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Determination of biotic aetiology of tapping panel dryness (TPD) syndrome of rubber tree (*Hevea brasiliensis*) by return-polyacrylamide gel electrophoresis (R-PAGE) technique

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Tapping panel dryness (TPD) syndrome affecting rubber tree (*Hevea brasiliensis*) is known to reduce natural latex production. Its aetiology remains ambiguous despite long years of research. A low molecular weight RNA similar to viroid RNA was isolated from TPD-affected samples of rubber trees. In the present study, a modified return-polyacrylamide gel electrophoresis procedure was standardised. The viroid-like low molecular weight (LMW) RNA was found associated with leaf, bark and root tissues and rubber seedlings. The technique was employed to detect LMW RNA in different clones of rubber planted in different locations and in bud-grafted plants. The LMW RNA isolated from TPD-affected trees was found infectious on seedlings of tomato cv Pusa Ruby. The LMW RNA was reisolated from symptomatic tomato leaves but not from control plants. This is for the first time that a biotic agent, a viroid RNA, is found consistently associated with the syndrome. The technology developed can be useful to demonstrate the onset of TPD in untapped trees in the absence of other methods such as nucleic acid hybridisation.

Keywords: rubber tree; *Hevea brasiliensis*; tapping panel dryness; R-PAGE; low molecular weight (LMW) RNA

Introduction

Tapping panel dryness (TPD) syndrome affecting rubber trees (*Hevea brasiliensis*) is widespread in all rubber growing areas and is a matter of serious concern to rubber industry. It was first reported in Malaysia affecting mostly high-yielding clones of rubber (Sharples 1936). Since then, it became important in other South Asian countries like China, India and Sri Lanka. The disorder derives its name from the effect it causes on the rubber tree, namely reduction in latex yield leading to partial and eventually total dryness of the tapping panel (Figure 1). As rubber is a perennial tree crop with a production span of nearly 25 years, the loss due to TPD is enormous. Once the trees are affected, TPD persists in the rubber plantation, without being influenced by changes in climate or geographical regions. Since the trees are affected at random, removal of affected trees and replanting is cumbersome and not feasible practically. Rubber is a vegetatively propagated crop. One of the causes that have impeded progress in management

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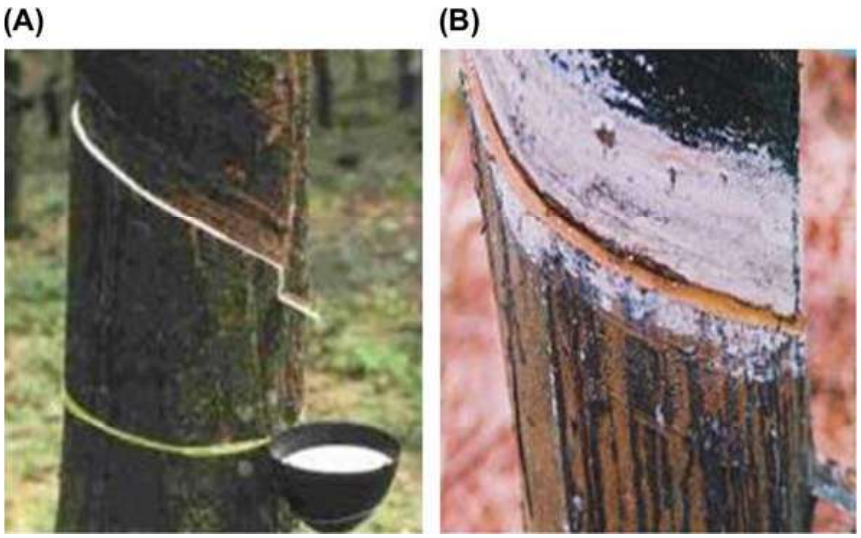

Figure 1. Tapping panel disease syndrome affected rubber plants showing (A) Healthy – free latex flow and (B) TDP affected – Total drying of latex.

of TPD is the fact that the syndrome becomes evident, about six to seven years after transplanting, only when the trees reach maturity for tapping.

