6
No. of blocks in each year of tapping	10	1
No. of trees in each block	100	100
Total no of trees studied	144000	1800
Frequency of observation	Single observation	Monthly

3.2.1.3 Recording of observations

Detailed study on the incidence of TPD in trees in all stages of tapping in clone RRII 105 was performed to quantity the actual extent of TPD. The pattern of latex flow was observed at the time of tapping. The dry area affected by TPD was marked with a chalk piece and the length of the dry area and the total panel length were measured.

The per cent of tapping panel dryness in each tree was calculated using the following formula.

$$\text{Per cent of TPD} = \frac{\text{Length of cut affected by TPD (dry area)}}{\text{Total panel length}} \times 100$$

Based on the intensity of TPD, trees were grouped into five categories i.e., no incidence (0%), low TPD (0-25%), medium TPD (25-50%), high TPD (50-75%), and very high TPD (>75%). TPD incidence in a particular panel was calculated by taking the average of TPD incidence in each year of tapping on that panel (total six years). The occurrence of TPD trees one after another along the direction of tapping (clustering) was also recorded.

3.3 RESULTS

3.3.1 Incidence of TPD in small holdings

Among the Nedumangad, Adoor, Kanjirappally, Pala, Mannarkadu and Thaliparampa regions in which observation were recorded from small holdings, Adoor and Nedumangad region showed the highest number of TPD trees and Kanjirappally the lowest (Table 3.2). Out of the 18900 trees observed at Adoor region 3508 trees (18.56%) showed more than 50% TPD, while it was 2425 out of 13700 (17.70%), 2153 out of 12700 (16.95%), 2821 out of 17300 (16.30%), 3106 out of 20200 (15.37%) and 2273 out of 15100 (15.05%) at Nedumangad, Taliparampa, Mannarkad, Pala and Kanjirappally respectively.

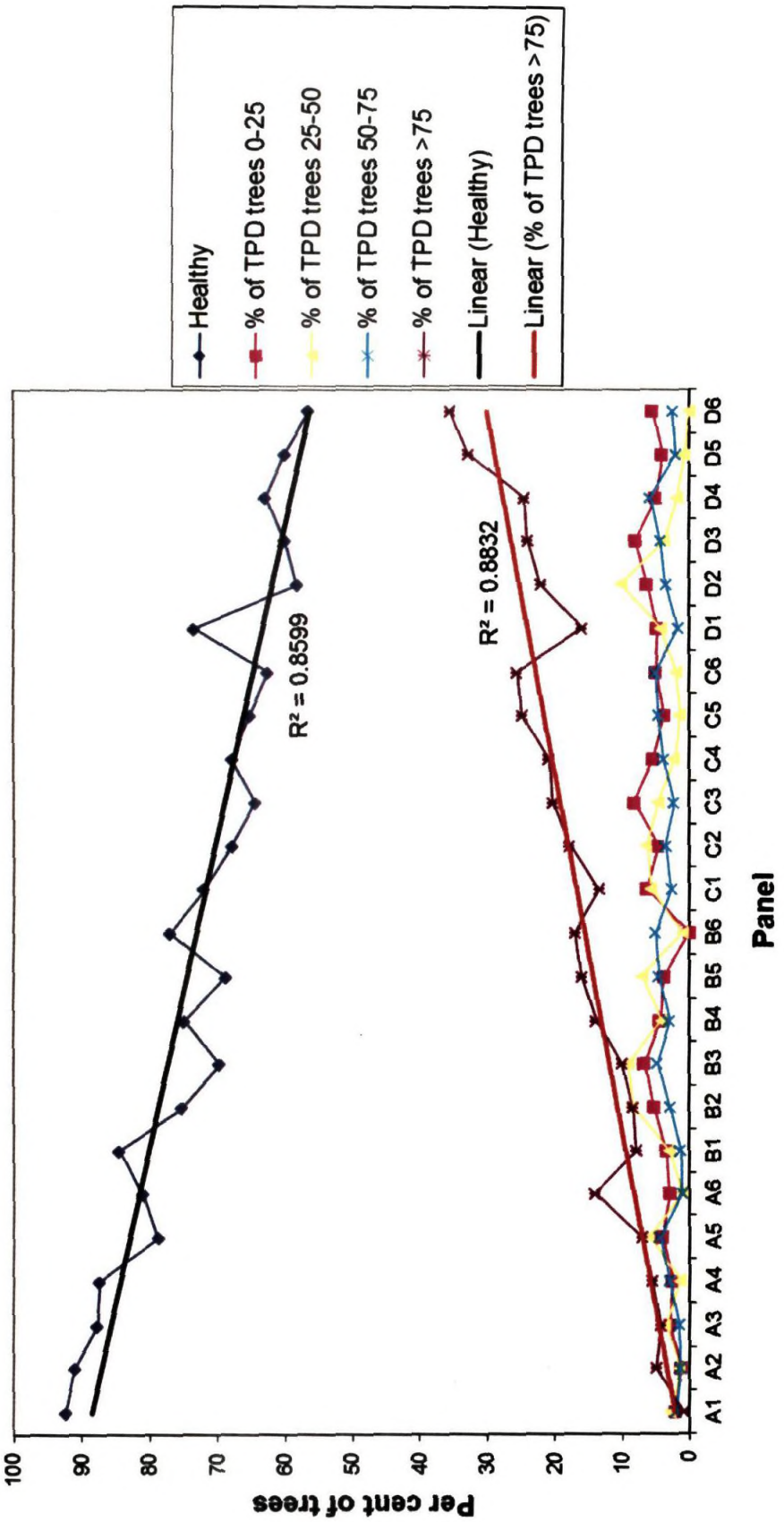
Table 3.2 Number of trees with more than 50% TPD at different locations

Location	Total trees observed	TPD trees	% of TPD trees
Adoor	18900	3508	18.56 [a]
Nedumangad	13700	2425	17.70 [ab]
Taliparampa	12700	2153	16.95 [bc]
Mannarkad	17300	2821	16.30 [c]
Pala	20200	3106	15.37 [cd]
Kanjirappally	15100	2273	15.05 [d]

3.3.1.1 *Incidence of TPD in different years of tapping*

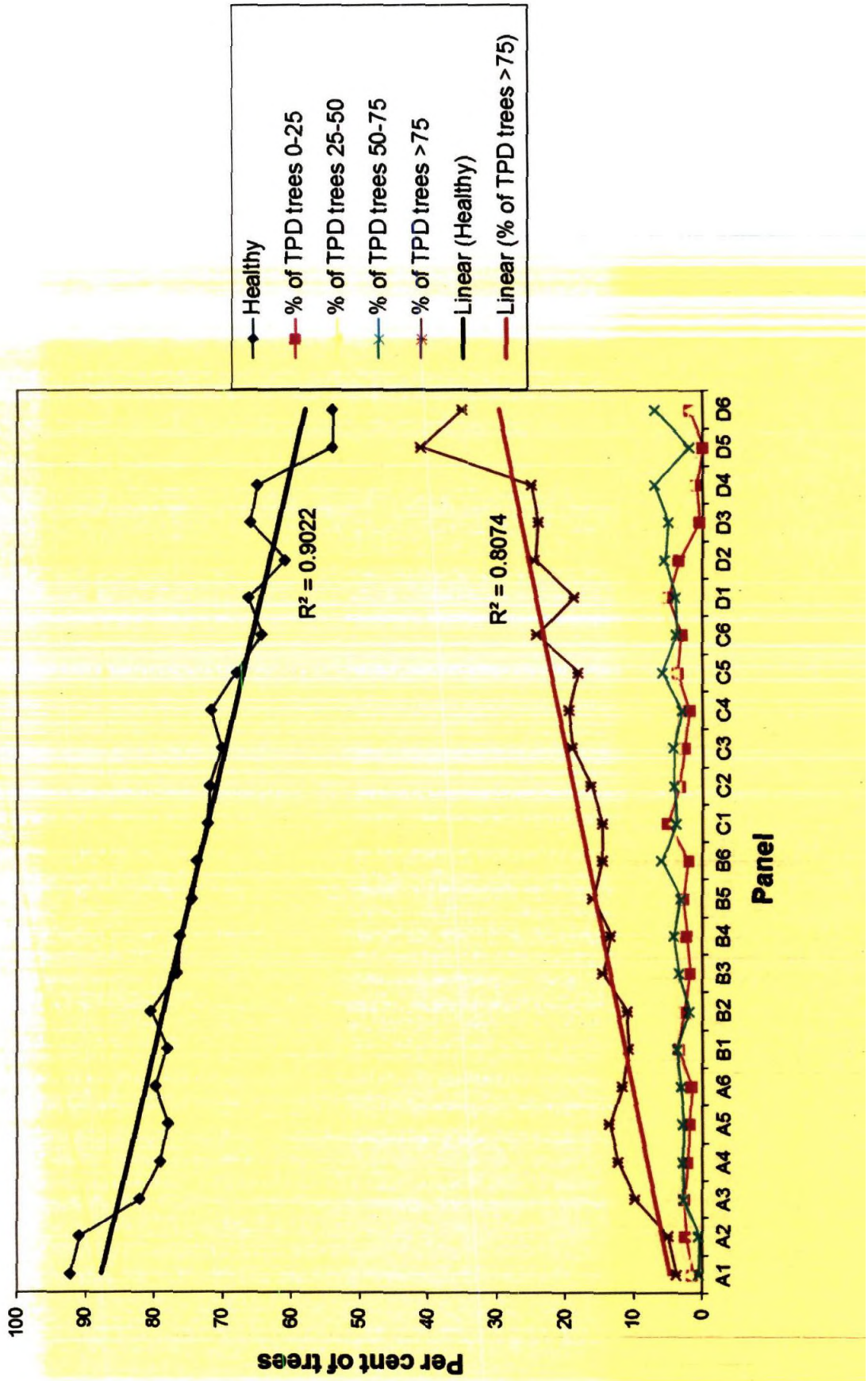
Percentage of TPD trees varied with the year of tapping and in general this increased as the year of tapping progress in all the locations (Fig. 3.3 to 3.8). When the linear trend line of per cent of healthy trees was plotted against year of tapping it showed a definite trend of decrease from the first year to the last year of tapping (Fig. 3.3 to 3.8). The percentage of trees in the category of very high TPD intensity showed a clear trend of increase from the first year to the last year of tapping at all the locations (Fig. 3.3 to 3.8).

The increase in the percentage of trees having more than 50% TPD leads to considerable loss of revenue to the grower and it increased with the age of trees, as evident from the above results.



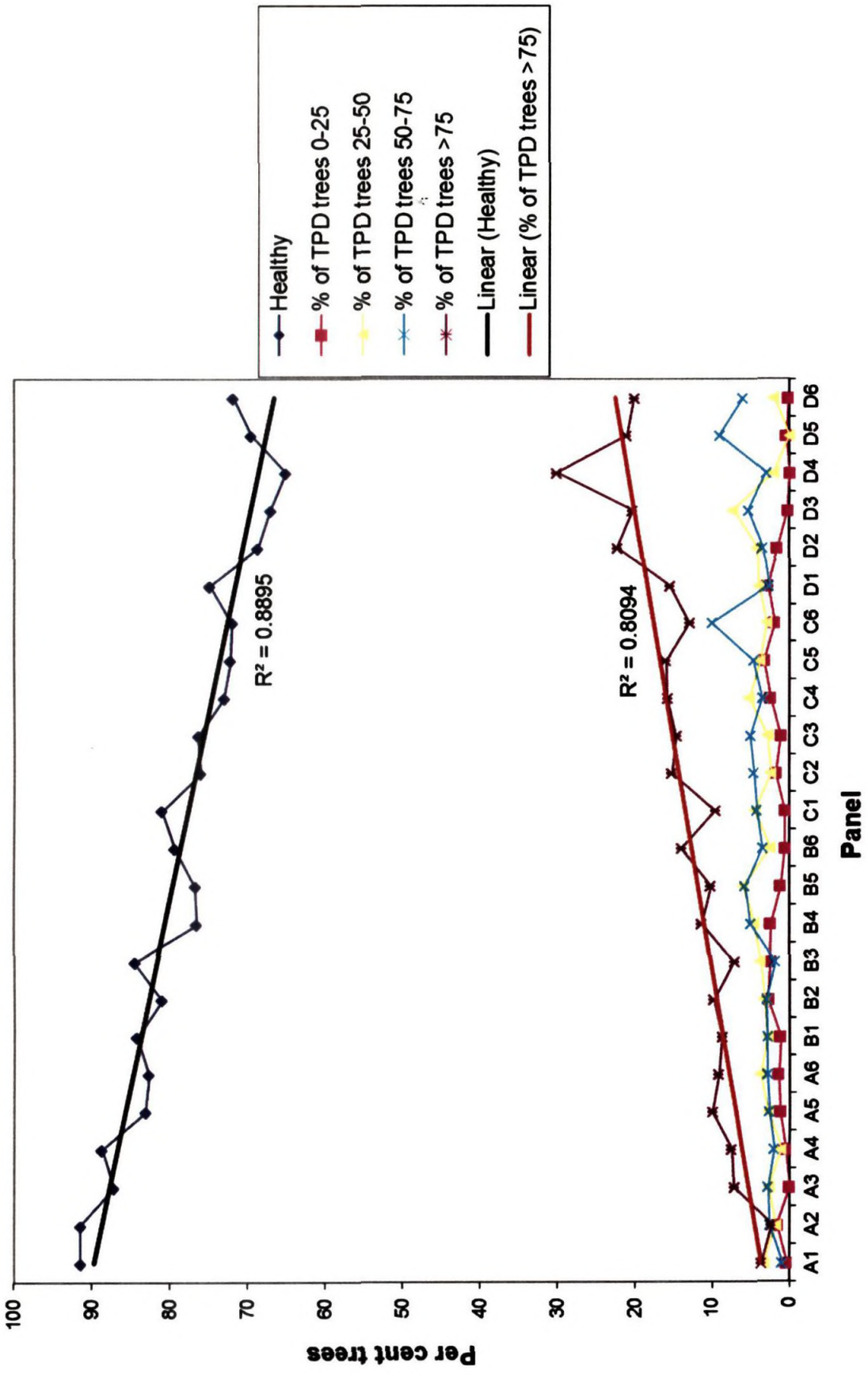
Panel A1 to A6 = BO 1, B1 to B6 = BO 2, C1 to C6 = BI 1, D1 to D6 = BI 2

Fig. 3.3 TPD incidence at Nedumangadu



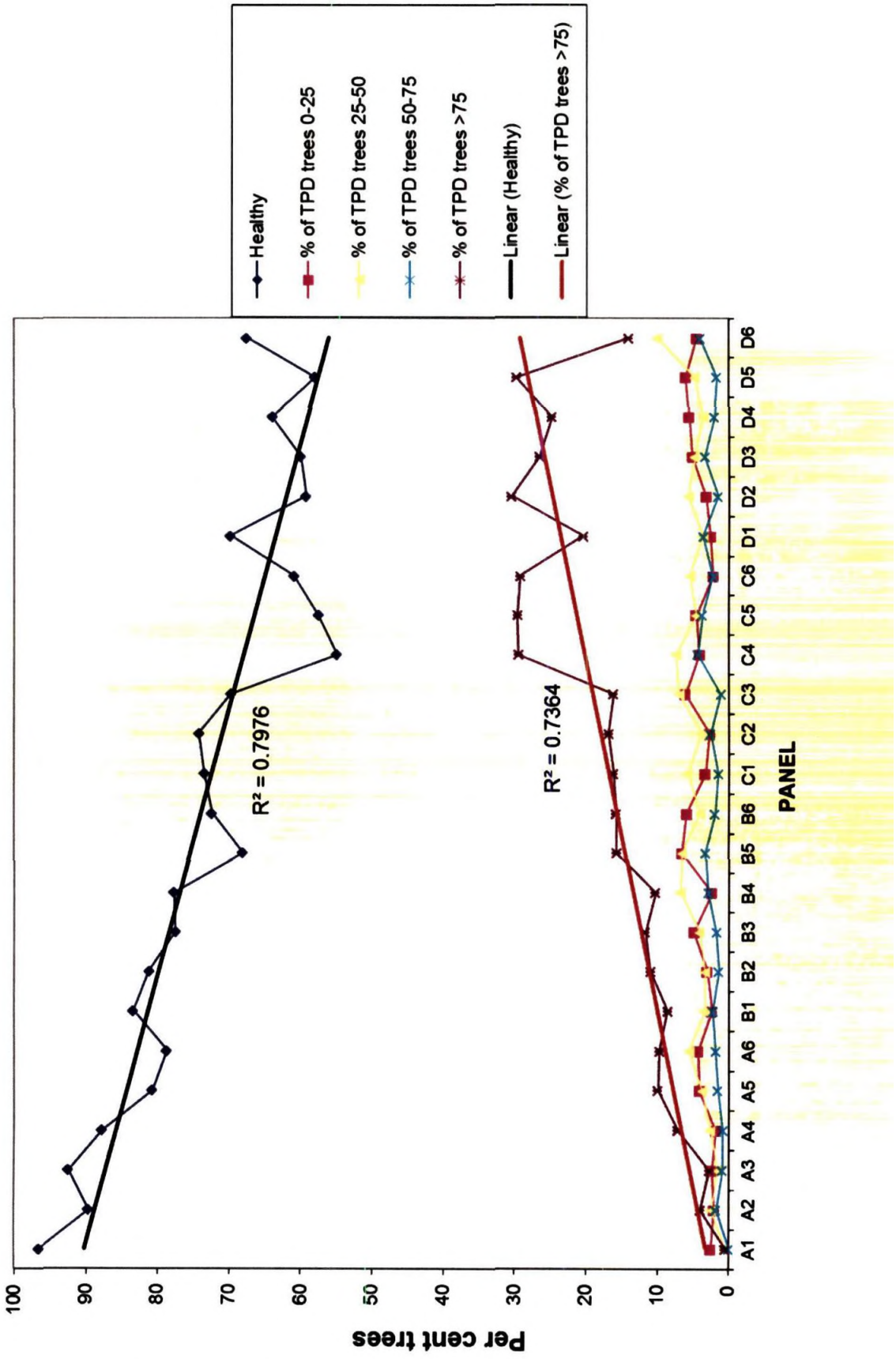
Panel A1 to A6 = BO 1, B1 to B6 = BO 2, C1 to C6 = BI 1, D1 to D6 = BI 2

