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# Serological and morpho-molecular characterization of papaya ringspot virus (PRSV) infecting the papaya (*Carica papaya*) in Tamil Nadu

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ABSTRACT

Papaya ringspot virus (PRSV) is wreaking havoc on papaya cultivation, leading to significant crop losses. Present study was carried out during 2022 and 2023 at Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu aimed to identify and characterize PRSV in four districts of Tamil Nadu. The incidence of the PRSV ranged from 32–100% in the surveyed districts. Samples displaying symptoms such as mosaic patterns, mottling, yellowing, puckering, shoestring appearance, oily stains on stems and petioles, deformed fruits, and concentric rings on fruits were collected for PRSV confirmation. The preliminary diagnosis of samples using DAS-ELISA showed the presence of PRSV in leaf and fruit skin samples but the absence of the virus in flower and seed samples. The existence of flexuous, filamentous rod-shaped particles measuring 809 nm × 15 nm in the tested leaf sample showed the incidence of Potyvirus, as revealed by transmission electron microscopy (TEM). Molecular characterization of PRSV in leaf and fruit skin samples was carried out using PCR with PRSV coat protein-specific primers, resulting in the amplification of an 850 bp product. Sequencing of the amplified products showed that the partial sequences of isolates CBE1-TN and ANR1-TN were 99.99% identical. Early detection of PRSV is imperative for implementing effective management strategies.

**Keywords**: DAS-ELISA, Electron microscopy, Molecular characterization, Papaya, PRSV

Papaya (*Carica papaya* L.) is the most commercially cultivated tropical fruit crop (Kaul *et al.* 2022). Nevertheless, the cultivation of this crop at a local and global level faces significant obstacles to realizing its maximum yield potential, mainly attributable to the prevalence of papaya ringspot virus (PRSV), which belongs to the genus *Potyvirus* and the family Potyviridae (Purcifull *et al.* 1984, Gorane *et al.* 2019). The PRSV is naturally transmitted through several aphid species (Gadhave *et al.* 2019) and artificially by mechanical inoculation (Gonsalves *et al.* 2010). In India, the area under papaya cultivation has been declining for the last 10 decades due to the occurrence of papaya ringspot disease (PRSD), which causes 85–90% yield loss, depending on the period of infection and stage of the plant (Thirugnanavel *et al.* 2015). All the growth stages of papaya are vulnerable to PRSV-P infection and exhibit symptoms on leaves within 2–3 weeks after infection, and spreading to the whole field within 3–7 months, often with 100% yield losses (Umer *et al.* 2022). The PRSV is prevalent across all countries where papaya cultivation occurs, exhibiting its highest level of diversity among Indian isolates (Premchand *et al.* 2023). Based on their main host range, PRSV is classified as serologically indistinguishable strains, such as biotypes P and W. The strain P affects Caricaceae, Chenopodiaceae, and Cucurbitaceae, referred to as papaya-infecting pathotype-P (PRSV-P). In contrast, strain W affects *Chenopodiaceae* and *Cucurbitaceae*, referred to as non-papaya-infecting pathotype-W (PRSV-W), reported as a watermelon strain (Tripathi *et al.* 2008). To mitigate plant virus outbreaks and develop resistant cultivars, it is necessary first to identify and detect the specific virus using genomic and phenotypic characteristics. Several serological and molecular approaches have been independently studied in PRSV (Kumar *et al.* 2019, Mishra *et al.* 2019); though, the majority of research relies on serological means for the detection of PRSV in diseased hosts (Roy *et al.* 1999), specifically DAS-ELISA (Thirugnanavel *et al.* 2015). Present study investigated serological, phenotypic and genotypic techniques for detecting PRSV in Tamil Nadu (India).

## MATERIALS AND METHODS

*Plant material*: Present study was carried out during 2022 and 2023 at Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. Symptomatic leaf samples expressing typical mosaic patches and shoe strings were collected from 4 districts of Tamil Nadu during December

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2022, notably fields in Tamil Nadu Agricultural University Orchard Coimbatore; Anthiyur, Ayyalur; Kannadiputhur; and Kannivadi that were severely affected by papaya ringspot virus (PRSV). The samples were stored in a deep freezer at -20°C for further PRSV confirmation. Random observations were made on at least 100 plants/field. The disease incidence was recorded based on the external symptoms and documented using the formula given by Salam *et al.* (2011).