In India, TPD is known to affect rubber plantations since the early years of rubber cultivation but became important as all the high-yielding clones, including Rubber Research Institute of India (RRII) 105 (developed indigenously by the RRII, Kottayam, Kerala) and cultivated in more than 85% of the area, succumbed to the disease. In Indian rubber plantations, the average incidence of TPD is found to be about 15% which results in a loss of approximately 18–20 billion rupees annually. Considering the importance of natural rubber, it is essential to check enormous losses to the rubber industry. In this regard, the first step is the determination of the aetiology of this syndrome.

Several decades of research from different parts of the world were focused to investigate the cause of the syndrome but no clear picture has emerged so far. Many evidences were put forward to show that TPD (earlier referred to as "brown bast of rubber") may be of abiotic nature particularly due to physiological changes arising due to nutritional deficiency, reduced turgor pressure, changes in minerals ratios in affected trees, in redox potential, ethylene stimulation and changes in protein pattern (Chua 1967; Bealing and Chua 1972; Pushpadas et al. 1975; Sethuraj et al. 1977; Chrestin 1984; de Fay 1988; Dian et al. 1955) or due to morphological, anatomical and histological changes arising due to physiological disorder (de Fay and Jacob JL 1989; Gomez et al. 1990; Gomez and Gandhimati 1990). Another disorder viz. tapping panel necrosis (TPN), very similar to TPD in terms of external symptom of dryness of tapping cut one to three years after the onset of tapping, was reported from Ivory Coast (Nandris et al. 1991). TPN was described as an irreversible disease arising from the collar region upward to tapping panel affecting the internal phloem and thus stopping latex production by latex vessels. TPN has been shown not to be affected by any biotic agent (Pellegrin et al. 2007).

There exist scanty reports in the literature about the involvement of biotic agent. From early days, the TPD syndrome has been considered to be due to "physiologic fatigue". In Sri Lanka, *Bacillus subtilis* was shown to be associated with the disorder, but its causal role was not proved. Other reports claiming biotic agents were that of a virus with bark cracking symptoms from the UK (Peries and Brohier 1965), rickettsia-like

organism (Zheng et al. 1988) and a phytoplasma with TPD-affected rubber trees from China (Chen et al. 1999). However, the most significant among these is the evidence for association of a viroid-like RNA with TPD-affected rubber trees from this laboratory earlier (Ramachandran et al. 2000). A low molecular weight LMW RNA was observed in all rubber clones irrespective of the age, tapping stage, location, season and regions. The LMW RNA was found to be insensitive to DNase, heat and phenol treatment but sensitive to RNase, properties similar to viroid pathogens. Some of the apparently healthy trees also showed LMW RNA band. More important was the observations that many of the trees where LMW RNA bands were detected in return-polyacrylamide gel electrophoresis (R-PAGE) gels before the trees were tapped showed TPD after tapping (Ramachandran et al. 2006). Viroids are LMW RNA, are the most recent plant pathogens discovered in the last century and are known to incite serious maladies in important fruit and plantation crops (Walia et al. 2012). R-PAGE has been specifically used for viroid detection in plants before the emergence of nucleic acid hybridisation (Singh and Boucher 1987).

The present study reports the details of standardising the technique of R-PAGE for applying to rubber tissue with the specific aim of detecting viroid RNA in the absence of an alternate tool for TPD detection. Infectivity of the isolated LMW RNA on tomato seedlings has been shown for the first time.

Materials and methods

Sample collection

In the years 2008–2010, plant samples used in the study were collected from rubber farms at RRII, Kottayam, Kerala, unless otherwise mentioned. Samples of bark (from tapping region) as well as tender leaves from healthy and TPD-affected trees were collected and stored at −80 °C. Before use, the samples were washed, blotted dry and crushed to a fine powder using liquid nitrogen in previously autoclaved pestle and mortar. Samples of root from healthy and TPD-affected trees were also similarly stored and powdered before use. Leaf samples (only 50 per clone) were collected as mentioned above from RRII farms and rubber plantation at the different districts Malankara, Vaniampara, CES Chethackal, Pala, Punnalur in the state of Kerala and Nagercoil in the state of Tamilnadu and tested for the presence of LMW RNA by R-PAGE.