Fig. 3.4 TPD incidence at Adoor



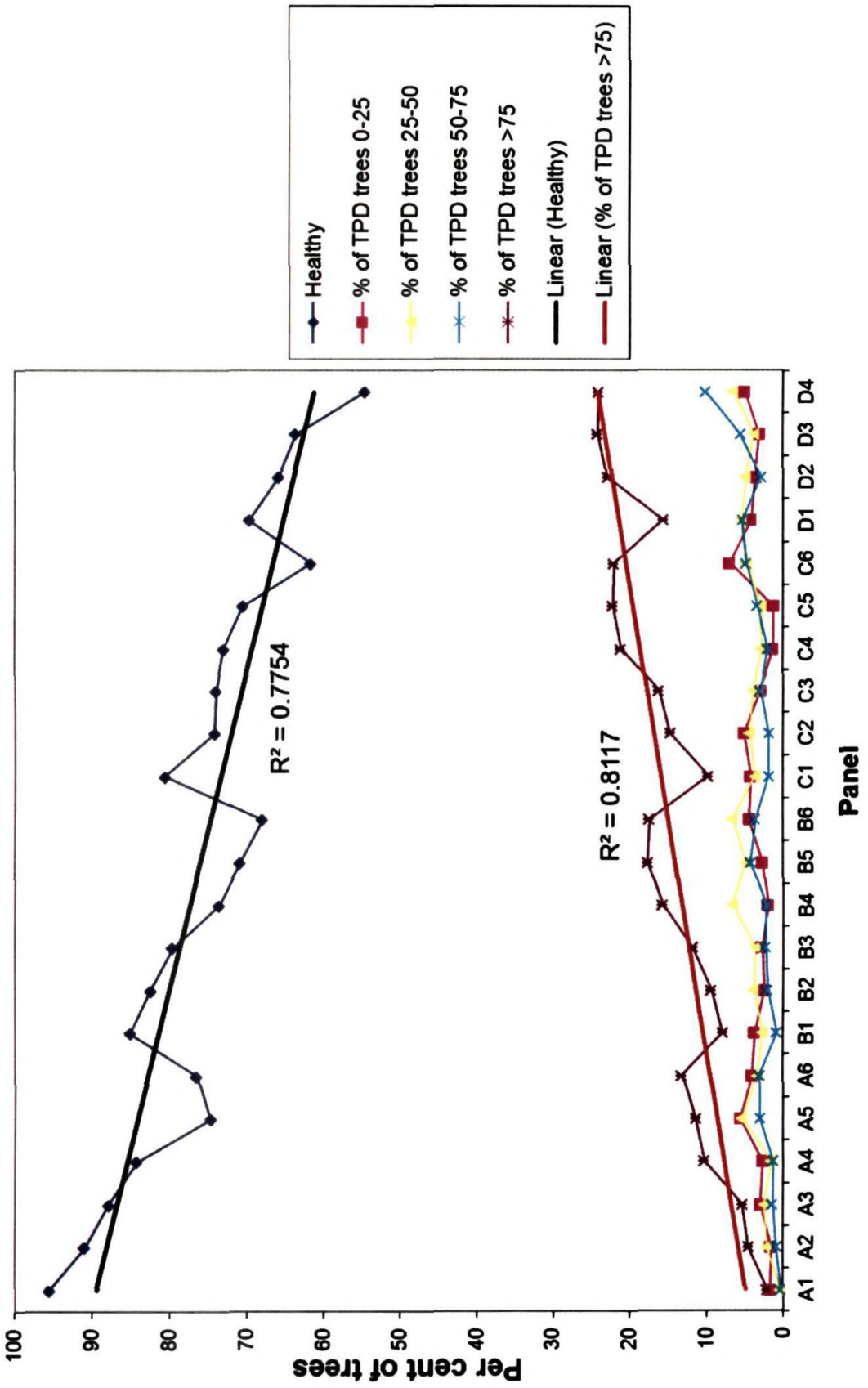
Panel A1 to A6 = BO 1, B1 to B6 = BO 2, C1 to C6 = BI 1, D1 to D6 = BI 2

Fig. 3.5 TPD incidence at Kanjirappally



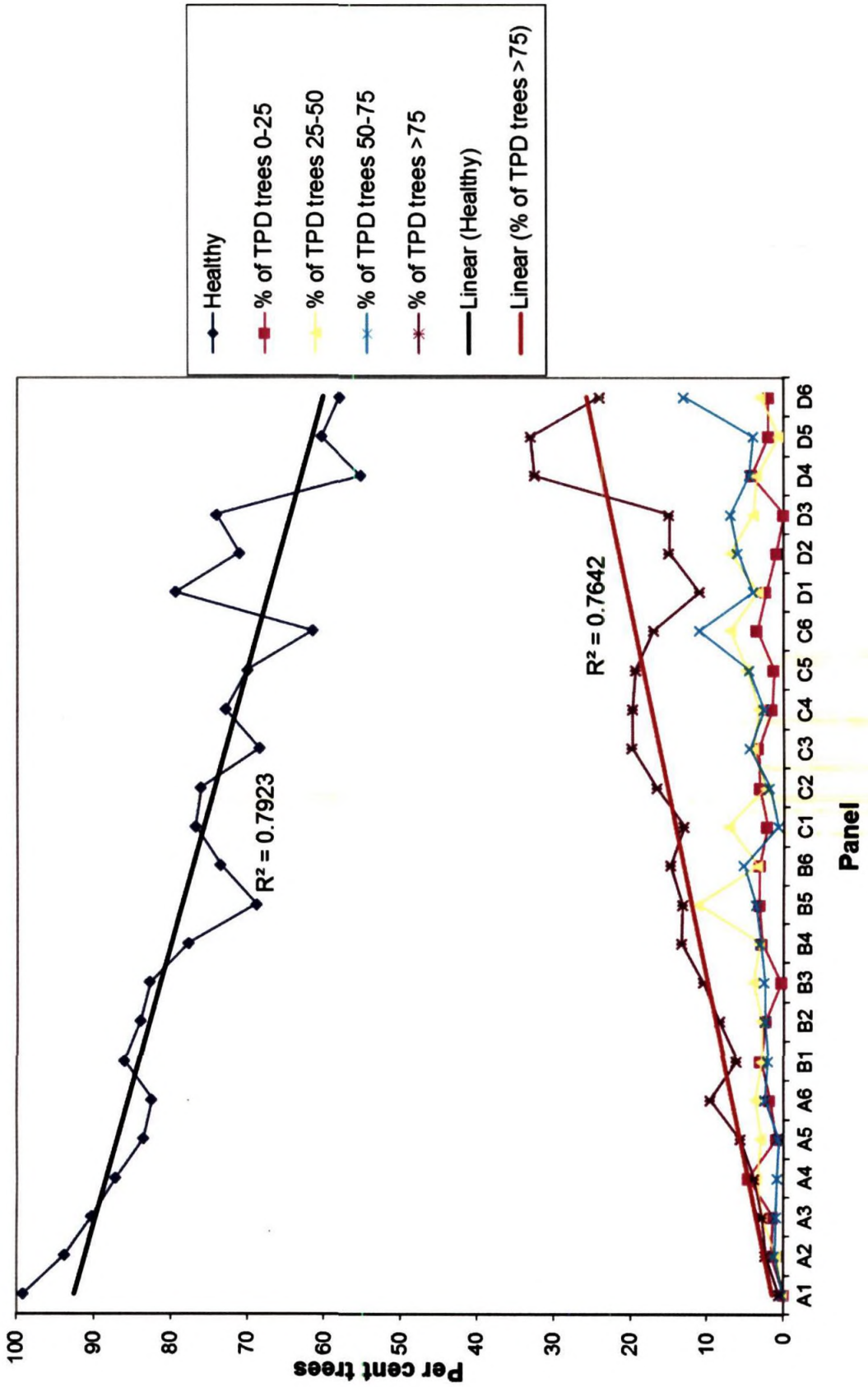
Panel A1 to A6 = BO 1, B1 to B6 = BO 2, C1 to C6 = BI 1, D1 to D6 = BI 2

Fig. 3.6 TPD incidence at Pala



Panel A1 to A6 = BO 1, B1 to B6 = BO 2, C1 to C6 = BI 1, D1 to D4 = BI 2

Fig. 3.7 TPD incidence at Mannarkad



Panel A1 to A6 = BO 1, B1 to B6 = BO 2, C1 to C6 = BI 1, D1 to D6 = BI 2

Fig. 3.8 TPD incidence at Thaliparampa

3.3.1.2 Incidence of TPD in different panels

Panel wise comparison of TPD incidence in more than 50% of panel length is provided in Table 3.3. χ^2 test of goodness of fit showed that there is significant difference in TPD incidence between the panels in each location and it showed increasing trend from BO 1 panel to BI 2 panel (Table 3.3). Adoor region showed the highest number of TPD trees in the category of >50% in panel BO 1, BO 2 and BI 2 which is 11.4, 17.4 and 30.1 respectively. Nedumangadu region also showed comparatively high incidence of TPD in all panels. Kanjirappally region showed lowest number of TPD trees in the category of >50% in panel BO 2, BI 1 and BI 2 which is 13.9, 19.4 and 26.4 respectively. The number of trees in the category >50% showed an increasing trend from BO 1 to BI 2 panel at all locations.

Table 3.3 Percentage of TPD incidence in more than 50% of panel length on various panels

Panel	Nedumangad	Adoor	Kanjirappally	Pala	Mannarkad	Taliparampa
BO 1	8.13	11.4	9.0	6.8	9.5	5.2
BO 2	15.73	17.1	13.9	14.4	15.4	14.2
BI 1	24.32	22.7	19.4	25.4	20.1	21.7
BI 2	29.02	30.1	26.4	27.0	26.5	28.2
χ^2	13.33 **	9.41*	9.76*	14.87**	8.71*	16.98**

3.3.1.3 Forward spread of TPD

Clustered occurrence of TPD trees

In order to study the spread of TPD in the forward direction of tapping in plantations, clustered occurrence of TPD trees corresponding to the direction of tapping was recorded. In some cases there were clusters of 6 to 8 TPD trees in the experimental plots (Fig. 3.9). In one of the holdings two trees planted in the same pit was observed to show TPD symptoms (Fig. 3.10).

3.3.3 Management of TPD trees

3.3.3.1 *Smallholdings, Location : Meenachil Taluk*

Only 23.8% of the small holders give rest to trees by leaving them untapped in all the panels when TPD is observed (Table 3.7). 29.5% of the small holders give rest to the trees when TPD is observed in BO 1 panel and follow the same in BO 2 panel also but do upward tapping when TPD is observed in BI 1 panel.

Table 3.7 Management of TPD trees in smallholdings

Sl. No.	Group	No of blocks	Percentage
1.	BO 1 → above BO 1 downward → BO 2 → above BO 2 downward → rest → BI 1 → UT	37	17.6
2.	BO 1 → above BO 1 downward → BO 2 → above BO 2 downward → UT	30	14.3
3.	BO 1 → BO 2 → UT on BO 1	19	9.0
4.	BO 1 → BO 2 → UT on BO 2	12	5.7
5.	BO 1 → rest → BO 2 → rest → BI 1 → UT on BI 1	62	29.5
6.	BO 1 → rest → BO 2 → rest → BI 1 → rest → BI 2 → rest → slaughter tapping	50	23.8
	Total	**210**	**100**

UT – Upward tapping

Fig. 3.9 A continuous row of TPD trees in the direction of tapping

Fig. 3.10 Trees planted in the same pit, simultaneously (at same tapping stage) showing the TPD symptoms.

It was interesting to note that the number of single occurrence of TPD trees did not show a remarkable increase from the first panel to the last although the total number of TPD trees increased as the year of tapping increased. The number of TPD trees in clusters of two or more showed a remarkable increase compared to the single TPD trees, from the first year of tapping to the last (Table 3.4 & Fig. 3.11 to 3.16) showing that there is a chance of spread of TPD from one tree to the neighbouring tree.

Nedumangadu region showed the maximum number of trees (28.47%) and Kanjirappally showed the minimum number of trees (21.81%) in cluster in BI 2 panel.

Fig. 3.20 Tree showing TPD in BO 1 panel and shifted tapping to BO 2 panel

Fig. 3.21 Tree showing TPD in BO 1 panel, tapped downward above BO 1 and currently tapped on BO 2 panel

Fig.3.22 Trees showing TPD in BO 1 panel, tapped upward above BO 1

Fig. 3.23 Tree rested when both BO 1 and BO 2 panels showed TPD

Fig. 3.24 Trees showing TPD both on upward and downward panels

3.3.3.2 *Management of TPD in estate by adopting upward system of tapping*

When the TPD affected trees were tapped in the upward system of tapping, more than 50 per cent of the trees showed dryness after four months of tapping (Fig. 3.25). The occurrence of TPD trees in the category of very high intensity TPD was 31% (Fig. 3.25).

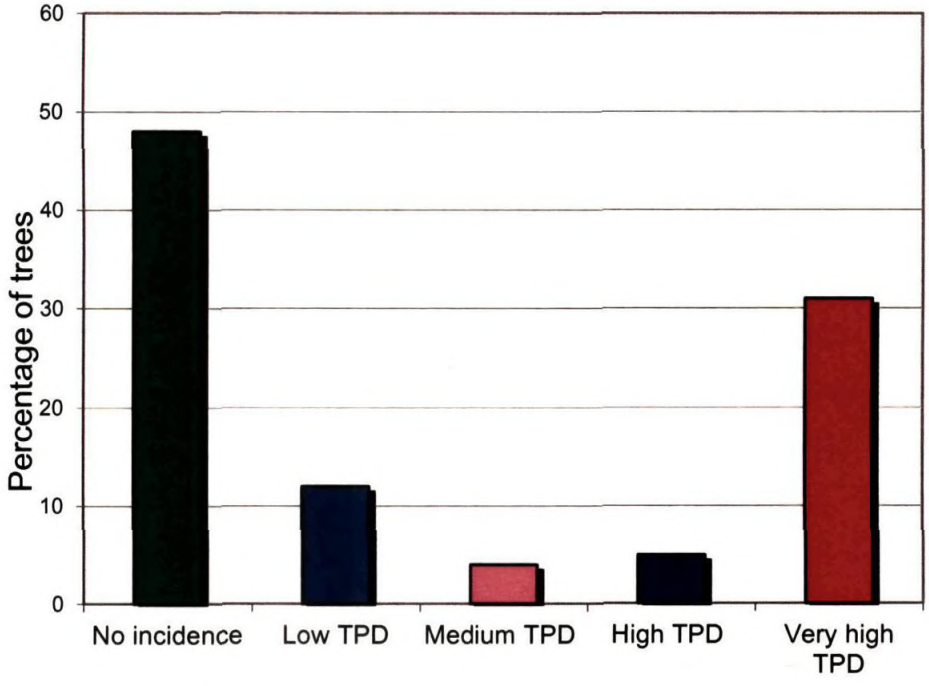


Fig. 3.25 Incidence of TPD in the TPD affected trees four months after initiation of upward system of tapping

3.4 DISCUSSION

It is essential to have an exact knowledge about the extent of the prevalence of TPD in a particular clone for selection of clones for planting and for adopting suitable management practices. When the occurrence of TPD trees in different years and on different panels were analysed it was observed that the number TPD affected trees increased as the year of tapping progressed at all the locations both in small holdings and in estates. The linear trend line of per cent of healthy trees plotted against year of tapping showed a definite trend of decrease from the first year to the last year of tapping. The results clearly showed that maximum TPD incidence is seen in older panels as observed by Chan (1996). He observed 2.1% TPD in the first year of BO 1 panel and 8.5% in the sixth year for the rubber clone PB 260 while on panel BO 2, it was 16.0 and 18.6% in the third and sixth years, respectively. The maximum dryness in individual fields reached 23.5% on panel BO 1 and 44.6% on panel BO 2.

Percentage of trees without TPD symptoms was high when the panel was changed but it again decreased a year after the panel change. TPD trees appeared as healthy without any panel dryness for a short period when the panel was changed, but dryness symptoms appeared again as tapping further advanced. This shows that once a tree succumbs to TPD, it does not recover from it, though symptom remission for short periods can sometimes be noticed.

The percentage of trees in the category of very high TPD intensity (>75%) showed a clear trend of increase from the first year to the last year of tapping at all the locations. The number of TPD trees in the other categories (low, medium and high) did not show such a remarkable trend of increase from BO 1 to BI 1. This could be since such trees were converted to more than 75 per cent TPD during the course of time as the age of the tree advanced and tapping progressed. The increase in the percentage of trees with more than 75% of panel affected by TPD leads to considerable loss of revenue to the grower. Therefore, it is very important to give the best management care at a young age.

The increase in incidence within a period of 16 months was 4% in BO 1, 9% in BO 2, 18.7% in BI 1 when trees with >50% TPD was considered. This shows that the scale of increase in TPD incidence is more in older trees than in trees at the initial stages of tapping.

Such a definite trend of increase was not observed in trees with less than 50% TPD. In BO 1 and BI 1 panel the percentage of trees in that group actually got reduced as some of the trees did not show symptoms of dryness or turned to show more than 50% TPD. It was interesting to note that reversion of TPD symptoms was observed only at a younger age as evidenced by higher number of symptomless trees at the final stage in BO 1 panel. This finding also suggests that it is very important to give the best management care at a younger age. de Fay and Jacob, (1989) reported that certain forms of bark dryness are transitory and do not display the characteristic symptoms of the formation of tylosoids or activation of the phenolic metabolism. Remission of symptoms have been reported in virus diseases. In pepper plants infection with *Pepper golden mosaic virus*, the newly emerged leaves showed a reduction in severity of symptoms (Carrillo-Tripp *et al.*, 2007).

Among the Nedumangad, Adoor, Kanjirappally, Pala, Mannarkadu and Thaliparampa regions in which observation were recorded from small holdings, Adoor and Nedumangad region showed the highest number of TPD trees and Kanjirappally the lowest. The field management practices in Adoor and Nedumangad was in general poor compared to Kanjirappally. For instance, the planting density is in general higher than the recommended (450 to 500 trees /ha) in Nedumangad. The proximity of trees may have an influence on disease spread. The occurrence of TPD affected trees in clusters was maximum in Nedumangad indicating such probability.

Progressive occurrence of TPD in clusters suggests the possibility of tree to tree spread of a biotic agent. De Fay and Jacob (1989) reported the occurrence of rows of four or five TPD trees in rubber plantations. The transmission of a biotic agent through the tapping knife could be the reason for the occurrence of TPD trees in clusters and the spread of TPD from one tree to the neighbouring tree. On continuous monitoring of the trees tapped immediately next to the TPD affected trees, it was observed that some of the healthy trees tapped immediately after tapping a TPD tree turned to TPD after a few months. This observations shows that, though at a limited level TPD is spreading from one tree to the tree tapped immediately next. Viral contamination by the tapping tool and the existence of micro conditions in the soil were suggested to account for the rows of dry trees (De Fay and Jacob, 1989). In a study of TPD affected trees with bark scaling (RRIM 605) out of the ten healthy immediate next trees to the bark scaled, seven became TPD affected by the fourth year of tapping (RRII, 2003). Several field and greenhouse studies have demonstrated that viroids are easily transmissible by

mechanical inoculation and efficiently spread by contact with contaminated pruning tools, farm implements, clothing, and human hands (Barbosa *et al.*, 2005; J. Th. J. Verhoeven *et al.*, 2010). Earlier attempts on disinfection of tapping knife (Jacob et al., 2005) were by a dip in fungicides or disinfectants that has little effect on molecular pathogens.

The most commonly recommended practice to manage TPD is taping rest (leaving the trees untapped) (de Silva, 1961). But, in the present study, it was observed that only 23.8% of the small holders give rest in all the panels when TPD was observed. This could be due to their experience that resting cannot not cure TPD. Nair, (2004) reported the re-occurrence of TPD in majority of the TPD affected trees left untapped for varying periods. Paranjothy and Yeang (1977) observed that the practice where TPD affected trees are rested and periodically re-opened is not considered beneficial and it was shown that any latex obtained from trees which 'recover' after tapping rest is derived largely from regenerated bark beneath diseased tissues. It was therefore recommended that TPD trees be exploited on yielding bark without a tapping rest. But, in the present study it was observed that when the TPD affected trees were tapped in the upward system of tapping, more than 50 per cent of the trees showed dryness after four months of tapping. Hence, this system is not a long term solution for TPD management as also evident from the work of Lukman (1992) in which panel change resulted in high percentage of TPD incidence. Eschbach *et al.*, (1994) also reported the increase in the intensity of bark necrosis with panel change. Krishnakumar *et al.*, (2002) reported that in almost all cases when one panel is fully affected, the other panel also gets affected upon tapping.

3.5 CONCLUSIONS

TPD incidence increased as the year of tapping progressed at all the locations both in small holdings and estates. The results clearly showed that maximum TPD incidence is seen in later panels of tapping. The percentage of trees in the category of very high TPD intensity showed a clear trend of increase from the first year to the last year of tapping at all the locations. The number of TPD trees in the lower intensity categories (low, medium and high) did not show such a remarkable trend of increase from BO 1 to BI 1 panels. The percentage of trees showing no symptoms of TPD increased in the first year of a new panel but again showed decreasing trend a year after the panel change showing the irrecoverable nature of TPD. The pattern of increase in the incidence within a period of 16 months in different panels showed that the scale of increase in TPD incidence in the category of more than 50% TPD is more in older trees than in trees at the initial stages of exploitation. Reversion of TPD symptoms in the category of less than 50% was observed only at a younger age as evidenced by higher number of symptomless trees at the final stage in BO 1 panel. This finding also suggests that it is very important to give the best management care at a younger age.