*Serological detection*: DAS-ELISA (Double Antibody Sandwich-Enzyme Linked Immuno Sorbent Assay) was performed for all collected samples, including the Tamil Nadu Agricultural University isolate (CBE1-TN), Anthiyur isolate (ANR1-TN), Ayyalur isolate (AYR1-TN), Kannadiputhur isolate (KPR1-TN) and Kannivadi isolate (KVI1-TN). The presence of PRSV was tested in four parts of infected papaya including the leaf, flower, seed, and fruit skin, using PRSV antiserum (DSMZ, Germany) following the manufacturers protocol. Healthy papaya plants grown in a glass house were used as controls. Each sample was tested in a 1:1 dilution with three replications. Each well of the microtitre plate was coated with 100 µl of PRSV antiserum in carbonate buffer (*p*H 9.6) at a dilution 1:500 and incubated at 37°C for 240 min. The plate was washed three times with washing buffer (1X PBS-T) at 3 min intervals. PRSV samples were ground with extraction buffer (PBS-T + 2% PVP + 0.2% BSA) and centrifuged at 6000 rpm for 10 min. The obtained supernatant was put into new tubes, and 100 µl of the supernatant was added to the plate, which was then stored overnight at 4°C. After washing, 100 µl of enzyme conjugate diluted 1:500 was added to each well and incubated at 37°C for 240 min. Then, 100 µl of p-Nitro Phenyl Phosphate substrate was added, and the mixture was left to incubate for 30 min in the dark. The absorbance was measured using a microplate reader (Sunrise™, Tecan Group Ltd, Switzerland) at 405 nm. Absorbance values that were twice those of the healthy control indicated a positive reaction (Diallo *et al.* 2008).

*Electron microscopy*: The CBE1-TN isolate was partially purified as per the procedure of Gonsalves and Ishii (1980). The purified virus particles were observed using a transmission electron microscope (TEM) TECNAI SPRIT G2 (FEI, Netherlands) to determine their shape and size. The virus preparation was set on the grids with a droplet of uranyl acetate, and grids were dried in desiccators for 15–30 min and examined under the TEM at different magnifications.

*Total RNA extraction from plant samples*: RNA was extracted from infested leaves and peels of fruit using the TRIzol® Reagent method (Gogoi *et al.* 2019). The extract was spun up at 13000 rpm for 10 min at 4°C. The filtrate was moved to a new 1.5 ml centrifuge tube, then 250 µl of chloroform was added, and it was kept at room temperature for 15 min until stratification, after which it was centrifuged for 10 min at 13000 rpm. The aqueous layer was moved to a new tube, and 250 µl of ice-cold isopropanol was added, and the mixture was incubated on ice for 10 min before being centrifuged at 13000 rpm for 15 min. The supernatant was discarded, and the pellets were washed at 13000 rpm for 8 min with 300 µl of 70% ethanol. The final particles were air-dried for 5 min, and 50 µl of RNAase-free water was then added to dissolve them. The total RNA from a healthy plant served as a negative control. The integrity of the isolated total RNA was evaluated by electrophoresis of individual samples of RNA on an agarose gel.

*cDNA synthesis*: Using a Thermo Scientific Revert Aid™ First Strand cDNA Synthesis Kit (CAT. #K1622), the total RNA isolated from PRSV-infected samples was converted to complementary DNA (cDNA) (Sawwa *et al.* 2018). In two steps, reverse transcription was conducted. In the initial step, 4 µl of RNA was diluted with 7 µl of nuclease-free water and 1 µl of gene-specific reverse primer before being denatured at 65°C for 5 min to disrupt the secondary structure. Then, 1 µl of riblock RNase inhibitor and 1 µl of Revert Aid M-MuLV Reverse transcriptase were added. The samples were then incubated for 45 min at 42°C and terminated for 5 min at 70°C using Thermocycler (Eppendorf), and the resulting cDNA was stored at -20°C.

*PCR amplification*: The 5 µl of synthesized cDNA was amplified by PCR using the specific primer pairs-HRP52f 5' TCCAARAATGAAGCTGTGGATGT 3' and RKJ3r 5' GTTGCGCATACCCAGGAGAG 2' (Sharma *et al.* 2005, Dhanam *et al.* 2011). PCR was done under the specified conditions (Sharma *et al.* 2005). The PCR product was visualized on a 1% agarose gel prepared in 1X TAE buffer (*p*H 8.8) and stained with ethidium bromide (0.5 g/ml). The electrophoresis pattern images were obtained using a gel documentation system (AlphaImager™ 2000 from Alpha Innotech, US).