Extraction of total nucleic acid

Finely powdered samples were homogenised in two extraction buffers, viz. EB1 and EB2 with following compositions at pH 8.5 and a sample:buffer ratio of w/v 1:3. EB1 contained 0.1 M Tris, 0.01 M EDTA, 0.1 M NaCl, 1% SDS with 5 mM DTT and DIECA, while EB2 had 0.1 M Tris, 0.1 M NaCl, 0.075 M HCl, 10 mM EDTA, 2-mercaptoethanol. The homogenate was then treated with chloroform: isoamyl alcohol mixture (24:1) 1:5 v/v. This mixture was mixed by gentle shaking before adding Tris-saturated phenol (containing 0.1 g of 8-hydroxyquinoline per 100 ml) 1:2 v/v and vortexed (at low) for 30 s and left at room temperature for 30 min, gently mixing in between. It was then centrifuged at 5000 g for 20 min at 4 °C in Sorvall RC-5C centrifuge. In the case of EB1, the aqueous layer was collected and subjected to re-extraction by repeating steps from adding of extraction buffer. Total nucleic acid was precipitated from the aqueous layer by addition of half volume of 0 4 M sodium acetate pH 5.2 and

2.5 vol ethanol (cold) and incubated at −20 °C overnight. The nucleic acid was recovered by centrifugation at 5000 g, 15 min, 4 °C. The pellet obtained was washed with 70% ethanol, vacuum dried, dissolved in 300 µL of sterilised non-ionic double-distilled water and stored in 4 °C. In the case of EB2, the nucleic acid in the aqueous layer was precipitated using half volume of ammonium acetate (7.5 M, pH 7.4) and two volumes of ethanol at −20 °C overnight. Total nucleic acid was recovered by centrifugation as above. The precipitate was dissolved in 350 µL of sterilised non-ionic double-distilled water and transferred to an Eppendorf tube. This was again precipitated using half volume of ammonium acetate and two volumes of ethanol at −20 °C for 4 h and total nucleic acid was recovered by centrifugation. To the precipitate, after 70% ethanol wash, 1 ml of ice cold aqueous 2 M LiCl (filter sterilised) was added. The tube was put in a shaker at 100 rpm, 4 °C for 1 h. and then centrifuged at 15,000 rpm, 4 °C for 30 min. The supernatant was recovered and stored at 4 °C. The pellet was re-extracted as above with 2 M LiCl and a second supernatant recovered. The two fractions were pooled and precipitated as previously at −70 °C for 1hr to recover total RNA. The final precipitate was washed in 70% ethanol and vacuum dried and dissolved in 50 µL sterile non-ionic double-distilled water to test by R-PAGE.

R-PAGE

The total nucleic acid samples were resolved using the technique of R-PAGE as reported earlier (Singh and Boucher 1987). Electrophoresis was carried out in a slab gel (16 × 14 × 0.15 cm) 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, 4 µL of dye containing 0.25% each of xylene cyanol and bromophenol blue (prepared in 60% sucrose) was added and applied to the slots. The first electrophoretic run was carried out under native conditions using high salt buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH: 8.3) at 20 °C and 46 mA constant current. The run was terminated after 2–2.15 h 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 5 min without disturbing. The gel was rerun at 70 °C, 46 mA current with reverse polarity for 1.5 h. The bands in the gel were resolved by silver staining following the protocol described by Mishra et al. (1991).

Infectivity test

Tomato seedlings (cv Pusa Ruby) were raised in 6″ earthen pots with standard potting mixture. The pots were maintained in an insect proof glasshouse and inoculated with the total nucleic acid extract isolated from both healthy and TPD-affected rubber trees at the first leaf stage (5 µL/leaf/plant) using carborundum as abrasive. Tomato seedlings inoculated similarly with buffer served as a control. After inoculation, the seedlings were sprayed with a fine jet of water and plants were observed regularly. Eight weeks later, the symptomatic leaves of TPD sample-inoculated plants and symptomless leaves from buffer-inoculated plants were analysed by R-PAGE as above.

Results

Standardisation of extraction procedure for total nucleic acid

Between the two different procedures for extraction of total nucleic acid, the one using EB1 buffer containing 0.1 M Tris, 0.01 M EDTA, 0.1 M NaCl, 1% SDS, 5 mM DTT

and DIECA, followed by re-extraction of the first precipitate for recovering the total nucleic acid from leaf and bark tissues of rubber, gave better resolution of bands when run by R-PAGE. Further analysis was done using this extraction procedure.