Higher number of TPD trees were observed in locations where field management practices were poor. Progressive occurrence of TPD in clusters suggests the possibility of tree to tree spread of a biotic agent. The observation that some of the healthy trees tapped immediately after tapping a TPD tree turned to TPD after a few months shows that, though at a limited level TPD is spreading from one tree to the tree tapped immediately next. It was observed that only 23.8% of the small holders give rest when TPD was observed. It was also observed that when the TPD affected trees were tapped in the upward system of tapping, more than 50 per cent of the trees showed dryness after four months of tapping. Hence, this tapping system is not a long term solution for TPD management.

Chapter 4

MOLECULAR STUDIES ON THE BIOTIC ETIOLOGY OF TPD

4.1 INTRODUCTION

Tapping panel dryness (TPD) syndrome is of serious concern in all rubber growing areas since, there are no proven methods to prevent or cure the disorder as the cause is not yet identified. There have been efforts to investigate the cause of the syndrome, but so far, no clear picture has emerged.

4.1.1 Anatomic studies

Since over-exploitation (too frequent tapping) was considered as a causal factor for TPD it was assumed that the exhaustion of food reserves leads to TPD. But several anatomical studies have shown that starch reserves are not run down in dry bark (Sanderson and Sutcliffe, 1921; Chua, 1967), except in the initial stage of TPD (Sanderson and Sutcliffe, 1921) and soluble carbohydrate reserves are not used by plants (Chua, 1967). Other investigations reported that starch grains are still abundant at all depths in *Hevea* wood, even in trees where tapping panel is fully devoid of latex. *In situ* coagulation of latex in the laticifers of the dry bark was noticed in TPD trees (Rands, 1921; Sanderson and Sutcliffe, 1921). In most of the laticifers in dry bark, the latex appears homogeneous and shiny (sudan red or oil red), rather than granular and mat, similar to latex from healthy bark.

According to de Fay (1982) the typical dryness started with internal coagulation of the latex and invasion of laticifers by thylosoides (bud bursts) from the associated parenchyma cells. Other abnormalities also appear of which the most characteristic are accumulation of lignified gums on the sieve tubes and laticifer cells. Finally the bark gets disorganised by the development of abnormal tissues.

The existence of endogenous NAD(P)H oxidase in lutoids which generates toxic form of oxygen responsible for the peroxidative degradation of latex cell organelles of diseased trees was reported by Chrestin (1984 & 1985). The combination of increased peroxidative activities and considerably diminished quantities of scavengers in latex of affected trees results in destabilization and lysis of lutoids leading to coagulation and degeneration of the latex cells of the stressed trees.

In dry bark, the number of tannin cells also increases in particular, there is increase of tannin in parenchyma cells associated with parts of the mantles where latex has coagulated *in situ* (Gomez *et al*, 1990; de Fay 1982; de Fay and Hebant, 1980; Paranjothy *et al.*, 1975). This accounts to a great extent for the appearance of brown patches in the bark. Globular inclusions can be observed in certain parenchyma cells of the phloem rays in the dry bark (Sanderson and Sutcliffe, 1921; de Fay, 1981).

Wu and Hao (1994) using transmission electron microscopy observed the diseased laticifers and the cells near to such laticifers in brown bast trees of *Hevea* in which the rubber particles did not coagulate. The various organelles in the laticifers showed abnormal changes. One of the important changes was membrane turnover disorder. On one hand, the membranes, especially the boundary membranes of some lutoids, Frey-Wyssling complexes, nuclei and other organelles were disorganized and on the other hand, the membrane materials appearing as myelin-like structures abnormally accumulated in diseased laticifers. One of the significant characteristics of the nucleus in diseased laticifer was the frequent presence of bundles of straight microfibrils about 5nm in diameter. Besides, reduction in nuclear contents and partial disorganization of nuclear membrane were observed.

Premakumari *et al.*, (1997) using correlation studies on ten *Hevea brasiliensis* clones showed that the factors detrimental to girth increment on tapping favours the occurrence of tapping panel dryness. Laticifer area index was an exception that showed positive correlation with both. The associations of characters indicated that a very high number of latex vessels rows in high yielding clones leads to high incidence of TPD. Hence selection based on high girth with good quantity of intraxylary and balanced number of latex vessel rows was suggested for reducing TPD incidence.

Certain forms of bark dryness are transitory and do not display the characteristic symptoms of the formation of tylosoids or activation of the phenolic metabolism (de Fay and Jacob, 1989).

4.1.2 *Climatic factors*

There are few reports of influence of climate and growth period on the incidence of brown bast (Vollema, 1949; Compagnon *et al.*, 1953; Bealing and Chua, 1972). However, this disorder is seen in almost all rubber growing countries, irrespective of the nature of soil, climate, geography, seasons etc. Even in India, irrespective of variation in soil texture and structure, climate, rain fall pattern, duration of dry period, altitude, latitude, water availability and other such factors, TPD prevails in all rubber clones. The incidence of TPD in North Eastern region of India which is a non-traditional rubber growing area experiencing chilling low temperature (less than 10^0C) during winter (Das *et al.*, 1998a), is comparable to that in the traditional area. This disorder is present in the plantations in Karnataka, Tamil Nadu, Orissa and Maharashtra in the same manner

where the climatic conditions are entirely different (10-40^{0}C) (Das *et al.*, 2006; Abraham *et al.*, 2006; Chandrasekhar *et al.*, 2006; Dey, 2006).

4.1.3 *Clonal variation/genetic characters*

Studies were conducted by Venkatachalam *et al.*, (2006) to identify genes, which are differentially expressed in rubber tree during TPD development. The expression of two selected gene transcripts was examined by Northern blot analysis using plant tissues of both healthy and TPD trees which confirmed that the expression of these two genes was down regulated in TPD trees. Sathik *et al.*, (2006) reported that genes involved in apoptosis and senescence are triggered and the genes involved in metabolic activities are down regulated under TPD conditions.

A large-scale evaluation trial of 21 clones of *Hevea brasiliensis* with respect to yield, girth and incidence of tapping panel dryness (TPD) over nine years of tapping (Mydin *et al.*, 1999) showed that TPD is a distinct clonal characteristic with high heritability and low genetic advance. A significant positive correlation of TPD with girth and girth increment over the period of tapping was observed. It was postulated that TPD is a highly heritable trait inherited through non-additive gene action, which results in non-fixable variance. However, the distribution of TPD affected trees in the fields was non-random in most of the clones studied. Hence, the high heritability value observed could also be due to influence of environment. Non-random distribution of brown bast affected trees was reported earlier by De Soya *et al.*, (1983).

Variations in occurrence of TPD within a clone are noticed under field conditions. In a plantation, some trees show early occurrence of TPD while others remain healthy and high yielding for 20 to 25 years of regular tapping in spite of identical growth conditions and management.

4.1.4 *Edaphic characters*

There have been several studies on the agronomic and nutritional aspects of TPD, but no clear trends were reported (Pushpadas *et al.*, 1974; Sivakumaran *et al.*, 1997; Zainab and Sivakumaran, 1996). Inadequate organic resources (Schweizer, 1949; Dijkman, 1951; Chua, 1966), Cu & K deficiency (Compagnon *et al.*, 1953), changes in mineral ratios, especially of K_2O/CaO and Mg/P (Beaufils, 1954) were reported to favour TPD. An increase in K content and K/Ca, K/P ratios in latex was observed in TPD (Pushpadas *et al.*, 1975). The effects of manuring practices, in particular imbalance in Mg/P ratios on dryness incidence have also been reported (Beaufils, 1954; Pushpadas

et al., 1975). Yeang *et al.*, (1994) reported the relationship of latex Cu content to the onset of TPD. The presence of large quantities of copper in the latex was detected prior to the onset of TPD syndrome. TPD susceptible clone had a higher concentration of copper in the latex during the early stages (Yeang *et al.*, 1994; Yusof *et al.*, 1995).

The observations carried out in fertilizer trials revealed either beneficial effect of potassium fertilizer on reduction of the disease (Compagnon *et al.*, 1953), or an opposite effect (Pushpadas *et al.*, 1975). In the latter case, analysis of soil, leaves and latex have shown that unbalanced mineral nutrition favours the occurrence of TPD. Application of higher levels of potassium reduced incidence of TPD in agroclimatically dry and poor lateritic soils (Sivakumaran *et al.,* 1994). When soil properties and nutrient concentration of the feeder roots, leaf, bark and latex of normal and TPD affected rubber trees were compared, the concentration of P, K, and Mg were found to be high in the leaves and bark of completely dry trees (Joseph, 2006). Manganese concentration was almost double in the latex from partially dry trees when compared with the normal trees. As the comparisons in most of the studies were between dry untapped trees and trees being tapped, the values observed may not be conclusive.

4.1.5 Biomass

There is a general observation that the girth increment in TPD affected trees is more than that of healthy trees (Mydin *et al.,* 1999). This could be because such trees are left untapped and they grow as any normal tree would have grown, if not tapped (Sethuraj, 1992). There are also reports that there is no significant positive correlation between girth increment and TPD incidence (Premakumari *et al.*, 1991).

4.1.6 Stock-scion incompatibility

Reports of TPD even on trees raised from seedlings shows that TPD may not be the result of stock-scion incompatibility. The theory of genetic distance contributing to stock-scion incompatibility and predisposing trees to TPD (Sobhana *et al.*, 1999) fails to explain the occurrence of TPD in test tapped seedlings, the mature seedling population and meristem cultured trees. Incidence of TPD was observed to be similar in meristem cultured trees as in budded trees (Thulaseedharan *et al.*, 2006). TPD was observed in seven trees out of 60 meristem cultured trees compared to nine trees out of 76 bud grafted control trees of same clone planted in an experimental location which received identical care.

4.1.7 *Impaired cyanide metabolism*

Chrestin *et al.*, (2004) hypothesised that possible cell decompartmentalization near stock scion junction results in local release and accumulation of toxic concentrations of highly diffusive cyanide that may cause necrosis and panel dryness.

4.1.8 *Tapping intensity*

Brown bast is generally recognized as a physiological disorder caused by excessive tapping or over-exploitation (Rands, 1921; Sharples and Lambourne, 1924; Paranjothy, 1979; Sivakumaran and Pakinathan, 1983; Yusof *et al.*, 1995). The intensive exploitation is reported to result in excess outflow of latex and consequent nutritional stress (Sharples and Lambourne, 1924; Taylor, 1926; Sharples, 1936; Schweizer, 1949; Chua, 1967). The proportion of dry trees in a plantation increases with tapping intensity and particularly with higher tapping frequency (Chua, 1967; Bealing and Chua, 1972; Paranjothy *et al.*, 1977). Sethuraj *et al.*, (1976) found that in intensively tapped trees initial flow rate and turgour pressure are reduced before the onset of dryness. TPD was considered as a physiological disorder affecting the laticiferous system, extending to the other bark tissues of the affected side (panel) and is related to intensity of tapping (Sethuraj *et al.*, 1977). Paranjothy (1980), however, maintained that intensive tapping tended to give rise to ionic imbalance and lower osmotic potential. That, in turn, damaged the lutoids and hence the occurrence of dryness. Tree dryness was also attributed to physiological fatigue (Van de Sype, 1984). The syndrome is reported to be an outcome of stress imposed on rubber tree due to excessive extraction of latex (Paardekooper, 1989). Wenxian *et al.*, (1994) considered tree dryness as a strong wound-response for self-defence. Occurrence of tree dryness in *Hevea* was observed to be closely related to stimulation and exploitation intensities and even tapping patterns (Wenxian *et al.*, 1994).

Siwei and Shaoqiong (1991) proposed that bark dryness due to over-exploitation and over-drainage of latex is a special and localized senescent malady in nature. Chrestin (1985) showed an abnormal production of toxic oxygen molecules in *Hevea* trees when over stimulated with ethephon and also reported that over-exploitation of rubber trees with ethephon often leads to TPD.

Incidence of TPD was very high in trees in which injurious tapping was carried out. Among the systems of tapping ½ S d/2 led to maximum incidence of TPD (27.6%) while ½ S d/3 resulted in only 18.3%. During the first 5 years of tapping, 79% TPD

trees showed only drying of panel and no other symptoms were observed (Rejithkumar, 2003). When five-years average of TPD incidence with respect to the period of tapping was estimated in the clone RRII 105 under ½ S d/2 tapping, an increasing trend was noticed. In the BO 1 panel 12.98 per cent of trees were affected while B1 2 (after 15 years) it was 40.19 per cent (Rejithkumar, 2003).

Sulochanamma *et al.,* (1992) suggested that high intensity of tapping was associated with TPD. Increasing the intensity of tapping appears to increase the metabolic capacity of the latex tissue as well as incidence of TPD (Bealing and Chua, 1972). While supply of carbohydrate for the intrinsic metabolic capacity of the laticiferous system might not be the limiting factor for TPD (Bealing and Chua, 1972), ATP was in short supply (Krishnakumar *et al.*, 2001a).

Since, over-exploitation was suspected to lead to TPD various management practices were recommended for controlling TPD incidence. The ½ S d/3 tapping system with weekly one-day rest was recommended for RRII 105, to reduce the incidence of TPD (Sulochanamma *et al.*, 1992). The ½ S d/3 tapping system with stimulation also reduced the incidence of TPD (Nugawela *et al.*, 2000; RRII, 2003). Upward tapping along with puncture tapping was reported to give more yield, consume less bark and result in a less recurrent rate of TPD than conventional upward tapping or vertical puncture tapping (Xiaodi *et al.*, 1997).

Occasionally TPD symptoms appear in untapped trees in some plantations. It was reported that even untapped trees were also affected by brown bast from Asia several years ago (Petch, 1921). About 1-3% trees become dry within 10 tappings and there are trees which show TPD at the first opening or immediately after (de Fay, 1981). Thus young *Hevea* can become dry during the first month of tapping, with browning at a depth and even at the surface near the base of trunk (de Fay, 1981). The bark displays the characteristic anomalies of typical dryness. Leaving trees untapped (resting) could not always cure TPD affected trees (Paranjothy and Yeang, 1977; Le Shizong *et al.*, 1984). But there is a tendency for increase in the intensity and incidence of TPD with increase in tapping intensity and exploitation level. Stress is known to aggravate diseases and therefore tapping stress or excessive removal of latex may make the tree more vulnerable.

4.1.9 *Physiological factors*

Comparison of biochemical and physiological parameters of TPD affected and unaffected trees have been attempted in several studies. Krishnakumar *et al.*, (1996,

1997, 1998, 1999, 2001, 2001a, 2002, 2003, 2003a, 2006) observed various distinct physiological changes in TPD trees in comparison with healthy trees. It was observed that TPD adversely affects the ability to synthesize rubber. TPD syndrome increases bark respiration. ATP concentration in the cytosol decreased, although respiration rate increases in TPD affected bark. Wound-induced ethylene produced in the bark tissue of TPD affected trees was significantly higher than that of healthy trees. Overproduction of endogenous ethylene was shown to inhibit rubber biosynthesis. The normal plants showed significantly higher Trans-zeatin riboside (t-ZR) content than the TPD affected plants. The higher t-ZR content in normal trees was negatively correlated with their peroxidase activity and phenol content. Alternative respiration is reported to increase in the TPD affected tissue. The various reactive oxygen species (ROS) can cause peroxidative damages in the tissue (Krishanakumar *et al.*, 2003a). The TPD affected bark tissue contained comparatively higher levels of sugars, phenols and soluble proteins than the healthy tissue (Krishnakumar *et al.*, 1999) ruling out starvation. The higher peroxidase activity and the accumulation of phenols in the TPD affected bark tissue indicate possible oxidative stress and the oxidative damage of the laticiferous vessels may be responsible for the complete shutdown of rubber biosynthesis (Krishnakumar *et al.*, 1998). The simultaneous occurrence of high concentrations of sucrose and P in the latex indicates a possible inhibition in the metabolic conversion of sucrose to rubber due to poor energy status of laticifer tissues of the tree that are affected by TPD (Thomas *et al.*, 1996).

Wound induced endogenous ethylene production is more in bark tissues of TPD affected trees along with an increase in the hydrogen peroxide (H_2O_2) and cyanide contents compared to the normal healthy tissue. Similarly a high peroxidative activity and malondialdehyde (MDA) was also noticed in the bark tissue of TPD affected trees indicating the oxidative stress in the tissue (Krishnakumar *et al.*, 2006). TPD affected bark tissue showed both increased carbohydrate content and respiration but no rubber biosynthesis (Krishnakumar *et al.*, 2001a). A reduction in the ATP levels of the C-serum of TPD affected trees was also observed. This reduced ATP content and the increased tissue respiration was attributed to the enhanced alternative pathway (cyanide resistant respiration) operating in the TPD affected bark tissue (Krishnakumar *et al.*, 2001a). Intermediates of isoprene pathway such as HMG-CoA and mevalonate were found in large quantities in TPD affected bark (Krishnakumar *et al.*, 1999). It was

therefore suggested that there is poor conversion of mevalonate to IPP in TPD trees possibly due to the inadequate supply of ATP (Krishnakumar *et al.*, 2001b).

There are indications that the metabolic breakdown in TPD affected tissues may be related to damages inflicted to cellular components by free radicals and active oxygen species. The equilibrium between the oxidative stress and the ROS scavenging activity of the tissue determines the level of oxidative damage. Ethepon application which causes "chemical damage" increases ROS generating activity of NADPH oxidases and simultaneous decrease in the level of scavengers such as thiols, super oxide dismutase and ascorbate (Das *et al.,* 1998b; Chrestin, 1989). This causes oxidative stress which may to lead to TPD. In another study, it was observed that with progression of time, the percentage of TPD was more in the frequently tapped trees than in stimulated ones (Das *et al.*, 2002). The study also revealed that the amount of free radicals in frequently tapped trees (where the wounding was excessive) was less than that of in the stimulated trees, though the damage to the scavenging system (SOD) was higher in the frequently tapped trees (Das *et al.*, 2002).

4.1.10 *High yield and TPD*

Observations from China (Shaoqiong, 1989), Malaysia (Sivakumaran and Haridas, 1989) and Sri Lanka (Samaranayake and Yapa 1989) revealed clonal differences in susceptibility and indicated a correlation between intensity of tapping and incidence of bast symptoms (Chua, 1967; Bealing and Chua, 1972). TPD incidence was positively correlated with rubber yield and over-exploitation (Sethuraj 1988; Premakumari *et al.*, 1991). The observation that high rubber yield is positively correlated with TPD (Premakumari *et al.*, 1991) and that high yielding clones are more vulnerable to TPD (Sivakumaran *et al.*, 1988) suggest that TPD is not merely a result of wound reaction alone.

TPD was observed in the second year of tapping in 30 trees out of 320 trees belonging to 80 accessions in the germplasm collection of Rubber Research Institute of India which was planted in 1995. On an average 5-10% TPD could be observed in germplasm accessions (C.P. Reghu, Rubber Research Institute of India, personal communication). It is interesting to note that these wild germplasm materials are poor yielders. Early reports of TPD (from 1887 to 1924) on trees raised from seedlings show that TPD was present in low yielding seedling trees as well.

4.1.11 *Bark grafting*

The transmission of TPD through bark grafts was attempted and the healthy bark grafted on the scion portion of TPD affected tree produced latex (Premakumari, *et al.*, 1996). The observation after three months on the grafted bark revealed that only two out of six grafted bark yielded latex in the entire cut. The sample size and duration of the experiment was not large enough to draw precise conclusions on the transmissibility of the disease through bark grafting.