*Sequencing and phylogenetic analysis*: The amplified products underwent partial sequencing at Eurofins Genomics India Pvt. Ltd., Bengaluru. Following the sequencing process, homology searches were conducted using the NCBI-BLAST tool. The verified sequences were submitted to the NCBI Genbank. The phylogenetic analysis was done using MEGA11 software and ITOL online phylogeny software (Kumar *et al.* 2019). The phylogenetic analysis was subjected to 1000 bootstrap replications and subsequently condensed using a threshold value of 70%. The amino acid sequences were analyzed using BioEdit (Version 7.0.4.1) by performing multiple sequence alignment (MSA) and sequence identity matrix analysis.

## RESULTS AND DISCUSSION

*Occurrence and severity of PRSD*: In India, the papaya production has been decreasing in recent years due to the prevalence of PRSD, which causes yield losses from 40–90% in Tamil Nadu (Thiribhuvanamala *et al.* 2016). A comparable survey revealed that the prevalence of PRSD in the primary papaya-growing regions of Karnataka varied from 50.5–100% from 2019 to 2021 (Premchand *et al.* 2023). During a papaya field survey in Rangareddy district, Telangana, the leaves of infested plants appeared

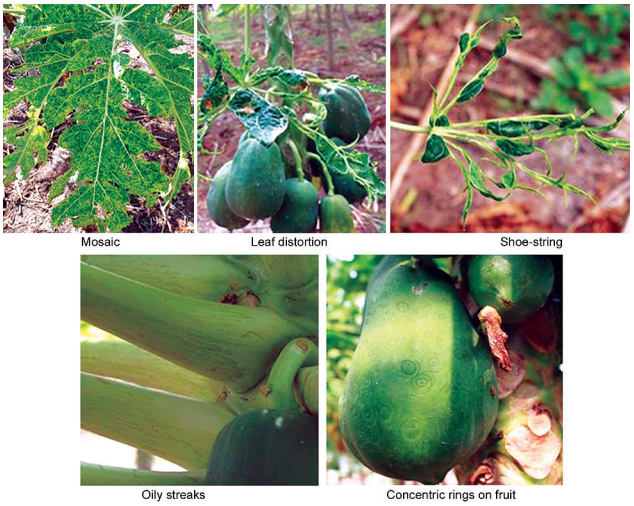


Fig. 1 Papaya ringspot virus symptoms on papaya.

yellowish, distorted, and exhibited a shoestring-like appearance (Gupta *et al.* 2023). During our survey, all the PRSV symptomatic papaya exhibited typical symptoms of mosaic, distortion, shoe-string of leaves, oily streak over petioles and deformation, and concentric dark green rings on fruit surface (Fig. 1). The incidence of PRSD ranged from 32–100% in the fields surveyed. Maximum incidence of 100% was recorded in two locations TNAU orchard, Coimbatore district, and Anthiyur, Erode district followed by Ayyalur (70%) and Kannivadi (65%) of Dindigul district (Table 1).

*Confirmation of PRSV through DAS-ELISA*: DAS-ELISA results of PRSV infected samples showed with significant development of yellow colour and increase in the absorption values over negative control was perceived for the samples collected. The absorption values (405 nm) of infested leaf (0.824, 0.812, 0.623, 0.773, 0.628) and fruit skin (0.578, 0.545, 0.513, 0.548, 0.571) samples of all the isolates (ANR1-TN, CBE1-TN, KPR1-TN, KVI1-TN, AYR1-TN) were twice and thrice than values of healthy control (leaf-0.236, fruit skin-0.241) (Table 2). Hence, the leaves and fruit skin showed positive reaction whereas flower and seed showed negative reaction for the presence of PRSV. Highest absorption value at 405 nm was recorded for leaf (0.824) and fruit skin (0.578) samples infected by ANR1-TN as compared to other isolates. This indicated that the virus concentration is more in ANR1-TN samples and lowest in KPR1-TN samples. Similarly, Akhter *et al.* (2013) reported that the skin of diseased papaya fruit and stem showed a positive reaction to DAC (direct antigen-coated)-ELISA. In contrast, flowers, latex, seeds, and roots of infected papaya showed a negative reaction. Chin *et al.* (2007) confirmed the PRSV in *Momordica charantia* leaf samples using DAS-ELISA in Jamaica of West Indies. Similarly, Diallo *et al.* (2008) confirmed the PRSV in leaf extracts made from dried samples of papaya using DAS-ELISA.

