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). Chen *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; Pacumbaba *et al*., 1994). 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; Verhoeven *et al*., 2004) 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 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

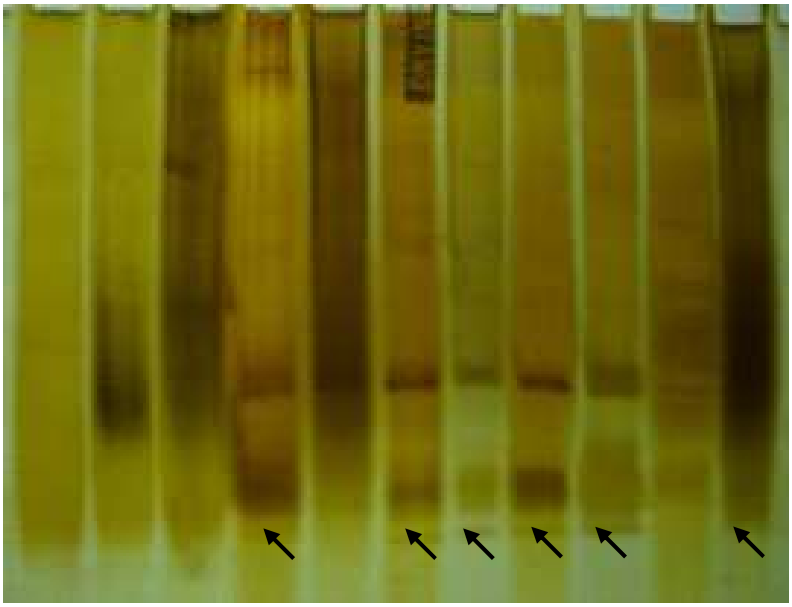

Fig. 5.3 R-PAGE of total RNA from inoculated tomato as well as rubber samples

(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).

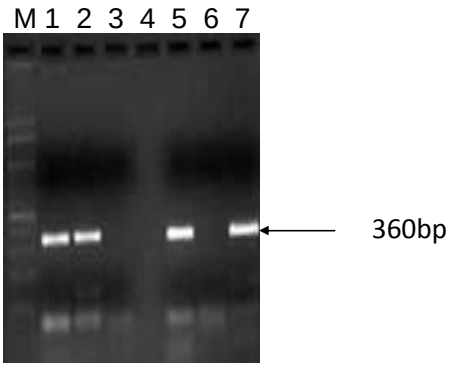

Fig. 5.4 Agarose gel electrophoresis of PCR products (obtained from tomato inoculated with total RNA from TPD trees) using different primers (M- marker (100bp), lane 1 & 5 Abutting primer, 2 & 7 - PSTV primer, lane 3 & 6 – Rao primer, lane 4- blank)

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).

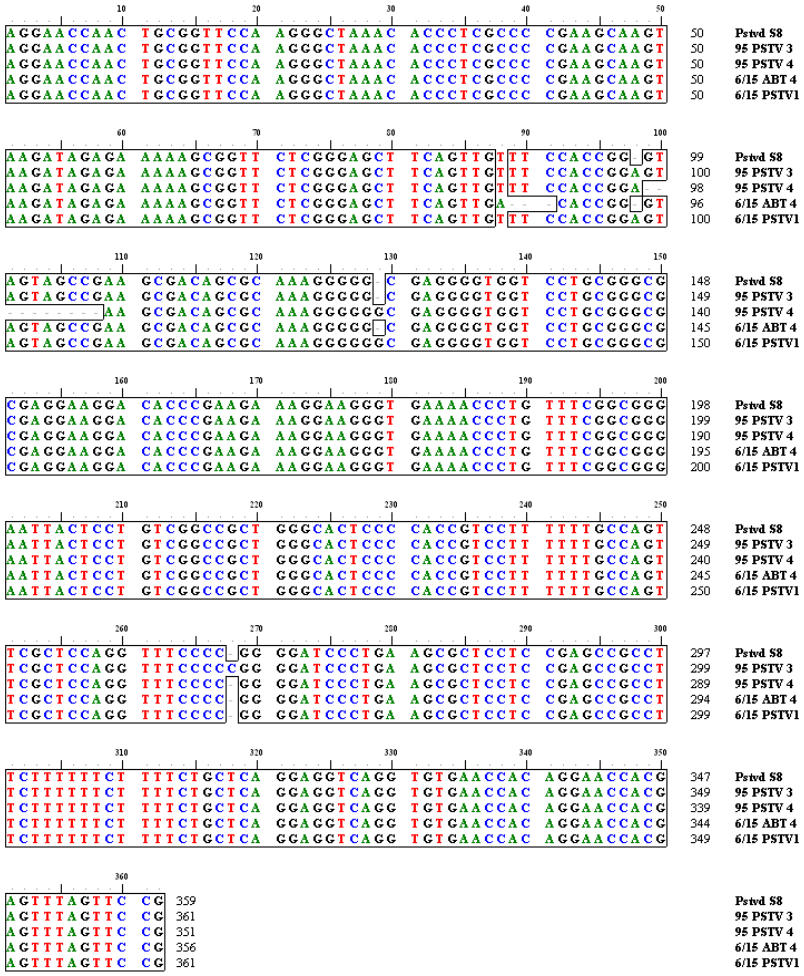

Fig. 5.5 Sequence alignment report

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.