4.1.12 Biotic etiology

As the TPD syndrome has, from the early days, been considered to be a problem of "physiologic fatigue" not much attention has been given to probe into possible biotic causes. Investigations were designed suspecting a physiological origin for the disease. Hence, there exist scanty reports in literature about the involvement of biotic agents with this disorder.

Peries and Brohier (1965) observed that bark-cracking symptom of rubber common in the eastern rubber growing countries could be associated with a virus. Since rubber trees are mainly propagated by bud grafting, use of budwood from plantations which had a history of bark cracking, is the most likely method of disseminating the disease. For this, they could observe evidence in the field records of some plantations in Sri Lanka. They also suspected a possible association of viruses with bark scaling symptom.

Detection of ricketssia like organism (RLO) in TPD affected trees was reported from China (Zheng *et al.*, 1982, 1988). Such trees were reported to be cured within a period of six months by the application of tetracycline and penicillin. RLO- like bodies in the phloem of trees with brown bast symptom was observed using electron microscope. Eschbach *et al.*, (1989) suspected that certain types of cortical necrosis, which lead to the stopping of latex flow, may have pathogenic causes.

4.1.13 *Stress induced by pathogens*

Anatomical abnormalities in TPD affected plants closely resembled those produced in the other virus infected woody plants (Peries and Satchuthananthavale, 1964). Rutgers (1917) proposed arguments in favour of a parasitic cause of TPD. He first confused the symptoms of brown bast with a type of canker caused by *Phytophthora faberi.*

The arguments in favour of a parasitic cause of TPD are local enrichment in phenolic compounds and other histological aberrations, occurrence of panel dried trees in continuous rows in plantations and the presence of dry rubber trees from the start of exploitation (de Fay and Jacob, 1989). The abundance of polyphenols (tannins and lignins), 'gum' and even hypoplastic phenomena are similar to the reactions triggered by pathogen infection of many plants (Schneider, 1973; Bell, 1981). TPD occurrence does not seem to be at random since row of four or five diseased trees are commonly observed in plantations (Taysum, 1960; de Fay, 1981). Murong *et al.*, (1994) also reported that TPD affected trees are not distributed randomly in the stand and that the disease is caused by pathogens like RLOs. Disinfection of the tapping cut and tapping knife did not appear to reduce the frequency of the disease (Rands, 1921).

The amplification of 16S rDNA of MLO/BLO associated with TPD in *Hevea* has also been reported. The observation that mycoplasma-like organisms (MLOs) and bacteria like organisms (BLOs) are present in the phloem of TPD affected trees implicated infectious agents associated with the altered physiology. This was supported indirectly by grafting test. In order to characterize the prokaryotic organisms and establish a detection method, a novel method was developed to amplify the prokaryotic 16S rDNA. Considering the conserved regions of the 16S rDNA sequences of the known MLOs, a pair of degenerate primers were designed and synthesized for polymerase chain reaction (PCR). The results showed that all the TPD affected samples gave a band approximately 865 bp in length, while the healthy ones gave none. This result further demonstrated that MLOs or BLOs are closely related with TPD (Zheng *et al.*, 1997).

R-PAGE analysis of TPD affected plants has indicated the association of low molecular weight RNA (suspected to be a viroid) with most cases of TPD (Ramachandran *et al.*, 2000).

4.1.14 *Occurrence of TPD in clusters*

From a limited number of sample, it was shown that occurrence of TPD in the field is not at random in many clones (Mydin *et al.*, 1999). In some cases there was a cluster of 6 to 8 TPD trees in the field. In another study scattered occurrence of affected trees in a holding was reported (Jacob and Krishnakumar, 2006). Viral contamination by the tapping tool and the existence of micro conditions in the soil were suggested to account for the rows of dry trees (de Fay, 1989).

4.1.15 *Forward spread of TPD in the direction of tapping*

Since four or five consecutive trees in a row are observed to be TPD affected in plantations, tree to tree spread was suspected (Taysum, 1960; de Soya *et al.*, 1983). It was interesting to note that the number of single occurrence of TPD trees did not show a remarkable increase when the linear trend was analysed from the first panel to the last although the total number of TPD trees increased with progressive tapping. But, the number of TPD trees in clusters of two or more showed a remarkable increase compared to the single TPD trees, from the first year of tapping to the last showing that there is a chance of spread of TPD from one tree to the neighbouring tree through tapping (Elsewhere in this thesis).

In a study of TPD affected trees with bark scaling (RRIM 605) out of the ten healthy immediate next trees to the bark scaled, seven became TPD affected by the fourth year of tapping (RRII, 2003). No evidence is now available for seed or pollen transmission.

4.1.16 *Increase in TPD intensity with age*

The number of trees in the category of very high TPD intensity (>75%) showed an increasing trend from BO 1 to BI 2 panel as well as with the year of tapping (Elsewhere in this thesis). The consistent increase in the incidence of TPD corresponding to the progress in the age of the tree also indicates that the organism that existed earlier in low titre becomes active on attaining favourable conditions, gets multiplied and expresses the symptoms.

4.1.17 *Change over tapping*

The percentage of trees showing no symptoms of TPD was more in the first year (opening) of a new panel (Elsewhere in this thesis). TPD trees appear healthy without any panel dryness for a short period when the panel is changed, but dryness symptoms appear again as tapping advances. In almost all cases when one panel is fully affected, the other panel also gets affected upon tapping (Krishnakumar *et al.*, 2002).

4.1.18 *Search for common pathogens*

The early studies on the biotic etiology of TPD showed that the pathogens then known were not associated with TPD. Molecular and other advanced tools for detecting the submicroscopic pathogens available now were absent at that time.

Many hypotheses have been put forward to show that the syndrome may be abiotic in nature particularly due to physiological changes or nutritional deficiency.

However, very few attempts have been made to investigate the involvement of biotic agents with this disorder. Possible association of viruses with bark scaling disease of rubber was reported from Sri Lanka (Peries and Brohier, 1965). Detection of Rickettsia-like organisms (RLO) in TPD affected trees was reported from China (Zeng *et al.*, 1997).

In order to investigate the possible association of the common pathogens, attempts were made to isolate the causative organism from the affected tissues (Mathew *et al.,* 2006b). But no pathogenic organisms namely fungi, bacteria, protozoa, phytoplasma, bacteria like organisms (BLO) or Rickettssia like organisms (RLO) were isolated from the affected tissues (Mathew *et al.,* 2006b). Remission of TPD symptoms were also not observed in spite of pressure injection with tetracycline, penicillin or carbendazim continuously for one year in partially affected trees, which again indirectly showed that phytoplasma, bacteria, protozoa and fungi are not associated with TPD (Mathew *et al.,* 2006b). Untapped trees, which received these chemicals prophylactically for one year, also expressed the symptoms of TPD when tapping was initiated indicating lack of association of these organisms with the syndrome (Mathew *et al.,* 2006b).

Ramachandran *et al.*, (2000) showed an association of a low molecular weight RNA (LMW RNA) with TPD affected trees through R-PAGE. The present study is an attempt to investigate on the association of LMW RNA in different tissues of TPD trees and to characterize the LMW RNA utilizing the molecular biological tools now available.

4.2 MATERIALS AND METHODS

4.2.1 Selection of plants

Trees at different years of tapping and unopened (untapped) plants at different years of planting were selected from various locations. In the case of tapped trees, both healthy and TPD affected plants were selected after confirming the TPD status by repeated observation for ten tappings. Plants were selected from two experimental farms of Rubber Research Institute of India (RRII), namely RRII Farm at Kottayam and Central Experiment Station, Chethackal, Ranni, Pathanamthitta district, Kerala. Plants were also selected from various large estates in Kerala state namely Malankara Estate at Thodupuzha in Idukki district and Vaniyampara Estate in Trissur district. Samples were collected from rubber growing areas outside Kerala state from New Ambadi Estate in Kanyakumari district of Tamil Nadu.

In order to understand the phenomenon of TPD, rubber plants were studied at different stages of development of the tree including seedling stage, before and after the commencement of tapping. A scale was used for identifying trees under different years of tapping. The trees untapped were designated with "-" numbers; i.e. "-1" means tree to be tapped one year later (Fig. 4.1). "0" is the year in which tapping started. Similarly, the plants were designated with "+" numbers where tapping has started earlier i.e. "+1" means the second year of tapping, "+2" the third, "+3" the fourth, "+4" the fifth and so on. This facilitated recording of symptoms as also other observations and to compare observations from different locations.

Trees tapped in the virgin as well as renewed panel were also observed to characterize the TPD affected plants. Plants with different symptoms like panel dryness alone, cracking, bulging, necrosis etc. were also studied.

-1 0 +1 +2 +4

Fig. 4.1 Rubber plants at different stages of tapping

4.2.2 Types of tissues

Samples of tender leaves as well as bark from root and shoot from healthy and TPD affected trees were collected in ice and immediately stored at -80^{0}C. Before use, the leaf samples were washed, blotted dry and crushed to a fine powder using liquid nitrogen in previously autoclaved pestle and mortar. Soft bark was cut and used from the collected bark samples from root as well as shoot. Leaf samples were comparatively easy to handle and better tissue for analysis as it showed more consistent results compared to bark samples.

4.2.3 Analysis of nucleic acid

4.2.3.1 *Extraction of total nucleic acid (TNA)*

Bark samples (trunk and root) and tender leaf samples collected from TPD affected as well as healthy plants were used for the isolation of RNA. In the case of bark samples only soft bark was used for extraction. The processing of the samples was done at 4^{0}C except in case mentioned other ways. Minimum 5g of leaf and soft bark was powdered in liquid Nitrogen and homogenised with extraction buffer (pH 8.5) (Tris 0.1M, EDTA 0.01M, NaCl 0.1M, 1% SDS containing 5mM DTT and DIECA) in the w/v ratio 1:3. To these three volumes of Chloroform isoamyl alcohol mixture (24:1) was added and the mixture was homogenized by gentle shaking. Tris saturated phenol (containing 0.1 g of 8- hydroxyquinoline per 100 ml) was added at the ratio 1:3 and mixed by vortexing and the mixture was kept at room temperature for 15 min. and centrifuged at 8500 rpm (Eppendorf centrifuge – 5804R) for 20 min at 4^{0}C. The

aqueous phase was collected and RNA precipitated by adding half volume of ammonium acetate (7.5 M, pH 7.4) and 2.5 volume of absolute alcohol (ice cold) and stored at –20^{0}C, overnight. Pellets were recovered by centrifugation at 10000 rpm for 30 min. at 4^{0}C and the steps repeated till a clear precipitate was obtained. Finally, pellets were washed in 70% ethanol and dissolved in 300µl autoclaved double distilled water in Eppendorf tube and used immediately for further analysis by R-PAGE, RT-PCR etc. or stored in deep freezer at -80^{0}C if required.

4.2.3.2 *Return Poly Acrylamide Gel Electrophoresis (R-PAGE)*

There are various methods available in literature to distinguish the Low Molecular Weight RNA (LMW RNA) from the host RNAs. A technique referred to as R-PAGE (Return Poly Acrylamide Gel Electrophoresis) has been used by many workers (Singh and Boucher, 1987) for detection of viroid RNA by using denaturing conditions of heat and low salt in the acrylamide gels that are used to resolve the total nucleic acid (TNA). In such gels LMW RNA appears as the lowest band since during the second run of electrophoresis all other forms of RNA run out of the gel matrix due to heat. The LMW RNA is heat resistant and due to its circularity, trails behind.

R-PAGE was carried out in a vertical electrophoresis unit (16x14x0.15cm) with the gel containing 7.5% acrylamide and 0.125% bis-acrylamide, 0.08% TEMED in high salt buffer and 0.07% ammonium persulphate. To 10 µl of test sample 4µl of dye containing 0.25% each of xylene cyanol and bromophenol blue (prepared in 60% sucrose - molecular biology grade) was added and applied to the sample loading slots. The first electrophoretic run was carried out under native conditions using high salt buffer (89mM Tris-HCl, 89mM boric acid, 2.5mM EDTA, pH 8.3) at room temperature and 46 mA constant current. The run was terminated after 120 – 135 minutes when xylene cyanol migrated to near bottom of the gel. After the first electrophoresis the buffer was exchanged for a low salt boiling buffer (1:8 dilution of high salt buffer). The chamber was left for 5min. without disturbance. The gel was re-run at 70^{0}C, 46 mA current with reverse polarity for 90 minutes. The bands in the gel were resolved by silver staining following Mishra *et al.*, 1991.

4.2.3.3 *Silver staining*

The gel was immersed in Fixative I and left at room temperature for one hour and then transferred to Fixative II for 30 min and kept in reciprocal shaker at 30-40 rpm. The Fixative II was drained off and the gel was stained with 0.25% silver nitrate for 30 min. The excess stain was removed by washing twice with distilled water. The gel was

placed in developer solution and fixed with Solution II and finally preserved in 5% glacial acetic acid. The gels were then dried on the gel drier.

Fixative I (Fixing Buffer)

Ethanol 20 ml

Acetic acid 1 ml

Distilled water 179 ml

Fixative II

Ethanol 5 ml

Acetic acid 1 ml

Distilled water 194 ml

Silver Stain

Silver nitrate 0.25 g

Distilled water 200 ml

Solution I (Developer Solution)

Sodium hydroxide 1.5 g

Formaldehyde 400 µl

Sodium borohydride 0.0088g

Made up to 200 ml with distilled water.

Solution II (Stop Solution)

Sodium carbonate 1.5 g

Distilled water 200 ml

4.2.3.4 *Elution of LMW RNA from gel*

In order to characterize the LMW RNA by RT-PCR, the band was eluted from the gel. For this five smaller wells were merged by Parafilm tape and used as a single well and 100µl of total RNA mixed with 30µl of loading dye and the samples were electrophoresed in R-PAGE. The gel was stained in ethidium bromide and the specific LMW RNA bands in the viroid region were cut from the gel and placed in eppendorf tubes at 4^0C. 1 ml of gel elution buffer was added to these tubes. They were then kept overnight in a shaker incubator at 37^0C at 100rpm. The tubes were placed on ice and with the help of sterile glass rod the gel pieces were crushed and mixed with the elution buffer. The contents were then passed through plastic columns packed with sterile glass wool which allows the elution of LMW RNA from the gel pieces. Buffer containing the viroid RNA was collected in Eppendorf tubes and precipitated. The LMW RNA was then dissolved in 20-30µl of sterile double distilled water.

4.2.3.5 Amplification of LMW RNA

4.2.3.5.1 ***Design of viroid specific primers***

Primers specific for viroids were selected from the commonly used viroid primers. New primers were also designed based on the already available viroid genome sequences using the biological sequence alignment editor and sequence analysis programme, BioEdit® and primer design application - PrimerSelect® in Lasergene™ software of DNASTAR® and also manually based on the conserved region in the viroid sequences.

4.2.3.5.2 ***cDNA synthesis***

The isolated RNA from untapped and tapped apparently healthy as well as TPD affected trees were used for cDNA synthesis. Quality and quantity of total RNA obtained was tested using Thermo Scientific NanoDrop™ 1000 Spectrophotometer. The template RNA was mixed with the synthesized specific primers in a fixed volume of 5µl in nuclease free water. The reaction mixture was heated at 70^0C for 5 min to remove the secondary structures, and then quick chilled at 4^0C for 5min.

4.2.3.5.3 ***RT-PCR***

RT-PCR was carried out using M-MuLV Reverse Transcriptase (Fermentas). The components of the reaction mixture are as follows.

RT-PCR reaction mixture

Components	**Volume**
Template RNA	4.0µl
Complementary primer	1.0µl
10X RT buffer	2.0µl
dNTP mix (10mM)	2.0µl
Ribonuclease inhibitor	1.0µl
Nuclease free water	9.0µl
M-MuLV reverse transcriptase	1.0µl
Total reaction volume	20.0µl

The reaction mix was prepared for multiple reactions and dispersed into 15µl aliquots and kept chilled in ice. Added 5µl of the denatured chilled RNA and complementary primer combination to each reverse transcription mix on ice. The reaction mixture was subjected to the following PCR conditions. The tube was chilled on ice and then started PCR at 25^0C for 5 min. First strand cDNA synthesis was

performed at 42^{0}C for 60 minutes and finally heat inactivated the reverse transcriptase at 70^{0}C for 10 min.

4.2.3.5.4 *Specific PCR*

The cDNA was further amplified with viroid specific primers. PCR amplification was carried out using 12µl of cDNA to which the following reactives were added to make a total volume of 50µl.

Template (cDNA)	12.0µl
10X PCR buffer	5.0µl
25mM $MgCl_2$	3.0µl
10mM dNTPs	1.0µl
Forward primer	1.0µl
Reverse Primer	1.0µl
Taq polymerase (5U/µl)	1.0µl
Sterile distilled water	26.0µl
Total volume	50.0µl

PCR amplification was performed in a thermal cycler (Biorad - PTC 100) with the following cycles and temperature profile. Initial denaturation of DNA at 94^{0}C for 2 minutes, 35 cycles which had 3 segments, denaturation at 94^{0}C for 30 sec., annealing at 60^{0}C for 30 sec., chain extension at 72^{0}C for 30 sec. The final extension was performed at 72^{0}C for 10 min and the amplified product was stored at 4^{0}C.

4.2.3.5.5 *Agarose gel electrophoresis*

The amplified PCR product was resolved on 1.5% agarose gel as follows. 1.5g of agarose was added to 100ml 1XTAE (Tris-acetate EDTA) buffer, boiled, cooled upto 50^{0}C and 2µl of Ethidium Bromide (0.5g/ml) added before pouring on casting unit. The products for analysis were mixed with 6X loading dye and electrophoresis was carried out at 50-60v for 60 to 90 minutes. An aliquot (500ng) of 1 kb DNA ladder was similarly mixed with the dye and electrophoresed to serve as molecular weight marker. Result was evaluated on a Gel Doc (BioRad).

4.2.3.5.6 *Purification of amplified product*

The gel band was eluted from the gel using QIAquick PCR purification kit from QIAGEN. Protocol was followed as per manufactures instructions.

4.2.3.5.7 *Molecular cloning*

The amplified product was excised from the gel, purified and cloned in pGEM®-T Easy Vector using Promega cloning kit. The protocol used was as follows

- Added 1.0µl of purified DNA, 10.0µl of 2x ligation buffer, 5ng pGEM-T vector, 1.0µl T4 DNA ligase and added sterile distilled water to makeup the total volume to 20µl in an eppendorf tube.
- Mixed well by short spin
- Stored the ligation mixture overnight at 4^0C

4.2.3.5.8 *Preparation of competent cells*

- Picked single $DH_5\alpha$ colony from a LA plate
- Inoculated into 50ml of LB medium and incubated it at 37^0C for overnight in a rotary shaker at 200rpm
- Taken 500µl of overnight grown $DH_5\alpha$ culture and re-inoculated it in 50ml of LB medium and incubated it at 37^0C for 1.5 to 2hrs.
- Pelletized the bacterial cells by centrifugation at 6000rpm for 10min at 4^0C
- Re-suspended the pellet in 10ml of cold (4^0C) 0.1M $MgCl_2$
- Centrifuged for 10min at 4^0C at 6000rpm
- Suspended the pellet in 10ml pre-cooled (4^0C) 0.1 M $CaCl_2$
- Incubated on ice for 1hr
- Centrifuged for 10min at 6000rpm
- Re-suspended the pellet in 1ml of cold (4^0C) 0.1M $CaCl_2$
- Incubated on ice for 1hr
- Prepared an aliquot of 200µl/eppendorf tube
- Kept at 4^0C and used within one day

4.2.3.5.9 *Preparation of transformation plates with selective media*

100ml LA containing 100 µl of 50mg/ml ampicillin, 20 µl of IPTG and 200 µl of X-gal. 25ml LA media was spread into each Petri plate.