Detection of LMW RNA in different plant parts

Total RNA from leaf, bark and root samples of TPD-affected trees were run on R-PAGE. For the purpose of detecting LMW RNA in different plant parts, leaf, bark and root of the same tree, were collected from 30 different TPD-affected and apparently healthy trees of the clone RRII 105 from RRII farms. Presence of LMW RNA similar in electrophoretic mobility to viroid RNA was observed in leaf, bark and root samples, indicating that LMW RNA is systemically present in the tree, thus establishing its biotic nature and association with TPD syndrome. More number of samples collected from TPD-affected trees show the LMW RNA specific bands as compared to the apparently healthy ones (Table 1, Figure 2(a)).

Detection of LMW RNA in different rubber clones from different locations

Results of R-PAGE analysis (Table 2) of leaf samples taken from prominent clones under different stages of tapping from different locations showed that the percentage of disease was highest in the clone RRII 105 in RRII farms followed by CES Chethackal, Malankara, Vaniampara and Nagercoil. It was the least in clones Rubber Resaerch Institute of Malaysia (RRIM) 600 (Nagercoil) and PB 217 (Malankara) (70%) followed by RRIM 605 (Pala) (75%). The Clones GT 1 and PB 260 (Malankara) showed disease percentage similar to the clone PB 28/59 (Nagercoil) and the clone RRIM 605 (Punnalur) but the clone PB 235 (Malankara) showed high disease incidence (88%) near similar to RRII 105 from RRII farms (Figure 2(b)). Based on these results, it is inferred that TPD incidence is irrespective of rubber clone in cultivation or its geographical location. However, it is evident that the high-yielding clone RRII 105 is the most susceptible even under different locations.

Detection of LMW RNA in different trees of the clone RRII 105 in RRII farm

Since the highest percentage of TPD incidence was observed in the clone RRII 105 and that at RRII experimental farms, a high percentage of this clone is in use, a set of both TPD expressing as well as apparently healthy tress were randomly selected and analysed by R-PAGE using their leaf and bark tissues. It was observed that all the six samples from TPD-affected trees showed the presence of LMW RNA (Table 3). Of the five apparently healthy plants tested, only one leaf sample from plant No. 69 showed the presence of LMW RNA band. Recently, when incision was made on this tree, it showed the partial drying of latex. These findings clearly reveal the proneness of the

Table 1. Results of R-PAGE analysis of leaf, bark and root samples from RRII farm.

Plant part	TPD-affected trees	Apparently healthy
Root	25/30*	3/30
Bark	22/30	5/30
Leaf	27/30	3/30

*no. showing band in R-PAGE/no. tested. (Root, bark and leaf of same tree)

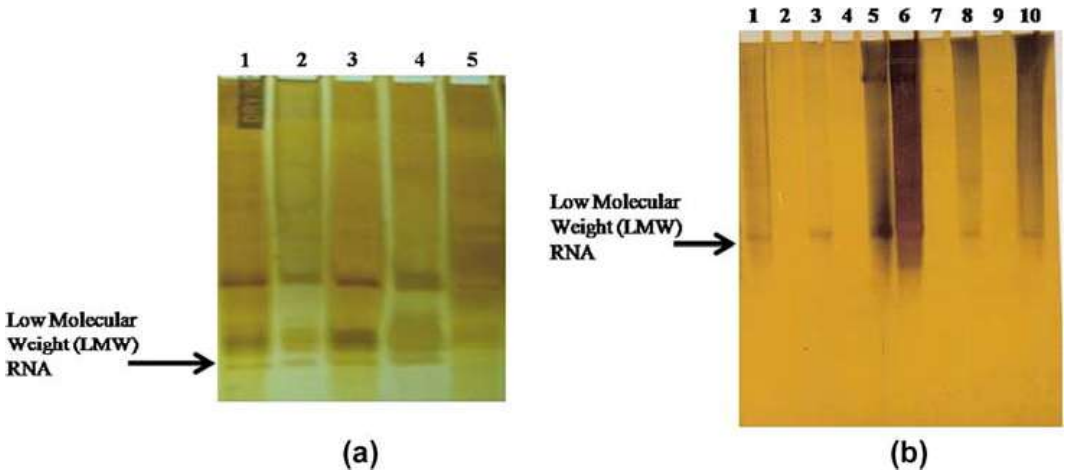

Figure 2. (a) R-PAGE gel showing LMW RNA bands in TPD-affected rubber. Lane 1 – RRII 6/15leaf (control), 2– leaf 4/4, 3 – bark 4/4, 4 – root 4/4 and 5 – healthy. (b) R-PAGE gel showing LMW RNA bands in TPD-affected rubber. Lane 1 – RRII 6/15, 3 – RRII 4/5, 5,6 – Chetthakal, 8 – Vaniampara and 10 – Malankara (PB235).