*Electron microscopy*: The purified and stained PRSV sample was observed as flexuous rod-shaped particles under at 80000X magnification in TEM. The size of the virus particle isolated from infected papaya leaves measured 809 nm × 15 nm (Fig. 2). The flexuous particles indicated that the virus associated with the disease belongs to the *Potyvirus* genus. Tripathi *et al.* (2008) reviewed that the PRSV particle was rod-shaped, non-enveloped, flexuous and filamentous, quantifying 760–800 nm and 12 nm. Similarly, the partially purified virus particles of PRSV were observed as 740–780 × 11 nm-sized flexuous rod-shaped in TEM studies (Muske *et al.* 2014). The PRSV was ultra-purified from systemically

Table 1 Papaya ringspot virus incidence in different districts of Tamil Nadu

| Location | Isolate | GPS latitude and longitude | Area (acre) | Variety grown | Stage of the crop | Disease incidence (%) |
|---|---|---|---|---|---|---|
| TNAU, Orchard Coimbatore | CBE1-TN | 11.006°N, 76.92°E | 4.0 | CO8 | Fruiting | 100 |
| Anthiyur, Coimbatore | ANR1-TN | 10.58°N, 77.24°E | 5.0 | Red Lady, Sapna and Zinda | Flowering | 100 |
| Ayyalur, Erode | AYR1-TN | 11.45°N, 77.438°E | 3.0 | Red Lady | Flowering | 70 |
| Kannadiputtur, Tiruppur | KPR1-TN | 10.584°N, 77.25°E | 2.0 | Red Lady | Fruiting | 32 |
| Kannivadi, Dindigul | KVI1-TN | 10.379°N, 77.830°E | 2.0 | Red Lady | Fruiting | 65 |

Table 2 Screening for the presence of papaya ringspot virus in samples by DAS-ELISA

| | OD value A405 nm | | | | |
|---|---|---|---|---|---|
| Isolate | Leaf* | Flower* | Seed* | Fruit skin* | Buffer* |
| ANR1-TN | 0.824 ± 0.009 | 0.322 ± 0.01 | 0.235 ± 0.004 | 0.578 ± 0.06 | 0.215 ± 0.007 |
| CBE1-TN | 0.812 ± 0.102 | 0.264 ± 0.008 | 0.232 ± 0.01 | 0.545 ± 0.04 | |
| KPR1-TN | 0.623 ± 0.05 | 0.253 ± 0.01 | 0.226 ± 0.004 | 0.513 ± 0.01 | |
| KVI1-TN | 0.773 ± 0.03 | 0.333 ± 0.01 | 0.236 ± 0.004 | 0.548 ± 0.055 | |
| AYR1-TN | 0.628 ± 0.032 | 0.278 ± 0.001 | 0.225 ± 0.005 | 0.571 ± 0.04 | |
| Healthy | 0.236 ± 0.015 | 0.236 ± 0.03 | 0.259 ± 0.015 | 0.241 ± 0.004 | |
| Antigen at 1:1 dilution | | | | | |

*Average A405 absorption value of 3 replications.

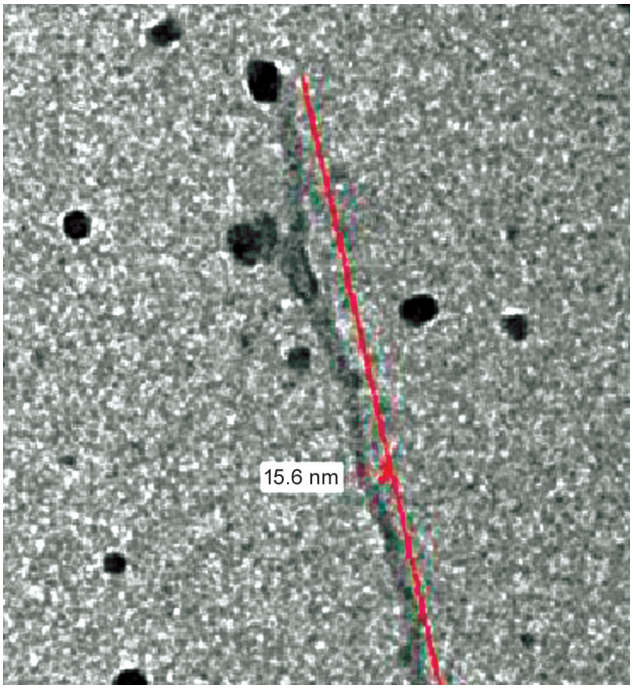


Fig. 2 Electron micrographs of papaya ringspot virus.

diseased papaya and confirmed by an EM (Navanath *et al.* 2017). These literatures are in confirmation with the nature of the PRSV responsible for sampled specimens.