Transformation of ligation mixture

- Taken 200 µl of competent cells and mixed with the ligation mix
- Incubated on ice for 1hr
- Given heat shock at 42^0C for 2min
- Incubated on ice for 3-5 min
- Added 800 µl of LB medium to competent cells

- Incubated at 37^0C with shaking at 200rpm for 1hr
- Subjected to a short spin at 8000rpm
- Re-suspended the bacterial pellet in fresh 100 µl of LB medium
- Spread it with glass spreader on selective LA plate
- Kept the plates for incubation at 37^0C, overnight
- After 15-16hr of plating, blue and white bacterial colonies appeared on the LA plates

4.2.3.5.10 ***Master plating***

- Selected only the white colonies and picked it with micropipette tips
- The bacterial colony was streaked in a new antibiotic (ampicillin) plate
- 25-35 individual colonies were spread on one master plate
- Incubated the master plate overnight at 37^0C
- Screened the positive colonies by colony PCR

The presence of insert in the colony was confirmed by colony PCR. For these cells from each colony was taken using micropipette tips and used as template DNA. PCR was performed as mentioned above with the same primers used for amplification of the product. Successfully cloned colonies showed the bands with the same molecular weight as that of the original PCR product and the successfully cloned colonies were sent for sequencing.

For reconfirmation of successful cloning, plasmids from the colony were harvested using readymade kits by following the manufactures protocol and the plasmid was cut with specific restriction enzymes to liberate the insert. The restricted plasmid was run on 1% agarose gel to confirm the molecular size of the insert.

4.2.3.5.11 ***Sequencing***

Successfully transformed colonies were picked from the master plate using micropipette tip and streaked it in zigzag way on antibiotic plate. Incubated the plates overnight at 37^0C. The whole plate was sent for sequencing. The sequencing was performed by external agency.

4.2.3.5.12 ***Sequence analysis***

The transformed colonies were sequenced and the sequence obtained was BLAST analysed in NCBI database to identify the sequence similarity with known viroids.

4.3 RESULTS

4.3.1 Analysis of nucleic acid

4.3.1.1 *Extraction of RNA and R-PAGE analysis*

A number of protocols were tried for TNA isolation. As the tissues are full of tannins and a number of other cell constituents that may interfere with the extraction process, it was quiet difficult to arrive at a good preparation devoid of impurities and therefore, often no two extractions of the same sample gave consistent result. This gave some difficulty in consistently reproducing the same result from a single tree. As the rubber trees contain large quantities of tannin and other interfering materials, special protocols were standardized for extraction of RNA from the tissues. The procedure developed in the present study was quiet good and was found very useful in detecting the presence of a low molecular weight RNA in TNA.

Analysis of nucleic acid using R-PAGE technique was carried out to observe the presence of LMW RNA similar to viroid in electrophoretic mobility using samples from affected and apparently healthy trees. Total nucleic acid extracts isolated from leaf, bark and root were subjected to polyacrylamide gel electrophoresis under denaturing condition of low salt and high temperature (R-PAGE). Presence of LMW RNA similar to potato spindle tuber viroid (PSTVd) were observed in TPD affected samples (Fig. 4.2) but not in majority of apparently healthy samples.

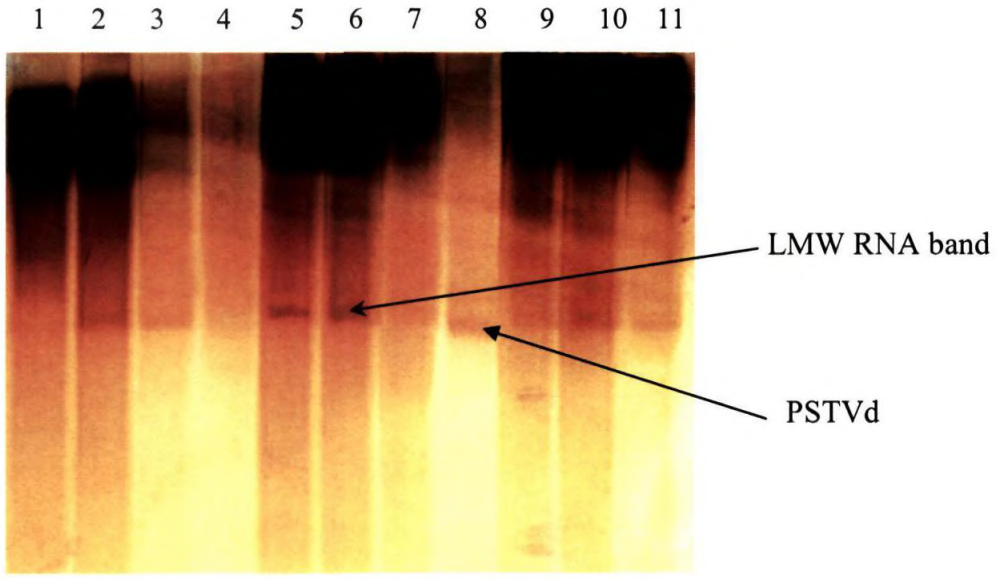


Fig. 4.2 R-PAGE analysis – Electrophorogram showing viroid bands from different rubber trees in comparison to PSTVd (Lane 1 to 6 leaf samples from rubber - 1 & 4 healthy, 2, 3, 5 & 6 TPD. Lane 7- healthy tomato leaf, lane 8 - PSTVd inoculated tomato. Lane 9 to 11 bark samples from rubber- 9 & 10 TPD, 11 healthy)

In non-denaturing gels the LMW RNA band appeared just above 7S host RNA in diseased samples but not in healthy ones. By this procedure it was possible to detect presence of LMW RNA in nucleic acid samples from a number of sources (Tables 4.1 to 4.8)

4.3.1.2 Viroid nature of the isolated RNA

Nuclease treatment: In order to know whether the bands observed were due to RNA or not, TNA samples were subjected to RNase and DNase treatment and then resolved by non-denaturing PAGE. The bands were not observed in the RNase treated samples indicating their RNA nature. When treated with DNase, the bands were observed reconfirming that the band is RNA.

Heat Treatment: The TNA samples from diseased and healthy trees were boiled for 5 min. in a water bath, dye added immediately, quick spinned and loaded on the gel for R-PAGE. After staining the gel, the viroid –like RNA bands were visible clearly in diseased samples as also in the positive control nucleic acid of PSTVd but not in the healthy ones (Fig. 4.3). The lack of sensitivity to heat treatment showed that the RNA is a LMW RNA, similar to viroid.

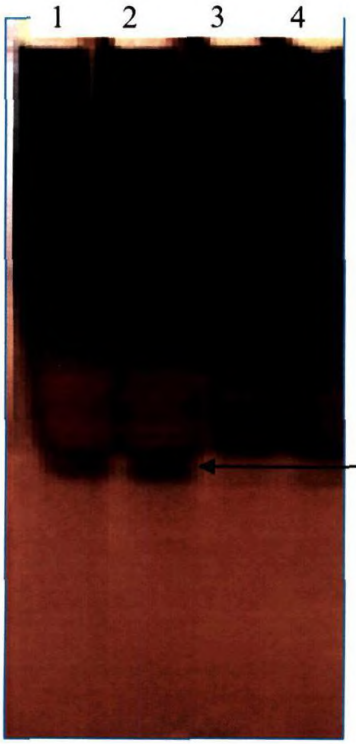


Fig. 4.3 R-PAGE gel run after heat treatment of samples
Lane 1 - TNA from TPD affected sample
Lane 2 - PSTVd NA
Lane 3&4 - Apparently healthy trees

4.3.1.3 Presence of LMW RNA in different plant parts

RNA from leaf, bark and root of TPD affected plants when analyzed using R-PAGE showed the presence of LMW RNA in 56 to 65 trees out of 80 trees tested (Table

4.1). Among the TPD affected trees 65 trees showed presence of LMW RNA in leaf while 60 trees showed LMW RNA in bark and 56 in root (Table 4.1). Apparently healthy trees also showed the presence of LMW RNA ranging from 27 to 32% of the trees. This was confirmed when some of the LMW RNA +ve healthy plants turned to TPD later (Table 4.7 & 4.8).

Table 4.1 Presence of LMW RNA in different plant parts

Sample	No. tested	TPD trees		No. tested	Apparently healthy trees	
		Positive	Negative		Positive	Negative
Leaf	80	65	15	80	24	56
Bark	80	60	20	80	26	54
Root	80	56	24	80	22	58

4.3.1.4 R-PAGE tests on trees under various tapping stages at different locations

Leaf samples were taken from plants under various tapping stages grown at different locations. Majority of the samples tested showed the presence of LMW-RNA in affected plants (Table 4.2) irrespective of the location.

Table 4.2 Presence of LMW RNA in leaf samples of TPD affected trees from different locations

Clone	Location	No. tested	LMW RNA	
			Positive	Negative
RRII 105	Vaniyampara	50	42	8
	Malankara	50	43	7
	Nagercoil	30	25	5
	CES Chethackal	40	35	5
	RRII Farm	50	45	5

4.3.1.5 Repeated R-PAGE tests on trees under tapping at RRII Farm

Trees confirmed as TPD affected/healthy by continuous observation for 10 tapings were subjected to R-PAGE analysis. Different tissues of the same tree were tested by R-PAGE to study the presence of LMW RNA in different plant parts. Leaf, bark and root samples were used and the results are given in Table 4.3. Trees which were tested +ve and –ve for LMW-RNA by R-PAGE at RRII Farm maintained its status of presence/absence of LMW-RNA on periodic testing every year. All the affected plants showed the presence of LMW RNA while all the apparently healthy ones showed its absence. In the TPD trees LMW RNA was present in all the tissues tested.

Table 4.3 Results of R-PAGE on trees under tapping in RRII Farm

Plant No.	Status of the tree	Type of sample	R-PAGE result		
			2008	2009	2010
4/4	TPD	Leaf	+	+	+
		Bark	+	+	+
6/15		Leaf	+	+	+
214		Leaf	+	+	+
6		Leaf	+	+	+
		Bark	+	+	+
		Root	+	+	+
95		Leaf	+	+	+
		Bark	+	+	+
		Root	+	+	+
123		Leaf	+	+	+
		Bark	+	+	+
5	Apparently healthy	Leaf	-	-	-
24		Leaf	-	-	-
		Bark	-	-	-
		Root	-	-	-
122		Leaf	-	-	-
		Bark	-	-	-
103		Leaf	-	-	-
		Bark	-	-	-

4.3.1.6 Presence of LMW RNA in trees showing different TPD symptoms

To study the presence of LMW RNA in trees with different symptoms of TPD, leaf and bark samples from trees with different symptoms of TPD were collected and nucleic acid was isolated and run on R-PAGE. LMW RNA was detected from all the samples that showed both cracking and bulging symptoms (Table 4.4).

Table 4.4 Presence of LMW RNA in plants with different symptoms

Symptoms	No. tested	LMW RNA	
		Positive	Negative
Cracking	20	15	5
Cracking + bulging	15	15	0
Necrosis	20	16	4

4.3.1.7 Detection of LMW RNA in TPD affected trees of different clones

In order to find out the presence of LMW RNA in TPD affected trees of clones other than RRII 105, leaf samples were analyzed. Presence of LMW RNA was detected in the range of 66.6 to 80 percent of the affected trees across the clones (Table 4.5).

Table 4.5 Presence of LMW RNA in TPD affected trees of different clones

Clone	No. of trees tested	LMW RNA		% of samples showing LMW RNA
		Positive	Negative	
RRII 105	50	40	10	80
GT 1	15	10	5	66.6
RRIM 600	30	20	10	66.6
PB 28/59	20	16	4	80
RRIM 605	10	8	2	80
PB 260	25	20	5	80
PB 217	10	7	3	70
Seedling trees	20	16	4	80

4.3.1.8 R-PAGE tests on seedlings

Sixty percent of leaf samples of seedlings selected randomly showed LMW RNA bands. The seedlings raised using seeds collected from TPD affected trees also showed LMW RNA bands (70%) while only very less (25%) seedlings raised from apparently healthy trees showed LMW RNA bands in R-PAGE (Table 4.6).

Table 4.6 Presence of LMW RNA in seedlings

Source of leaf sample	No. tested	Positive	Negative
Seedlings selected randomly	20	12	8
Seedlings from TPD trees	20	14	6
Seedlings from apparently healthy trees	20	5	15

4.3.1.9 Evaluation of R-PAGE as a diagnostic tool

The technique of R-PAGE was evaluated for its usefulness as a diagnostic tool to detect presence of LMW RNA in nucleic acid samples from a number of sources (Tables 4.1 to 4.8). The LMW RNA bands were observed in the TNA of these plants throughout the year, irrespective of tapping period, in untapped tree ("-"stage), tree just opened ("0"stage) as also in trees which are in different years of tapping (i.e. +2,+3,+4,+6. +7 and +13).

4.3.1.10 Relationship between molecular evidence and field observations

Apparently healthy trees (trees with no panel dryness) which showed LMW RNA in R-PAGE were continuously monitored for TPD symptoms. Some of those trees developed TPD symptoms during the course of time (Table 4.7). Moreover, unopened trees ("-"stage) in which LMW RNA was present, developed TPD symptoms on opening for tapping (Table 4.8).

Table 4.7 Appearance of TPD symptoms on apparently healthy trees which were LMW RNA +ve

Location	Total no. of apparently healthy trees with LMW RNA	No. of Trees which later turned to TPD
Vaniyampara	8	4
Malankara	3	3
RRII	6	2
CES Chethackal	13	8

Table 4.8 Association between a LMW RNA and TPD

Name of Estate	Tree no	Status	R-PAGE result	Percentage of dryness of panel at the time of sampling	Percentage of the panel showing dryness after two to three years tapping
Malankara	31	'-1'	+	0	100
	34	'-1'	+	0	40
	35	'-1'	+	0	50
Vaniampara	52	'-1'	+	0	30
	58	'-1'	+	0	100
	56	'-1'	+	0	20

4.3.2 Properties of LMW RNA

4.3.2.1 *Amplification of LMW RNA*

To characterize the LMW RNA and to study its sequence similarity with already published viroids, attempts were made to amplify, clone and sequence the LMW RNA. RT- PCR was carried out using total RNA extracted from diseased and healthy samples by phenol extraction, gel extraction and using RNeasy Plant Kit from QIAGEN.

4.3.2.2 *Design of viroid specific primers*

Commonly available Random Hexamers as well as viroid specific primers were tried for first strand synthesis and PCR. Viroid specific primers used in the study included both specially designed and commonly available viroid primers. Newly designed primers based on the available viroid genome sequences is listed in Table 4.9.

Table 4.9 List of specially designed viroid primers.

Sl. No	Sequence (5'-3')
1.	ACTCGTGGTTCCTGTGGTT
2.	TTCCAAGGCTAAACACC
3.	CGGAACTAACTCGTGG
4.	AGGAACCAACTGCGG
5.	CCCTGAAGAAGCGCTCCTCGAG
6.	CCCGGCGCTGGCTGCTTCT
7.	CCCCGGGGCTCCTTTCTC
8.	CGCGACCCGGTGGAATCA
9.	CTGGATTCCGACGAGAGT
10.	NTCTCCTTCCTCCTGCTCCTG

Primers selected from the commonly available pool of viroid primers are listed in Table 4.10.

Table 4.10 List of common viroid primers used in the study.

Sl. No	Primer name	Sequence (5'- 3')
1.	Pospi R	5' CCC TGA AGC GCT CCT CCG AG '3
2.	Pospi F	5' ATC CCC GGG GAA ACC TGG AGC GAA C 3'
3.	Had 3	5' CTCCAGGTTTCCCCGGG 3'
4.	Rao2	5' GCGGATCCGGTGGAAACAACTGAAGC 3'
5.	Had 4	5' AGGGCTAAACACCCTCGCCC 3'
6.	Rao14	5' AGGGATCCCCGGGGAAACC 3'

Abutting primers were also designed to cover the upper and lower half of the viroid RNA (Abut UR and Abut LR) and used in RT-PCR reaction (Table 4.11).

Table 4.11 Abutting primers

Sl. No	Primer name	Sequence (5'- 3')
1.	AbtLC-F	5' CAACTGAAGCTCCCGAGAACCGCTT 3'
2.	AbtLC-R	5' TTTCCACCGGGTAGTAGCCGAAGCGAC 3'

Pospi viroid group specific primers were also designed and used in RT-PCR (Table 4.12).

Table 4.12 Pospi viroid group specific primers

Sl. No	Primer name	Sequence (5'- 3')
1.	PSTV- F	5' CCGGTGGAAACAACTGAAGCTCCCGAGAAC 3'
2.	PSTV- R	5' GTAGTAGCCGAAGCGACAGCGCAAAGGGG 3'

4.3.2.3 *cDNA synthesis*

The isolated RNA from untapped and also from tapped apparently healthy as well as TPD affected trees were used for cDNA synthesis. Good quality RNA with an OD 260/280 ratio of 1.8 to 2 and an OD 260/230 of 1.8 or greater was obtained from the *Hevea* leaves by the method already described.

4.3.2.4 *PCR amplification*

In order to amplify the LMW RNA, different PCR conditions were tried with varying annealing temperature depending upon the primer combination. When specially designed primers listed (Table 4.9) were tried, PCR amplification of a band of ~360 bp (Fig. 4.4) was observed in TPD affected plants with the primers no.6 & 7 when the following temperature profile was used. One cycle at 94^0C for 2 min. followed by 35 cycles at 94^0C for 30 seconds, annealing at 54^0C for 30 seconds, extension at 72^0C for 30 seconds and final extension at 72^0C of 8 min. The band was found to be faint when the annealing temperature was increased. The amplified band was purified from the agarose gel by gel band purification kit and carried out direct sequencing. However, the sequencing results showed non-specific amplification.

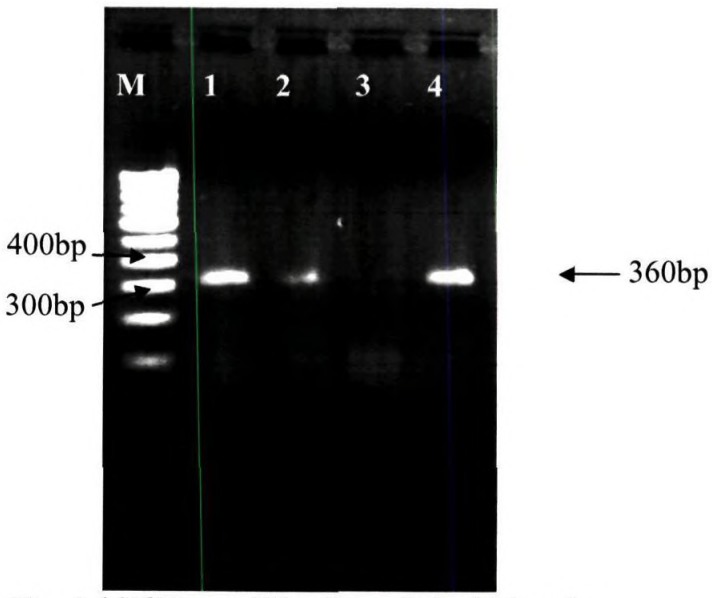


Fig. 4.4 PCR amplification of viroid bands
(M- 100bp DNA ladder; lane 1, 2 and 4- TPD affected trees; lane 3 – Healthy tree)

Since, primers with low annealing temperature were showing non-specific amplification, which could be due to high secondary structure as a result of the high CG content in viroid, primers with high annealing temperature were tried. PCR amplification was done using primers of Pospi viroid group-specific primers and PSTVd specific primers (Table 4.10). The PCR products in the range of viroid bands were cloned and sequenced. The results of BLAST (NCBI) gave two types of hits: one viroid specific and another viroid non-specific.