Table 2. Results of R-PAGE analysis of rubber trees of different clones growing in different locations.

Rubber clone	Location	R-PAGE	%TPD
RRII 105	Vaniyampara	42/50*	84
	Malankara	43/50	86
	Nagercoil	25/30	83.3
	CES Chethackal	35/40	87.5
	RRII Farm	45/50	90
GT 1	Malankara	12/15	80
RRIM 600	Nagercoil	21/30	70
PB 28/59	Nagercoil	16/20	80
RRIM 605	Punalur	8/10	80
	Pala	6/8	75
PB 260	Malankara	20/25	80
PB 217	Malankara	7/10	70
PB 235	Malankara	44/50	88

*no. showing band in R-PAGE/no. tested.

Table 3. Detection of LMW RNA in randomly selected trees of clone RRII 105 from RRII farms.

Tree no.	TPD Affected LB	Tree no.	Apparently HealthyLB
4/4	+…+	5	−…−
6/15	+…+	69	+…−
214	+…+	24	−…−
6	+…+	122	−…−
95	+…+	103	−…−
123	+…+		

"+" presence of LMW RNA in gel, "−"its absence; L- leaf B- Bark.

clone RRII 105 to TPD and the consistent association of LMW RNA with TPD syndrome. The technique of R-PAGE can detect the presence of LMW RNA in apparently healthy trees which show the symptom when tapped.

Detection of LMW RNA in rubber seedlings

Rubber is vegetatively propagated by grafting high-yielding clones on rubber seedlings used as rootstock. Rootstocks are important in disease transmission, and hence analysing the status of rubber seedling used in propagation of rubber was essential. Rubber seedlings were germinated each from TPD affected, apparently healthy and seeds collected from randomly growing trees maintained in the glass house at RRII, Kottayam. Total RNA was extracted from the leaves of the young seedlings for R-PAGE analysis. Among the TPD-affected trees, (14 of 20), randomly selected trees (12 of 20), and apparently healthy trees (5 of 20) when tested, showed the presence of the LMW RNA band in return gel (Table 4). These observations indicate the possibility of seed transmission of the biotic agent. These plants are under observation for grafting and subsequent tapping on maturity.

Presence of LMW RNA on bud-grafted plants

Bud grafting is a most common method of perpetuation in rubber. To study the transmission of LMW RNA by bud grafting, both stock and scion to be used were previously checked by R-PAGE analysis for presence (+) or absence (−) of LMW RNA. Four combinations of bud grafts were carried out, namely: (i) +ve stock and +ve scion, (ii) +ve stock and −ve scion (iii) −ve stock and + ve scion and (iv) −ve stock and −ve scion, with 50 plants in each group. The results of transmission through bud grafts showed that all the plants tested from the group (i) 100% showed bands in R-PAGE, in group (ii) 70% showed viroid band, in group (iii) 50% of plants while in group (iv) 30% of the plants showed viroid bands. All the grafts were not successful. Depending on the number of good samples available after grafting, different number of samples were analysed in each group by R-PAGE. The results in (Table 5) showed that group (i) showed bands in all the samples tested (100%); group (ii) showed bands in only 50% samples; group (iii) showed bands in 70% samples while group (iv), where both scion and stock grafted were negative, also showed bands in nearly 30% samples. At this stage, it is difficult to explain the result of group (iv); however, the plants are under observation waiting to reach tapping.

Infectivity test of LMW RNA on tomato seedlings

Total RNA isolated from healthy and TPD-affected rubber trees were rub-inoculated on seedlings of tomato cv Pusa Ruby an indicator host for most of the viroids. LMW RNA extracts from leaf and bark of two TPD-affected trees from RRII farm 4/4 and 2TPD were used. Eight weeks post-inoculation leaves in LMW RNA-inoculated plants

Table 4. Detection of LMW RNA in rubber seedlings.