Based on the viroid specific partial sequences obtained, a set of abutting primers to cover the upper and lower half of the viroid RNA (Abut UR and Abut LR) were designed and used in RT-PCR reaction (Table 4.11 & 4.12). The results of amplification were encouraging as a product ~300+bp was consistently amplified in R-PAGE positive TPD affected samples which was absent in most of the R-PAGE –ve apparently healthy samples. The PCR products in the range of viroid bands were cloned and sequenced. The results of BLAST (NCBI) again gave two types of hits, viroid specific as well as viroid non-specific. It was suspected that such kind of nonspecific amplification was due to the low purity of the total RNA extracted from the samples which was used as template in cDNA synthesis.

In order to maximize the purity of RNA in the extracted product an additional purification step of phenol extraction developed specifically for extraction of RNA from rubber samples was introduced. Total RNA was extracted from diseased and healthy samples by phenol extraction followed by another extraction using RNeasy Kit from QIAGEN. Thus extracted RNA was used as template for cDNA synthesis using viroid specific complementary Abutting primers. When PCR amplification of cDNA was done using primers of Pospi viroid group specific Abutting primers, products in the range of viroid (360bp) were observed only in TPD affected trees (Fig. 4.5) and absent in healthy plants.

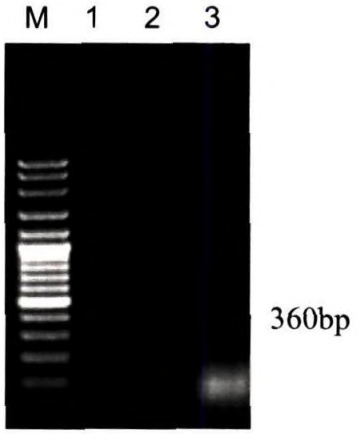


Fig. 4.5 Agarose gel electrophoresis of PCR products (M – Marker (100bp), Lane 1-TPD tree (6/15), 2 - TPD tree (95) 3 – Healthy tree (24))

4.3.2.5 ***Molecular cloning, sequencing and sequence analysis***

The direct sequencing of the PCR product obtained was performed and the sequence showed homology to Potato Spindle Tuber Viroid on BLAST analysis (Fig. 4.6).

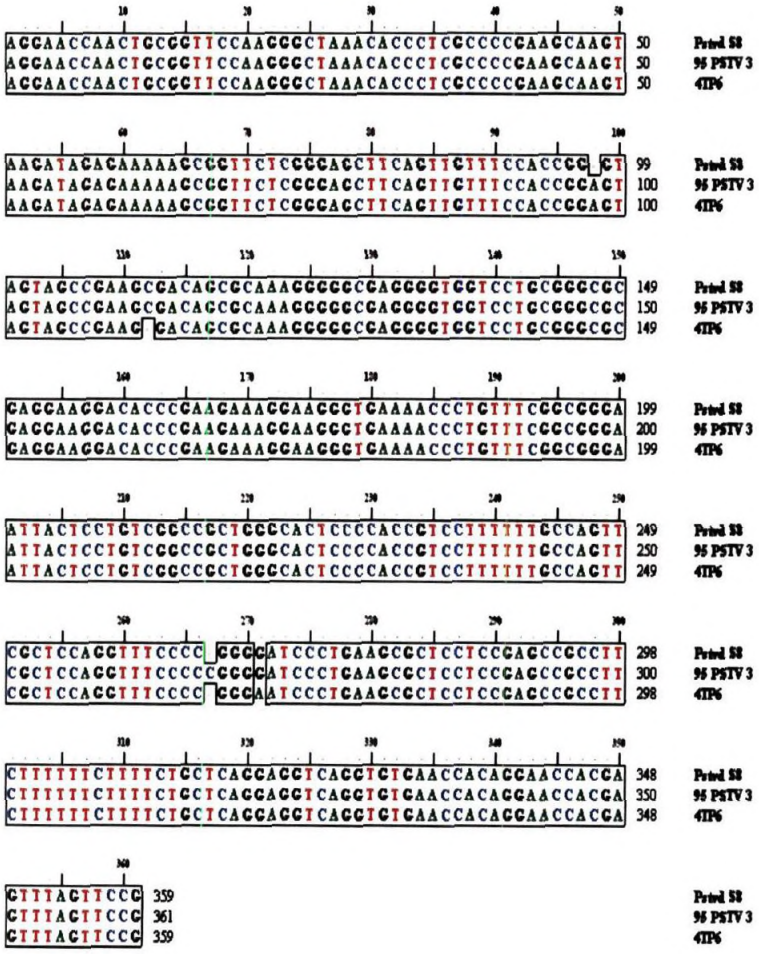


Fig. 4.6 Sequence alignment report (PSTVd & Rubber)

Cloning of the RT-PCR product was done and the sequencing of the clones showed homology to Potato Spindle Tuber Viroid on BLAST analysis. The sequence obtained showed 98% homology to PSTVd on BLAST (NCBI) analysis.

To reconfirm the above results, samples were collected from healthy as well as TPD trees which were also R-PAGE -ve and +ve respectively. Total RNA was extracted from diseased and healthy samples by phenol extraction followed by a second extraction using RNeasy Kit from QIAGEN. The extracted RNA was used as template for cDNA synthesis using viroid specific complementary primers. PCR amplification of cDNA was done using primers of Pospi viroid group specific and abutting primers. Products in the range of viroid (~360bp) were observed in TPD affected trees (which were R-PAGE +ve) and absent in apparently healthy plants (which were R-PAGE –ve) (Table 4.13 & Fig. 4.7). These products were cloned. The sequencing of the cloned product was performed which showed homology to Potato Spindle Tuber Viroid (PSTVd) on BLAST analysis (Fig. 4.8 & Table 4.13).

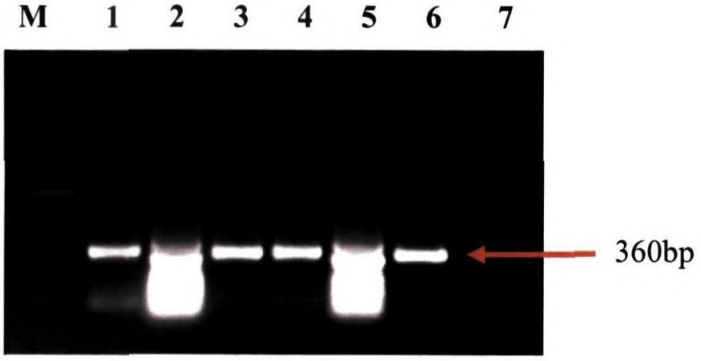
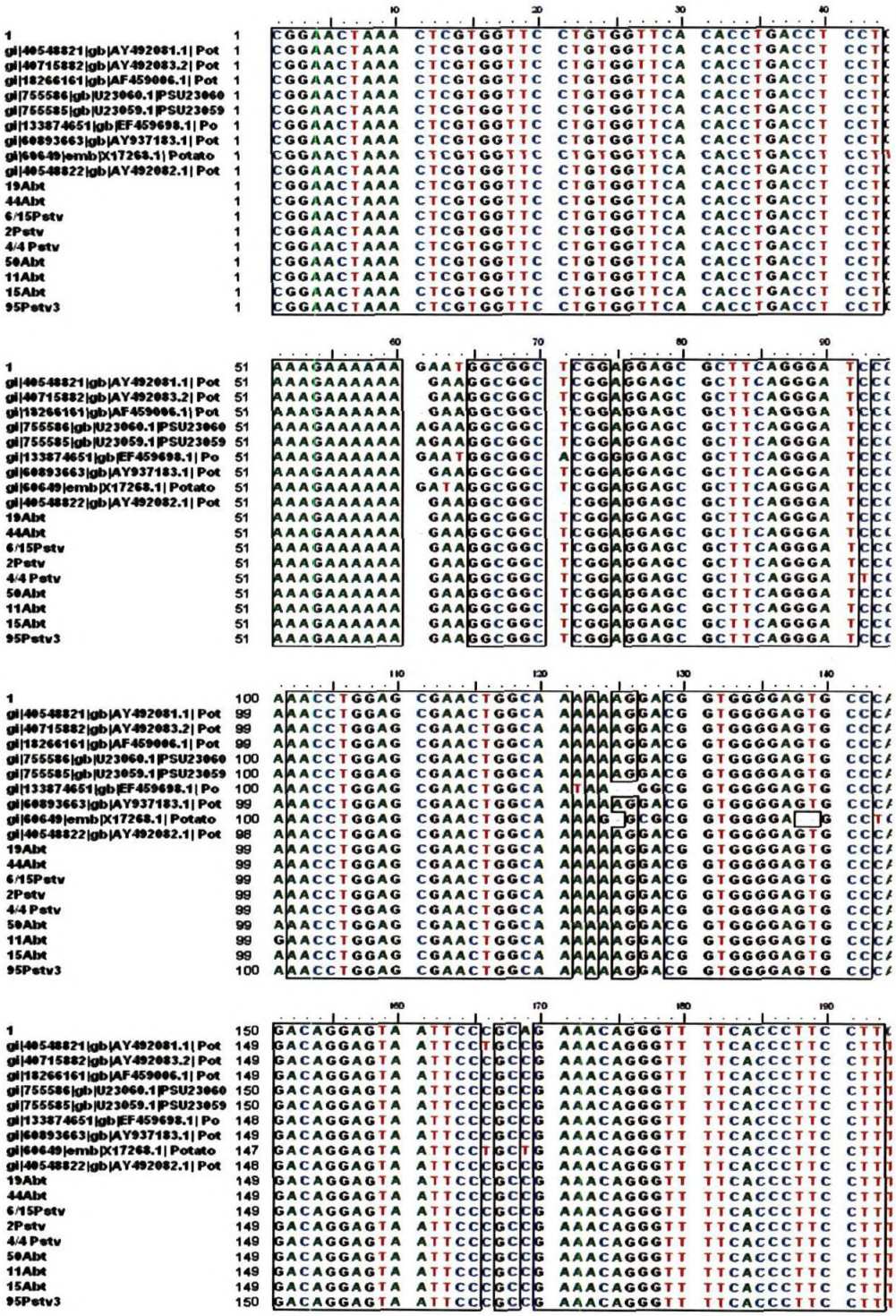


Fig. 4.7 Agarose gel electrophoresis of PCR products (M- marker, lane 1-6 TPD, lane 7- Apparently healthy)

Table 4.13 Presence of viroid in TPD affected trees and their absence in healthy trees evaluated by R-PAGE

	Tree No	TPD Status/ R-PAGE result	PCR	Sequencing
1.	4/4	TPD/ R-PAGE +ve	+ve	Viroid
2.	62		+ve	
3.	120		+ve	
4.	6/15		+ve	Viroid
5.	95		+ve	Viroid
6.	2		+ve	Viroid
7.	116		+ve	
8.	24	Healthy/ R-PAGE -ve	-ve	
9.	98		-ve	
10.	103		-ve	
11.	36		-ve	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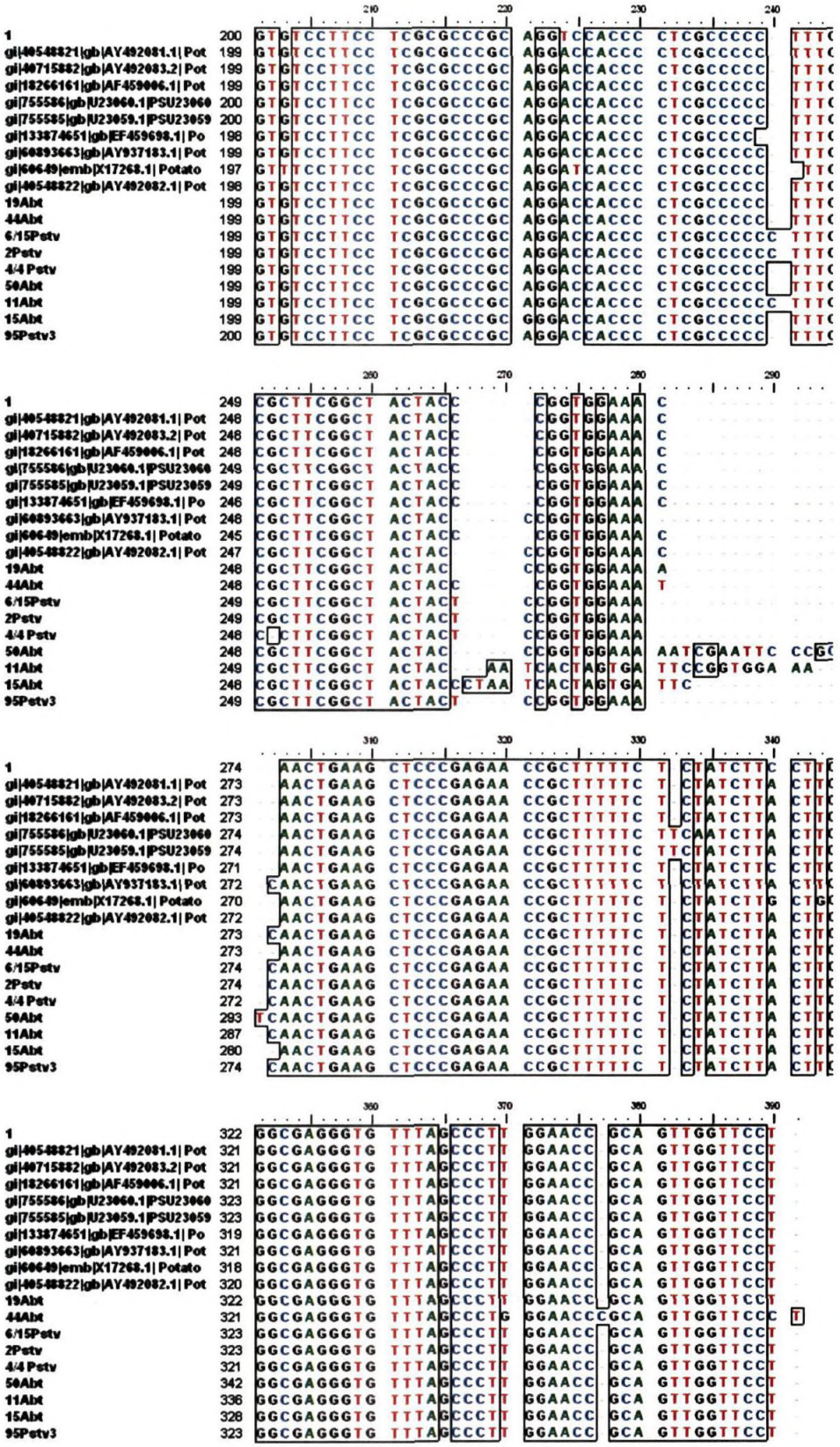

Fig. 4.8 Alignments of rubber viroid with PSTVd

4.4 DISCUSSION

There have been several efforts to investigate the cause of the TPD syndrome, but so far, no clear picture has emerged. The anatomical changes observed in TPD affected trees by several workers (Rands, 1921; Sanderson and Sutcliffe, 1921; de Fay, 1981; Gomez *et al*, 1990; de Fay, 1982; de Fay and Hebant, 1980; Paranjothy *et al.*, 1975; Wu and Hao, 1994) could be the aftereffect of various traumatisms (mechanical such as tapping, chemical or pathological infection) which cause formation of ethylene, the influence of which on biochemical, anatomical and histological phenomena is proved (Yang and Pratt, 1978). The observation that TPD was present in entirely different (10-40^{0}C) climatic conditions (Das *et al.*, 2006; Abraham *et al.*, 2006; Chandrasekhar *et al.*, 2006; Dey, 2006) disprove the role of climatic factors as the cause of TPD. Occurrence of TPD in almost all the clones with varying intensity (Mydin *et al.*, 1999) indicates that it may not be a clonal character. Since, rubber trees are vegetatively propagated through budding, variations in inheritance of genes between the healthy and TPD affected trees within one clone is ruled out. TPD occur on trees grown in soils of different nutrient status and under different levels of fertilizer applications (Pushpadas *et al.*, 1974; 1975; Sivakumaran *et al.*, 1997) and no definite correlation could be established.

Stock-scion incompatibility cannot be the cause of TPD as TPD could be observed even on trees raised from seedlings (RRII, 2003) and meristem cultured trees (Thulaseedharan *et al.*, 2006) as well as in the test tapped seedlings (RRII, 2003). The theory of impaired cyanide metabolism due to cell decompartmentalization near stock scion junction as the cause of TPD also fails to explain incidence of TPD on seedlings and meristem cultured trees. The observation of TPD in the first month of opening for tapping (de Fay, 1981) and failure to cure TPD by leaving trees untapped (Paranjothy and Yeang, 1977; Le Shizong *et al.*, 1984) raise questions on the validity of the theory that tapping stress causes TPD. However stresses are known to aggravate diseases. Presence of TPD in test tapped seedlings (RRII, 2003) and also in low yielding germplasm plants (RRII, 2003) rules out the theory of relation between high yield and TPD.

The hypotheses that TPD is the result of various physiological factors such as wound-induced ethylene, peroxidative damages due to various reactive oxygen species (ROS), reduced ATP content and the increased tissue respiration were based on

comparison of data recorded from healthy and fully dry trees. In the absence of data from the trees which are in the process of developing TPD symptoms, it is likely that all these biochemical changes are effects and not the cause. The comparisons in most of the studies were between dry untapped trees and healthy trees being tapped as control and hence the validity of conclusions is questionable. Moreover, comparisons of trees that are dry and left untapped for varying number of years also may not be appropriate. Reports shows that TPD developed in several trees irrespective of their initial latex yield. All physiological parameters of the latex preceding the onset of the TPD failed to predict its occurrence (Sivakumaran *et al.*, 2002). Many pathogens are known to induce physiological changes in plants due to their infection (Qi and Ding, 2003). Itaya *et al.*, (2002) showed that viroid (PSTVd) strains can alter expression of both common and unique genes. These genes encode products involved in defense/stress response, cell wall structure, chloroplast function, protein metabolism and other diverse functions.

Occurrence of TPD in adjacent trees in the same line (Taysum, 1960; de Soya *et al.*, 1983; Mydin *et al.*, 1999) is common in rubber plantations. In many cases the immediate tree next to an affected tree also gets affected. This phenomenon is seen in rubber trees irrespective of the clone, location, climate and type of planting materials namely seedlings, bud grafts and meristem culture plants. Transmission of a pathogen through either root contact or tapping knife can be suspected. The initial occurrence at random could be due to the activity of the pathogen that exists in the plant from the seedling stage itself or carried through planting material from an infected tree. All these point to a possible spread of the disorder by a biotic agent from an infected point.

The consistent increase in the incidence of TPD corresponding to the progress in the age of the tree also indicates that the organism that existed earlier in low titre becomes active on attaining favourable conditions, gets multiplied and expresses the symptoms. There are reports that molecular pathogens like viroids express the symptoms only when their concentration reaches a threshold titre (Szychowski *et al.*, 1995). Stresses like intensive tapping, severe pruning, drought, water logging, presence of rocks in the root zone etc. can make the tree vulnerable and the organism may become active triggering the appearance of TPD symptoms. It was reported that in Avocado severe pruning of symptomless carriers, and perhaps other severe causes of tree stress, are suspected of causing viroid (Avocado Sunblotch Viroid) to become active in the new growth, inducing previously symptomless trees, to exhibit symptoms (Steve, 2008).

The percentage of trees showing no symptoms of TPD was more in the first year (opening) of a new panel (Elsewhere in this thesis). TPD trees appear healthy without any panel dryness for a short period when the panel was changed, but dryness symptoms appear again as tapping advances. In almost all cases when one panel is fully affected, the other panels also gets affected upon tapping (Krishnakumar *et al.*, 2002). So, once the tree is infected, it continues to be in the same condition. Trees affected in basal panel often show TPD even when tapped upward. This phenomenon indicates the association of a molecular pathogen, where reversion or recovery from the disorder is not possible.

The stress developed as a result of an attack of a pathogen causes physiological and morphological malfunctioning (symptoms). Many plant pathogens show different symptoms on the same host (Diener, 1979; Visvader and Symons, 1985). All the symptoms present in TPD trees can be considered as the result of an attack of a pathogen. Symptoms noticed in TPD trees are similar to other virus and viroid diseases in other species (Peries and Satchuthananthavale, 1964; Diener, 1979; Visvader and Symons, 1985). But, investigations by Jacob *et al* (2006) did not reveal association of fungi, bacteria, virus, MLO or protozoa as biotic agents.

In this scenario, the evidence of a possible association of viroid with TPD syndrome (Ramachandran *et al.*, 2000) is to be seriously looked upon. As viroids contain neither capsid nor nonstructural proteins, ELISA was not an option. The technique of R-PAGE has been successfully used for detection of viroids infecting other plants like potato, apple, plum, citrus, coconut (Diener, 1987.). This method involves a combination of native and denaturing polyacrylamide gel electrophoresis that relies on the circular properties of the viroid RNA molecule to resolve it from other plant RNAs (Schumacher *et al*, 1986; Singh and Boucher, 1987). This method is still very useful for demonstrating the presence of new viroids in diseases of unknown etiology. The technique is specifically used for viroid detection since the viroid RNA molecules differ from host RNA in their electrophoretic mobility in denaturing gels. Electrophoretic analysis of nucleic acid of viroid infected samples in non-denaturing gel condition shows an additional band just above 7S RNA band when silver stained, but not in healthy ones. Whether this additional band was due to a low molecular RNA or not was established by running the gel under denaturing conditions by reversing the polarity. Such a method is very specific for detection of viroid since the denaturing conditions provided allow all other forms of RNA present to move out much faster, the viroid RNA trailing behind

due to its secondary structure and hence can be resolved as the lowest band in a return gel.

As rubber trees contain large quantities of tannin and other interfering materials, special protocols for extraction and purification of nucleic acid was essential. Besides, viroids are present in very low concentrations in the plants, which are mostly localised in the nucleoli of cells. Stringent procedures of purifications results in loss and reduces the viroid nucleic acid to non-detectable levels. With a protocols standardized, it was used to detect the presence of LMW RNA in leaf, bark and root tissues. The infectious LMW RNA was detected from different samples drawn from varying ages of trees from different locations. In many cases the trees where a LMW RNA was detected invariably showed the TPD syndrome. Therefore, it can be hypothesized that the presence of LMW RNA has some correlation with TPD.

The observation that majority of TPD affected trees from various locations showed presence of LMW RNA (Table 4.2) indicate association of the band with TPD irrespective of environmental conditions and methods and stages of tapping. Similarly the consistency in the presence of LMW RNA over three years of observation (Table 4.3) in TPD affected and its absence in the healthy trees also confirm the association of the LMW RNA with TPD. Most of the trees that showed advanced symptoms of TPD tested positive for LMW RNA (Table 4.4). More than two third of all the samples from TPD affected trees across seven popular rubber clones and seedlings tested positive for LMW RNA (Table 4.5) showing that the association of TPD with the LMW RNA is independent of the genetic constitution of the trees.

Pellegrin *et al.*, (2004) observed numerous viroid-like (between 250 and 400 nucleotides) and double strand virus-like (1,800 bp) low-molecular-weight RNAs but, no definite correlation was found with the bark necrosis status of rubber trees. They also reported that the sequencing of the various isolated RNAs only identified plasmids, nonpathogenic bacteria and yeasts, but none of the suspected pathogens. In addition, previous and recent transmission trials (tapping knife disinfection, bud grafting, bark implantation, and etc.) failed to confirm the involvement of a biotic agent. Hence, they concluded that a physiological disease is suspected that involves exogenous stresses, nonoptimal vascular relations at the rootstock/scion junction and impaired cyanide metabolism.

About 30 percent of the apparently healthy trees showed presence of LMW RNA band similar to the band observed in majority of TPD affected trees. Similar

observations on symptomless carriers are reported in other viroid diseases. The apparently healthy trees having the presence of LMW RNA are symptomless carriers as observed in Citrus exocortis viroid (CEVd), which can infect all varieties of citrus but is symptomless in most (Gillings *et al*, 1991). Disease symptoms develop when infected budwood is grown on susceptible rootstocks like *Poncirus trifoliata*, rangpur lime, and sometimes Swingle citrumelo and citrange. No symptoms of exocortis are seen on trees grown on rough lemon, sweet orange and mandarin rootstocks (Gillings *et al*, 1991).

Presence of bands in apparently healthy samples also indicated that the biotic agent can be detected in the tree much before the onset of TPD symptoms, thus showing that R-PAGE can be used as a diagnostic tool in the absence of other reliable procedures. The intensity of vein-banding symptoms was directly correlated with an enhanced titer of grapevine yellow speckle viroid GYSVd-1 and GYSVd-2 (Szychowski *et al.*, 1995). Most of healthy trees that tested positive for LMW RNA eventually became TPD affected (Table 4.7 & 4.8). Presence of LMW RNA in seedlings from different sources particularly those showing TPD symptoms is significant in vegetative propagation of rubber trees (Table 4.6).

Detecting a LMW RNA in TPD affected samples was not a mere conjecture. At the time TPD was being investigated very effectively world over, knowledge of molecular diagnostics with improved techniques was not available and hence there was a lot of conservatism to associate any biotic agent with the syndrome. Consistent observations on the association of LMW RNA band detected mostly with TPD affected trees prompt to suggest a possible biotic etiology for the syndrome that the agent could be a viroid is proposed due to following observations:

(a) Similarity of the band in electrophoretic mobility to PSTVd
(b) Its sensitivity to ribonulease
(c) Its insensitivity to phenol and heat
(d) Its single-strandedness and circularity
(e) Detection of LMW RNA in trees before opening in "-" stage of tapping
(f) Presence of bark cracking symptoms in affected trees
(g) LMW RNA +ve healthy plants turned to TPD later
(h) Absence of any other form of pathogenic agent
(i) Occurrence of the disease in higher proportion in the high yielding clone RRII 105

The PCR amplification of the cDNA using viroid specific primers consistently amplified products in the range of viroids in R-PAGE positive TPD affected samples which was absent in R-PAGE –ve apparently healthy samples. The sequencing results of amplified LMW RNA showed 98% homology to Potato Spindle Tuber Viroid (PSTVd) on BLAST analysis. Hammond and Owens (2006) were of the view that routine indexing of diagnostic samples has revealed that several viroids are more widely distributed than previously thought. There are lessons to be learned from both the discovery of novel viroids and the rediscovery of known viroids in new crop plants that can help prevent the outbreak of new infections. Viroids are also being detected in crop species where they were not previously known to occur. In some cases, these infections are latent, but in others, severe disease symptoms develop.

Although the primary natural host of PSTVd is potato, it is reported that this viroid also naturally infects a variety of other *Solanum spp.* It has a wide experimental host range, infecting 94 species in 31 families (Jeffries, 1998). In Peru, PSTVd has been detected in Avocado (*Persea americana*), which is a perennial tree species where infections are often latent unless the tree is co-infected with ASBVd (Querci *et al.*, 1995). It is to be recalled that *Hevea brasiliensis* also originated from South America. Bar-Joseph (2003) suggested that for woody perennials, old vegetatively-propagated crops should be located and evaluated as possible symptomless carriers and sources of viroids capable of causing disease in newly planted cultivars. An outbreak of PSTVd was reported in a tomato production nursery in the south-east of England (Mumford *et al.*, 2004). The origin of these infections is currently under investigation.

4.5 CONCLUSION

Various factors which were assumed to be the cause of TPD could not conclusively prove its etiology. Occurrence of TPD in adjacent trees in the same line, consistent increase in the incidence with age, similarity in symptoms and anatomical abnormalities to some known diseases, inability to revive the affected trees by resting and the presence of dry rubber trees from the start of exploitation prompted a biotic etiology for TPD. But, investigations by Jacob *et al* (2006) did not reveal association of fungi, bacteria, virus, MLO or protozoa as biotic agents. Hence, the report on the evidence of a possible association of viroid with TPD syndrome was seriously looked upon. A protocol was standardized to detect the presence of LMW RNA in leaf, bark and root tissues. The infectious LMW RNA was detected from different samples drawn from varying ages of trees from different locations. In most cases the trees where a LMW RNA was detected invariably showed the TPD syndrome. Therefore, it is hypothesized that the presence of LMW RNA has an association with TPD.

Presence of bands in apparently healthy samples indicated that the biotic agent also occurs as symptomless carriers and hence, this technique can be used as a diagnostic tool for detection of TPD much before the onset of symptoms. Majority of healthy trees that earlier found positive for LMW RNA eventually became TPD affected. Presence of LMW RNA in seedlings from different sources particularly those showing TPD symptoms is significant in vegetative propagation of rubber trees. The sequencing results of amplified LMW RNA showed homology to Potato Spindle Tuber Viroid (PSTVd). Although the primary natural host of PSTVd is potato, the reports that this viroid also naturally infects a variety of other crops and more importantly perennial tree species such as Avocado in South America from where *Hevea brasiliensis* also originated.

Chapter 5

TRANSMISSION STUDIES

5.1 INTRODUCTION

As the TPD syndrome has, from the early days, been considered to be a problem of physiological origin, not much attention has been given to probe into possible transmission of TPD from an affected tree to a healthy tree (mechanical) or by graft transmission and seed transmission. Hence, there exist scanty reports in literature about the studies on the transmission of TPD.

Transmission of TPD can be suspected due to the non-random or clustered occurrence of TPD trees in plantations. TPD occurrence does not seem to be at random since row of four or five diseased trees are commonly observed in plantations (Taysum, 1960; de Fay, 1981). Murong *et al.*, (1994) also reported that TPD affected trees are not distributed randomly in the stand and that the disease is caused by pathogens like RLOs. But, there are reports which show that disinfection of the tapping cut and tapping knife did not appear to reduce the frequency of the disease (Rands, 1921).

Peries and Brohier (1965) observed that bark-cracking symptom of rubber common in the eastern rubber growing countries could be associated with a virus. Since rubber trees are mainly propagated by bud grafting, use of budwood from plantations which had a history of bark cracking, is the most likely method of disseminating the disease. For this, they could observe evidence in the field records of some plantations in Sri Lanka. They also suspected a possible association of viruses with bark scaling symptom.

Viral contamination by the tapping tool and the existence of micro conditions in the soil were suggested to account for the rows of dry trees (de Fay and Jacob, 1989). In a study of TPD affected trees with bark scaling (RRIM 605) out of the ten healthy immediate next trees to the bark scaled, seven became TPD affected by the fourth year of tapping (RRII, 2003). No evidence is now available for seed or pollen transmission.

The transmission of TPD through bark grafts was attempted and the healthy bark grafted on the scion portion of TPD affected tree produced latex (Premakumari, *et al.*, 1996). The observation after three months on the grafted bark revealed that only two out of six grafted bark yielded latex in the entire cut.

Since, in the present study, viroid showing sequence homology to PSTVd was identified in TPD affected trees, the transmissibility of TPD as well as the causal organism from an affected plant to a healthy plant should be identified to prove the Koch's postulates and thereby the etiology of TPD. The transmissibility of the viroid to

an indicator host can also be an indirect method to prove the Koch's postulates. Several field and greenhouse studies have demonstrated that viroids are easily transmissible by mechanical inoculation and efficiently spread by contact with contaminated pruning tools, farm implements, clothing, and human hands. Viroids can also be spread by graft transmission and foliar contact between neighbouring plants. Seed transmission has been demonstrated for many, but not all, viroids, and pollen-borne transmission is also known to occur in tomato (Kryczynski, *et al.*, 1988). For potatoes, PSTVd may be introduced into a field by planting infected seed tubers or true potato seed (Hunter *et al.*, 1969; Singh, 1970; Hadidi *et al.*, 2003)

Reports of seed transmission of Chrysanthemum Stunt Viroid (CSVd) are contradictory, Monsion *et al.*, (1973) presented evidence for seed transmission, while Hollings and Stone (1973) reported that CSVd is not seed transmitted. Vertical transmission has been demonstrated for Avocado Sunblotch viroid (ASBVd) in avocado (Whitesell, 1952) and PSTVd in tomato and pepino (Benson and Singh, 1964; Hollings and Stone, 1973; Singh, 1970) but not *Tomato planta macho viroid* (TPMVd) in tomato (Galindo, 1987). Insect transmission was reported for TPMVd, where the aphid, *Myzus persica* was found to transmit the viroid from the wild host, *Physalis foetens,* to the experimental host, tomato. PSTVd can also be transmitted by insect vectors. De Bokx and Pirone (1981) reported a low rate of transmission of PSTVd by the aphid *Macrosiphum euphorbiae* but not by *Myzus persicae* or *Aulacorthum solani.* However, *Myzus persicae* is able to efficiently transmit PSTVd from plants that are doubly-infected with PSTVd and potato leaf roll luteovirus (PLRV) (Salazar *et al.*, 1995). PSTVd was subsequently shown to be heterologously encapsidated within particles of PLRV (Querci *et al.*, 1997), a phenomenon that may have important implications for the epidemiology and spread of PSTVd under field conditions.

PSTVd is transmitted in true potato seed (Fernow *et.al.*, 1970; Singh, 1970) via infected pollen or ovules (Grasmick and Slack, 1986; Singh *et al.*, 1992) and by contact, mainly by machinery in the field. Experimental acquisition and transmission of PSTVd by *Myzus persicae* from plants co-infected by Potato leaf roll virus has been reported (Salazar *et al.*, 1995; Querci *et al.*, 1996; Syller and Marczewski, 1996; Querci *et al.*, 1997), from a small percentage of plants (Singh and Kurz, 1997).

Transmission of viroid from one rubber plant to another by bud grafting and also to indicator plants my artificial methods were investigated in the present study to identify the infectious nature of the viroid.

5.2 MATERIALS AND METHODS

5.2.1 Transmission studies through bud grafting

5.2.1.1 *Both stock as well as scion as the source of TPD*

Seedlings to be used as stocks were screened for the presence/absence of LMW RNA by analyzing (R-PAGE) the leaf samples collected from seedling nursery at Central Nursery of Rubber Board, Karikkattoor before bud grafting. Healthy as well as TPD affected trees were selected based on both the symptoms and R-PAGE analysis. Bud woods collected from the screened trees were used as scion. Seedlings that are LMW RNA +ve or -ve were bud grafted (brown budding) with scion collected from LMW RNA +ve TPD affected trees and LMW RNA -ve healthy trees in the following four combinations with 100 plants in each group.

1. LMW RNA +ve stock with LMW RNA +ve scion,
2. LMW RNA +ve stock with LMW RNA -ve scion,
3. LMW RNA -ve stock with LMW RNA +ve scion,
4. LMW RNA -ve stock with LMW RNA -ve scion.

Bud grafted plants were planted in polybags and maintained in polybag nursery for sprouting. 50 plants were selected from each group at two whorl stage and field planted at RRII Central Experiment Station (CES) Chethackal, Ranni, Pathanamthitta District with recommended spacing. Three years after planting, 20 plants were selected at random from each group and leaf samples collected from each plant was tested by R-PAGE to study the presence/absence of LMW RNA. Girth of trees were recorded at a height of 30cm above bud union after three years of planting.

5.2.1.2 *Scion as the source of TPD*

In order to study the symptom development in the next generation when the source of bud (scion) was TPD affected/healthy, 250 seedlings (LMW RNA status unknown) each were bud grafted with scion taken from TPD affected trees (LMW RNA +ve) as well as healthy trees (LMW RNA -ve) and planted at CES Chethackal. Girth of the plants with TPD scion as well as healthy scion was recorded annually. Test tapping was performed after four years from planting to study the appearance of TPD symptoms. Out of 250 plants in each group, 80 plants from each group with girth ranging from 35 – 45 cm at a height of 30cm above bud union were selected for test tapping. The trees were tapped daily and observed for TPD at an interval of 30 days. Yield recording from all the tapped trees were carried out by collecting cup lumps on all

tapping days and weighing every month. DRC samples were collected from randomly selected 20 trees in each group.

5.2.2 Pathogenicity test on indicator plants (Infectivity test)

Viroid's can be detected by biological indexing (bioassay) of the suspect plant materials on a range of indicator hosts. Indicator hosts express diagnostic symptoms when infected by specific pathogens. For biological indexing to be successful, both the host range as well as the symptoms produced on that host by specific viroids must be known. Tomato plants were successfully used earlier as indicator for PSTVd. Hence tomato was used as indicator host.

a. Inoculation of RNA

Tomato seedlings (cv Pusa Ruby) which was used as indicator host were raised in earthen pots with standard potting mixture. The pots were maintained in an insect proof glasshouse and inoculated (5ul/leaf/plant) with the total nucleic acid extract isolated from both healthy and TPD affected rubber trees at the cotyledonary stage upto 2-4 leaf stage using carborundum (600-mesh) as abrasive. After inoculation the seedlings were sprayed with a fine jet of water and observed regularly for symptoms.

b. Re-isolation of LMW RNA from the inoculated plants

Eight weeks later the symptomatic leaves from the plants in which TPD sample was inoculated and leaves from control inoculated plants were analysed by R-PAGE as well as RT-PCR by following the same procedure for analysis of samples from rubber.

c. Characterisation of LMW RNA from the inoculated plants

The extracted RNA was used as template for cDNA synthesis using viroid specific complementary primers. PCR amplification of cDNA was done using primers of Pospi viroid group specific and abutting primers. The amplified PCR products were size fractioned on a 1.5% agarose gel. The amplified product was excised from the gel and cloned in pGEM®-T Easy Vector. The transformed colonies were sequenced and the sequence obtained was BLAST analysed to identify the sequence similarity if any, with the viroids.

5.3 RESULTS

5.3.1 Transmission studies through bud grafting

5.3.1.1 *Both stock as well as scion as the source of TPD*

The R-PAGE test of bud grafted plants under transmission studies showed that all the plants tested from the group in which both stock and scion were viroid +ve, maintained the viroid bands (Table 5.1). But, in plants where stock is viroid –ve and scion is viroid +ve, only 70% plants showed viroid band. Plants which had viroid +ve stock and viroid –ve scion showed viroid band in 50% of plants. This shows that viroid was transmitted from viroid +ve stock to viroid –ve scion. 25% of plants in which both stock and scion were viroid –ve showed viroid bands.

Table 5.1 Results of R-PAGE test on seedlings under transmission study through budding

Stock	Scion	Total plants studied	R-PAGE result	
			+	-
+	+	20	20	0
+	-	20	10	10
-	+	20	14	6
-	-	20	5	15

The girth of seedlings did not differ statistically between the treatments (Table 5.2).

Table 5.2 Girth of seedlings under transmission study through budding

Stock	Scion	Mean girth (cm)
+	+	35.8
+	-	31.6
-	+	32.7
-	-	34.0
CV%		21.93ns

5.3.1.2 *Scion as the source of TPD*

Plants budded with scion taken from TPD affected trees as well as healthy trees planted at CES Chethackal were test tapped to study the appearance of TPD symptoms. The average girth of the plants with TPD scion was more compared to trees with healthy scion (Table 5.3).

The results after one and half years tapping showed that TPD was observed in both group of plants, namely scion taken from TPD as well as healthy trees (Table 5.4). This shows that stock is also playing a role in the development of TPD. But, bark

cracking symptoms was observed only in plants with scion taken from TPD trees. The latex yield was comparatively high in trees with TPD scion (Table 5.5).