Source of leaf sample	R-PAGE analysis
Seedling selected randomly	12/20*
Seedling from TPD-affected tress	14/20
Seedling from apparently healthy trees	5/20

*no. showing band in R-PAGE/no. tested.

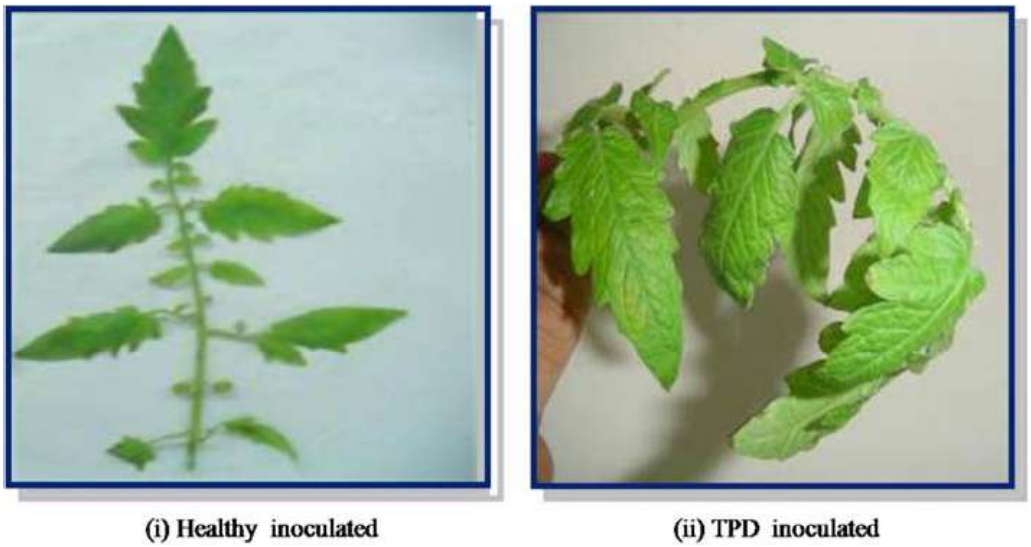

Figure 3(a). Infectivity test of LMW RNA on tomato seedlings (i) Buffer-inoculated tomato leaf and (ii) LMW RNA-inoculated tomato leaf showing epinasty.

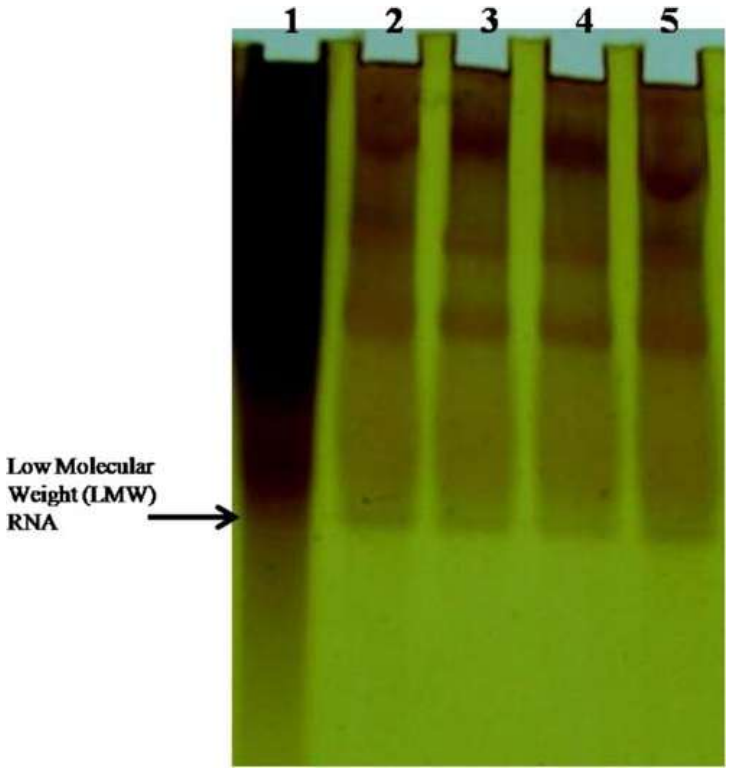

Figure 3(b). R-PAGE gel showing LMW RNA bands in symptomatic tomato leaves inoculated with LMW RNA from TPD-affected rubber plants. Lane 1 – 6/15 (rubber control), 2 – 4/4 (leaf), 3 – 4/4 (bark), 4 – 2 TPD (leaf) and 5 – 2 TPD (bark).