Table 5.3 Average girth of the plants (at 30cm height from the bud union)

Scion source	Girth (cm)
TPD tree	34.6
Healthy tree	30.9

Table 5.4 Incidence of TPD in test tapped trees (80 numbers)

Scion source	No. of TPD trees						
	Jun '10	Aug'10	Oct'10	Dec'10	Feb '11	Nov '11	Jan '12
Healthy tree	0	4	2	3	9	10	13
TPD tree	0	2	5	5	7	9	9

Table 5.5 Yield (g/t/t)

Scion source	Jun '10	Aug'10	Oct'10	Dec'10	Feb '11	Nov '11	Jan '12
Healthy tree	4.35	5.77	8.15	10.73	13.53	26.06	18.11
TPD tree	3.71	6.25	8.18	11.40	13.99	27.14	23.48

5.3.2 Pathogenicity test on indicator plants

Total RNA isolated from healthy and TPD affected trees were inoculated into indicator host Pusa Ruby variety of Tomato. After 8 weeks the plants inoculated with RNA from TPD trees showed epinasty (Fig. 5.1 & 5.2).

Fig. 5.1 Epinasty symptoms on tomato plants inoculated with RNA from TPD trees

a. **b.**

(a. Inoculated with RNA from TPD tree, b. Inoculated with RNA from healthy tree)

Fig. 5.2 Pathogenicity test on indicator plants

Reisolation of LMW RNA from the inoculated plants

Total RNA from inoculated tomato with epinasty symptoms showed LMW RNA band in R-PAGE (Fig. 5.3) indicating that the inoculated LMW RNA can be reisolated.

1 2 3 4 5 6 7 8 9 10 11

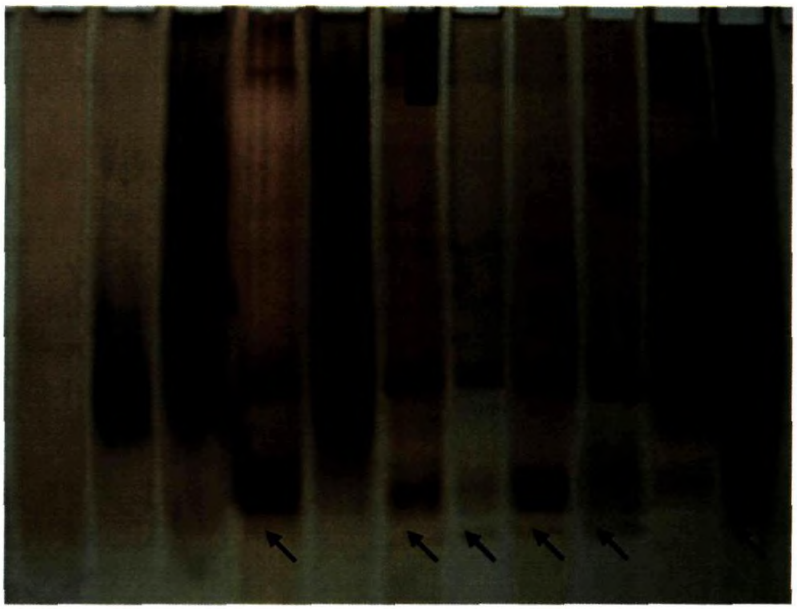

Fig. 5.3 R-PAGE of total RNA from inoculated tomato as well as rubber samples

(Lane 1 to 3 – Tomato inoculated with RNA from healthy trees, Lane 4 & 11 – TPD tree, Lane 5 & 10– Healthy tree, Lane 6 to 9 - Tomato inoculated with RNA from TPD trees)

RT-PCR from total RNA isolated from inoculated plants with viroid specific primers yielded product in the range of viroid. Amplification of a 360bp product was

observed in samples from tomato seedlings inoculated with RNA from TPD trees (Fig. 5.4).

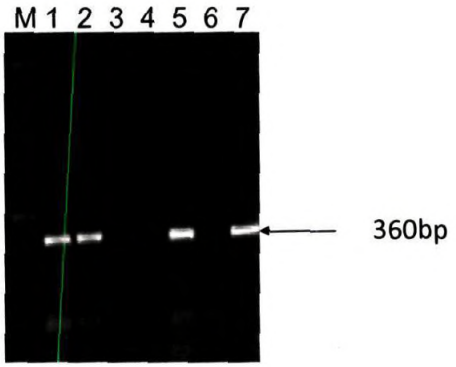

Fig. 5.4 Agarose gel electrophoresis of PCR products (obtained from tomato inoculated with total RNA from TPD trees) using different primers (M- marker (100bp), lane 1 & 5 Abutting primer, 2 & 7 - PSTV primer, lane 3 & 6 – Rao primer, lane 4- blank)

The direct sequencing of the PCR product was performed and the sequence showed homology to Potato Spindle Tuber Viroid on BLAST analysis (Fig.5.5).

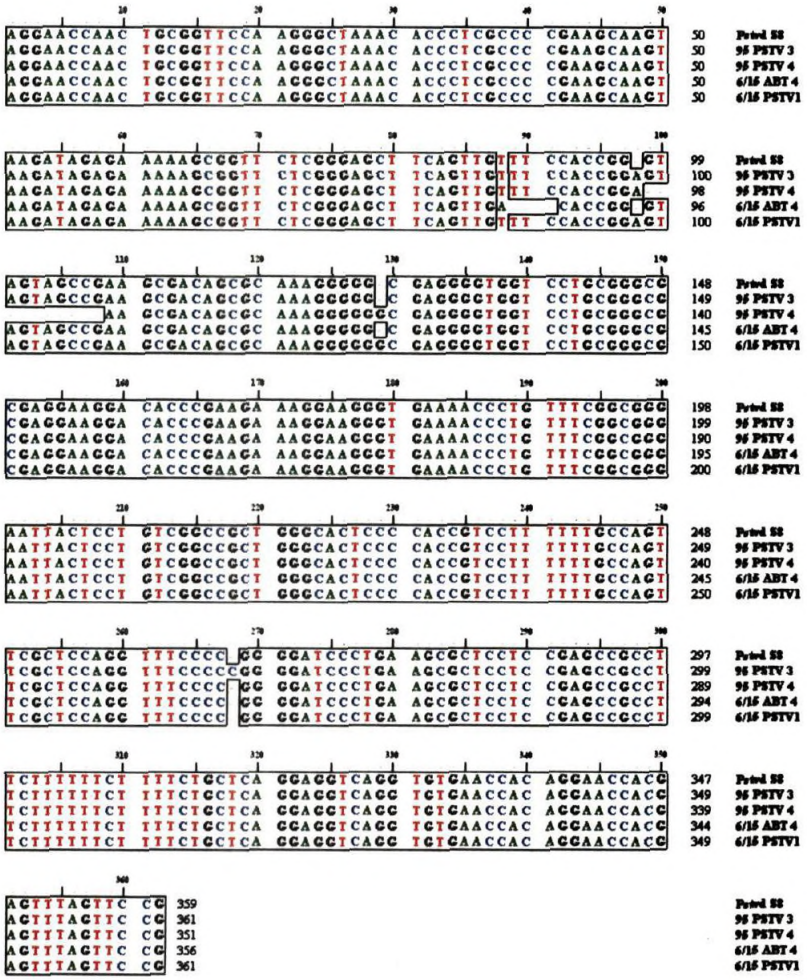

Fig. 5.5 Sequence alignment report

Cloning and sequencing of the RT-PCR product was also performed and the sequence showed homology to Potato Spindle Tuber Viroid on BLAST analysis.

5.4 DISCUSSION

The R-PAGE test of bud grafted plants under transmission studies showed that all the plants tested from the group in which both stock and scion were viroid +ve, maintained the viroid bands. But, in plants where stock is viroid –ve and scion is viroid +ve, only 70% plants showed viroid band. The reason for non-detection of viroids in the remaining 30% might be because the budwood and shoot cuttings from symptomatic trees sometimes do not contain viroid as reported by Steve (2008) in Avocado Sunblotch Viroid (ASBVd).

Plants which had viroid +ve stock and viroid –ve scion showed viroid band in 50% of plants. This shows that viroid was transmitted from viroid +ve stock to viroid –ve scion. 25% of plants in which both stock and scion were viroid –ve showed viroid

bands. This could be due to the buildup of viroid titer to a detectable level in the later stage which was below the detectable level in either stock or scion earlier. It was reported that the amount (titre) of viroid particles present in avocado trees varied a great deal. Viroids levels can vary by 1000 times between branches on the same tree and by 10000 times between trees (Steve, 2008). Rootstocks are known to cause disease transmission (Szychowski *et al.*, 1988). Since rubber is mostly vegetatively propagated by grafting buds of high yielding clones on random rubber seedlings used as rootstock, transmission of TPD through root stock is possible.

Test tapping showed TPD in both group of plants, namely plants budded with scion taken from TPD affected trees as well as healthy trees. This shows that root stock also plays a role in the development of TPD. Viroid present in the stock seedlings may have induced the healthy scion to show TPD symptoms. Szychowski *et al.*, (1988) demonstrated that infected rootstock was one of the major factors in the widespread distribution of viroids in Grapevines.

Epinasty symptom development on tomato plants inoculated with total RNA isolated from TPD affected trees and absence of any symptoms on plants inoculated with total RNA from healthy trees showed that the viroid present in rubber can be transmitted to an indicator host. Total RNA from inoculated tomato with epinasty symptoms showed LMW RNA band in R-PAGE showing that the inoculated LMW RNA can be reisolated. The sequence homology of the RT-PCR product obtained from the inoculated tomato with that of Potato Spindle Tuber Viroid proved its viroid relationship. These experiments confirmed that the LMW RNA could be transmitted to indicator host plants to express the characteristic epinasty symptoms and can be re-isolated thus indirectly satisfying the Koch's postulates.

The evidence for field or natural transmission of TPD from one tree to the other was observed in the present field studies. The observations that the number of TPD trees in clusters of two or more showing a remarkable increase compared to the single TPD trees, from the first year of tapping to the last indicates that there is a chance of spread of TPD from one tree to the neighbouring tree (Elsewhere in this thesis). The present study also shows that, at a limited extent TPD spreads from one tree to the tree tapped immediately next (Elsewhere in this thesis).

The present study clearly shows the transmission of rubber viroid through seedlings and bud grafts. Viroids can spread by graft transmission. Seed transmission has been demonstrated for many, but not all, viroids, and pollen-borne transmission is

also known to occur in tomato (Kryczynski, *et al.*, 1988). PSTVd is transmitted in true potato seed (0 - 100% of seed may be infected) (Fernow *et al.*, 1970; Singh, 1970) via infected pollen or ovules (Grasmick and Slack, 1986; Singh *et al.*, 1992).

For potatoes, PSTVd may be introduced into a field by planting infected seed tubers or true potato seed. For Avocado Sun blotch viroid (ASBVd) vertical transmission has been demonstrated (Whitesell, 1952). Such transmission is also observed for PSTVd in tomato and pepino (Hollings and Stone, 1973; Singh, 1970) but not *Tomato planta macho viroid* (TPMVd) in tomato (Galindo, 1987). PSTVd can also be transmitted by insect vectors (Salazar *et al.*, 1995; Querci *et al.*, 1997). But, in the present study we have not made any attempt to study the insect vector transmission of rubber viroid.

The evidence for field or natural transmission of TPD from one tree to the other was observed in the field studies. But, viroids are highly transmissible by mechanical inoculation and on contact with contaminated tools. As rubber trees in plantations are tapped using the same tapping knife, the expected incidence of TPD is much larger than what is observed in the field. Moreover, the roots of rubber trees are reported to get anastomosed or interlinked when canopy of the trees closes. It is evident from this study that many trees which are LMW RNA or viroid +ve do not show the TPD symptoms and may later turn to TPD. Severe pruning of symptomless carriers and other severe causes of tree stress, are suspected of causing Avocado Sun blotch Viroid to become active in the new growth, inducing previously symptomless trees to exhibit symptoms (Steve, 2008). Stannard *et al.*, (1975) reported that mechanical transmission varied with the citrus scion. It was easier to transmit CEVd between lemons, than lemon to orange or orange to orange showing that transmission of viroid is a complex phenomenon which involves many factors.

5.5 CONCLUSION

R-PAGE analysis of bud grafted plants clearly established that the RNA is transmissible by bud grafting. Observation of TPD in plants budded with scion taken from healthy trees shows that stock also plays a role in the development of TPD. Epinasty symptom development on tomato plants inoculated with total RNA isolated from TPD affected trees and absence of any symptoms on plants inoculated with total RNA from healthy trees showed that the viroid present in rubber can be transmitted to an indicator host. Total RNA from inoculated tomato with epinasty symptoms showed LMW RNA band in R-PAGE showing that the inoculated LMW RNA can be reisolated. The sequence homology of the RT-PCR product obtained from the inoculated tomato with that of Potato Spindle Tuber Viroid proved its viroid relationship. The observation of lesser incidence of TPD than the expected (since viroid is transmitted easily by mechanical means) could be due to occurrence of symptomless carriers of the viroid.

The present study is the first record of the infectious nature of the LMW RNA isolated from TPD affected rubber trees to a herbaceous host (tomato), thus establishing the biotic nature of the causal agent of TPD syndrome affecting rubber plantations. Reisolation of the RNA from symptomatic tomato leaves and confirmation of the viroid specific band on return gel partially proves the Koch's postulates to establish the biotic nature of the causal factor.

Chapter 6

GENERAL CONCLUSION

SUMMARY AND CONCLUSION

In this study it was observed that TPD is initiated mainly by two ways either with extreme fluidity of the latex with abnormally low DRC in which the latex flows only from the inner most layer of latex vessels or with very viscous latex with abnormally high DRC and partial dryness of varying degrees with vessels devoid of latex. When dryness was observed in the BO 1 panel, the cracks were seen extended to the opposite panel. Cracks and necrosis started from the tapping panel or from the region near the bud union, which later extended towards the tapping cut. The present study revealed that cracking and bulging of bark increased with the age of trees and with progression in period of tapping. Nearly 40% reduction in total latex volume was observed on trees in the category of less than 50% TPD and nearly 90% reduction was observed when the trees were in the category of more than 75% TPD in panel BO 1. Contrary to total latex volume, an increase in DRC was noted as the TPD intensity increased. Dryness of root system was observed along with necrosis, cracking and bulging (as observed on the trunk) in TPD affected trees. Although the roots originated from a root stock those on the side corresponding to the dry portion of scion showed dryness.

It was observed that the number TPD affected trees increased as the years of tapping progressed at all the locations both in small holdings and in large estates. The percentage of trees in the category of very high TPD intensity (>75%) showed a clear trend of increase from the first year to the last year of tapping at all the locations. The number of TPD trees in the other categories (low, medium and high) did not show such a remarkable trend of increase from BO 1 to BI 1 panels. The scale of increase in TPD was more in older trees than in trees at the initial stages of tapping. Percentage of trees without TPD symptoms was high when the panel was changed but it again decreased a year after such panel change. Reversion of TPD symptoms was observed only at a young age. The evidence for natural transmission of TPD from one tree to the other was observed in the present field studies as number of trees in clusters showed a significant increase with progress of tapping. When the systems for management of TPD were studied, it was found that only 23.8% of the small holders give rest when TPD was observed. When the TPD affected trees were tapped in the upward system of tapping, more than 50 per cent of the trees showed dryness after four months of tapping.

Various factors which were assumed to be the cause of TPD could not conclusively prove its etiology. Occurrence of TPD in adjacent trees in the same line, consistent increase in its incidence with age, similarity in symptoms and anatomical abnormalities to some known diseases, inability to revive the affected trees by tapping rest and the presence of dry rubber trees from the start of exploitation prompted investigation on biotic etiology for TPD. Earlier investigations did not reveal association of fungi, bacteria, virus, MLO or protozoa as biotic agents. Hence, possibility of association of viroid with TPD syndrome was investigated. A protocol was standardized to detect the presence of LMW RNA in leaf, bark and root tissues of rubber trees. LMW RNA was detected from different samples drawn from varying ages of trees from different locations. In most cases the trees in which LMW RNA was detected showed the TPD syndrome. Therefore, it was hypothesized that the presence of LMW RNA has an association with TPD.

Presence of bands in apparently healthy samples indicated that the biotic agent also occurs in symptomless carriers. Majority of healthy trees that earlier found positive for LMW RNA eventually became TPD affected. Hence, this technique can be used as a diagnostic tool for detection of TPD before the symptoms are visible. The amplified LMW RNA showed sequence homology to Potato Spindle Tuber Viroid (PSTVd).

The R-PAGE test of bud grafted plants under transmission studies showed that all the plants tested from the group in which both stock and scion were viroid +ve, maintained the viroid bands. Viroid was observed to be transmitted from viroid +ve stock to viroid –ve scion. Test tapping showed TPD in both group of plants, namely plants budded with scion taken from TPD affected trees as well as those from apparently healthy trees. This shows that root stock also plays a role in the development of TPD. Development of epinasty symptom on tomato plants inoculated with total RNA isolated from TPD affected trees showed that the viroid present in rubber can be transmitted to an indicator host indirectly satisfying the Koch's postulates. It was reisolated and the LMW RNA band was observed in R-PAGE analysis of reisolated sample. The sequence homology of the RT-PCR product obtained from the inoculated tomato with that of Potato Spindle Tuber Viroid proved its viroid nature.

Although the primary natural host of PSTVd is potato, it is reported that this viroid also infects a variety of other crops. It has a wide host range, infecting 94 species in 31 families (Jeffries, 1998). In Peru, PSTVd has been detected in Avocado (*Persea*

americana), which is a perennial tree species where infections are often latent unless the tree is co-infected with Avocado Sun blotch Viroid (Querci *et al.*, 1995). The spread of the viroid into new cultivars of woody perennials can occur through vegetative propagation as suggested by Bar-Joseph (2003).

The most appropriate method to establish viroid etiology for TPD is artificial transmission of the LMW RNA isolated from rubber to a viroid free healthy rubber plant to express TPD symptoms. However, the varying periods of latency of the viroid (over several years) prior to symptom expression, as observed in this study, poses problems. If viroid etiology is confirmed, bud wood and root stock certification for pathogen-free propagation materials can be adopted for developing TPD free plantations.

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List of publications from this work and Awards

1. Abraham T., Mathew J., Ramachandran P., Philip S., Jacob C. K. and Nair R. B. (2009). Tapping panel dryness in *Hevea brasiliensis*- Symptoms suggesting a possible pathogen involved? *Indian Phytopathology* 62 (3) : 398

2. Mathew J., Abraham T., Ramachandran P. and Jacob C. K. (2009). Tapping Panel Dryness: An Enigmatic Disorder in *Hevea brasiliensis*. *Indian Phytopathology* 62 (3) : 389

Conference papers

1. Abraham, T., Mathew, J., Ramachandran, P., Philip, S., Jacob, C. K. and Nair, R.B. (2008) Tapping panel dryness in *Hevea brasiliensis*- Symptoms suggesting a possible pathogen involved. *IPS-MEZ Annual Meeting & National Symposium on Advances in Microbial Diversity and Disease Management for Sustainable crop Production.* College of Forestry and Hill Agriculture, G B Pant University of Agriculture and Technology, Hill Campus, Ranichauri- 249199, Tehri Garhwal, Uttarakhand.13-15 Oct., 2008.

2. Mathew, J., Abraham, T., Ramachandran P. and Malathi V.G. (2012). Characterisation of the low molecular weight (LMW) RNA associated with tapping panel dryness of *Hevea brasiliensis*. *International Workshop on Hevea Diseases In Africa, 5th – 7th June, 2012, Benin City, Edo State, Nigeria*

Awards/recognitions

1. **Best Poster Award** – Thomson Abraham, Jacob Mathew, Padma Ramachandran, Shaji Philip, C. Kuruvilla Jacob and Ramesh B. Nair (2008) Tapping panel dryness in *Hevea brasiliensis*- symptoms suggesting a possible pathogen involved. *IPS-MEZ Annual Meeting & National Symposium on Advances in Microbial Diversity and Disease Management for Sustainable crop Production.* College of Forestry and Hill Agriculture, G B Pant University of Agriculture and Technology, Hill Campus, Ranichauri- 249199, Tehri Garhwal, Uttarakhand.13-15 Oct., 2008.