showed symptoms of epinasty, i.e. inward curling of leaf (Figure 3(a)). To confirm the presence of LMW RNA, total nucleic acid was extracted from symptomatic leaves as well as leaves of buffer control. R-PAGE analysis showed the presence of LMW RNA band only in symptomatic leaves confirming the transmissible nature of the LMW RNA (Figure 3(b)). The LMW RNA from symptomatic tomato leaves was reinoculated on

Table 5 Result of R-PAGE analysis of bud-grafted plants.

Group	Stock	Scion	R-PAGE result (%)	
(i)	+*	+	11/11**	(100)
(ii)	+	+	8/16	(50)
(iii)	−	−	7/10	(70)
(iv)	−	−	5/16	(30)

* "+" presence and "−" absence of viroid band in stock and scion at the time of grafting.
**no. of samples showing viroid band/no.tested.

tomato seedlings which also showed the epinasty symptoms. The observation confirmed the infectious nature of the LMW RNA isolated from TPD-affected trees, thus partially proving Koch's postulates of the biotic agent associated with TPD syndrome.

Discussion

Although the physiological manifestation of TPD has been investigated over the years, no causative connection could be established. Besides latex drying, TPD-affected trees show symptoms of bark scaling, cracking, drying, necrotic streaking and browning of internal bark leading to decay of internal tissues (de Fay 2011). Often, prominent bulges on the lower part of tree trunks occur where the first panel begins to dry. Amidst the attempts to examine a possible biotic aetiology, the short note on evidence of a possible association of viroid with TPD syndrome seems logical (Ramachandran et al. 2000). Their investigations showed absence of association of biotic agents such as fungi, bacteria, virus, mycoplasma like organism or protozoa. They detected a LMW RNA from total nucleic acid of TPD-affected rubber tissue and demonstrated its viroid-like nature by its sensitivity to RNase and insensitivity to DNase, phenol and heat by using the technique of R-PAGE. This technique has been successfully used for the detection of the viroids infecting other plants like potato, apple, plum, citrus, coconut and others (Singh 1989). However, they did not report the infectious nature of the isolated LMW RNA.

The R-PAGE technique gives an added advantage that such detection can be achieved even in the absence of a specific healthy sample. As rubber trees contain large quantities of tannin and other interfering materials, special protocols for extraction and purification of nucleic acid is essential (Cotter et al. 2009). Besides, viroids are present in very low concentrations in plant cells and that too mostly localised in the nucleoli of cells. Stringent procedures of purifying the nucleic acid result in loss of concentration of the nucleic acid to detectable levels (Ding 2009). This was the reason for choosing the EB1 extraction procedure, since it was sufficient for a routine detection of the LMW RNA in large number of samples.

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 showed an additional band just above 7S RNA band when silver stained (picture not shown), 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. A low salt molarity (1/8) of the high salt buffer used in the first run was boiled and used at 70 °C as running buffer in the same apparatus 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.

With a protocol standardised for the tissue under study, it was used to detect the presence of LMW RNA in leaf, bark and root tissues. The earlier reports did not detect the presence of LMW RNA in roots of TPD-affected rubber trees. The infectious LMW RNA was detected from eight different rubber clones from seven plantations other than RRII, Kottayam. The highest disease incidence by R-PAGE analysis was found in the highest yielding and popularly cultivated clone RRII 105. This observation is in conformation with the earlier reports of high incidence of TPD in this clone. 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, though a time-demanding procedure, can still be used as a diagnostic tool in the absence of other reliable procedures (Ramachandran et al. 2006). Presence of LMW RNA in seedlings from different sources is also a significant finding not reported earlier. R-PAGE analysis of bud-grafted plants clearly establishes that the RNA is mechanically transmissible. These plants are being monitored carefully at RRII, Kottayam, to confirm the onset of TPD after they reach tapping stage.

The present study constitutes 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 this century old syndrome affecting rubber cultivation in the country. Work on cloning and sequencing of the molecule is underway. In fact, reisolating the RNA from symptomatic tomato leaves and seeing the band on return gel partially proves the Koch's postulates to further establish the biotic nature of the disease causing molecule.

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