*Molecular characterization of PRSV*: The cDNA followed RT-PCR using a particular primer targeting the *CP* gene of PRSV. The PCR amplification product was successfully produced for all the isolates, yielding an amplicon of approximately 850 bp in size. Dhanam *et al.* (2011) reported comparable findings, where they utilized same PRSV *CP* gene-specific primers to identify PRSV-infected papaya, resulting in amplicons of 850 bp.

*Sequencing and phylogenetic analysis*: In BLAST analysis, the partial sequence of both isolates CBE1-TN and ANR1-TN showed 99% identity and concluded that the virus causing the disease was PRSV. The analysis demonstrated that two isolates, namely CBE1-TN and ANR1-TN, exhibited a 95% similarity in their *CP* regions. The isolates ANR1-TN and CBE1-TN were compared with different isolates retrieved from the NCBI database by forming a phylogenetic tree (Table 3).

The nucleotide sequence of the CP segment of PRSV isolates CBE1-TN and ANR1-TN was analyzed in comparison to the same region of 27 additional PRSV isolates and one SMV isolate, which is classified under the Potyvirus family. The major cluster comprises all the PRSV isolates and out group SMV, including our isolates, revealing their distinct nature (Fig. 3). Our PRSV isolates MH481855 and MH509739 along with the OQ301899 and KC149502 were grouped under the minor cluster. Similarly, Lima *et al.* (2002) conducted a study on the homogeneity of 12 PRSV Brazilian strains, the *CP* genes of all strains exhibit a nucleotide-level consistency of 97.3%. In addition, uniformity of 90.7% was observed when comparing

Table 3 Virus sequences retrieved from NCBI database for comparison

| Virus sequences | Origin | Accession no. |
|---|---|---|
| Papaya ringspot virus isolate MH-SN | Maharashtra | MG977151 |
| Papaya ringspot virus isolate MH-PN | Maharashtra | MG977150 |
| Papaya ringspot virus isolate MH-PL | Maharashtra | MG977149 |
| Papaya ringspot virus isolate UASBBLR1122 | Karnataka | KT248544 |
| Papaya ringspot virus isolate GOA3 | Goa | KY448329 |
| Papaya ringspot virus isolate GOA2 | Goa | KY448328 |
| Papaya ringspot virus isolate DWD-P | Karnataka | KU196792 |
| Papaya ringspot virus P isolate MP | Madhya Pradesh | KC149501 |
| Papaya ringspot virus isolate SR390-PAP | Vellayani, Kerala | KT825492 |
| Papaya ringspot virus strain Annur | Annur, Tamil Nadu | HM454197 |
| Papaya ringspot virus strain Avinashi | Avinashi, Tamil Nadu | HM454196 |
| Papaya ringspot virus isolate | Tamil Nadu | EF104919 |
| Papaya ringspot virus isolate B | Karnataka | AY238884 |
| Papaya ringspot virus isolate Trichy | Tamil Nadu | DQ077175 |
| Papaya ringspot virus strain Ellampillai | Tamil Nadu | HM754218 |
| Papaya ringspot virus P isolate PRSV-P-CP-Ranchi-1 | Jharkhand | JQ394693 |
| Papaya ringspot virus isolate ODS | Odisha | KY448331 |
| Papaya ringspot virus isolate UASBMAN1122 | Karnataka | KT248543 |
| Papaya ringspot virus P isolate Pune3 | Maharashtra | KC149502 |
| Papaya ringspot virus from India coat protein | Sikkim | DQ354072 |
| Papaya ringspot virus isolate Annur | Tamil Nadu | HM778170 |
| Papaya ringspot virus | Tamil Nadu | AY687386 |
| Papaya ringspot virus isolate PRSV-P | Tamil Nadu | OL450369 |
| Papaya ringspot virus isolate Andhra Pradesh | Andhra Pradesh | EF210197 |
| Papaya ringspot virus isolate AP40 | Andhra Pradesh | OQ301916 |
| Papaya ringspot virus isolate AP39 | Andhra Pradesh | OQ301915 |
| Papaya ringspot virus isolate AP23 | Andhra Pradesh | OQ301899 |
| Sugarcane mosaic virus CB671-1 (Outgroup) | Coimbatore | DQ343236 |

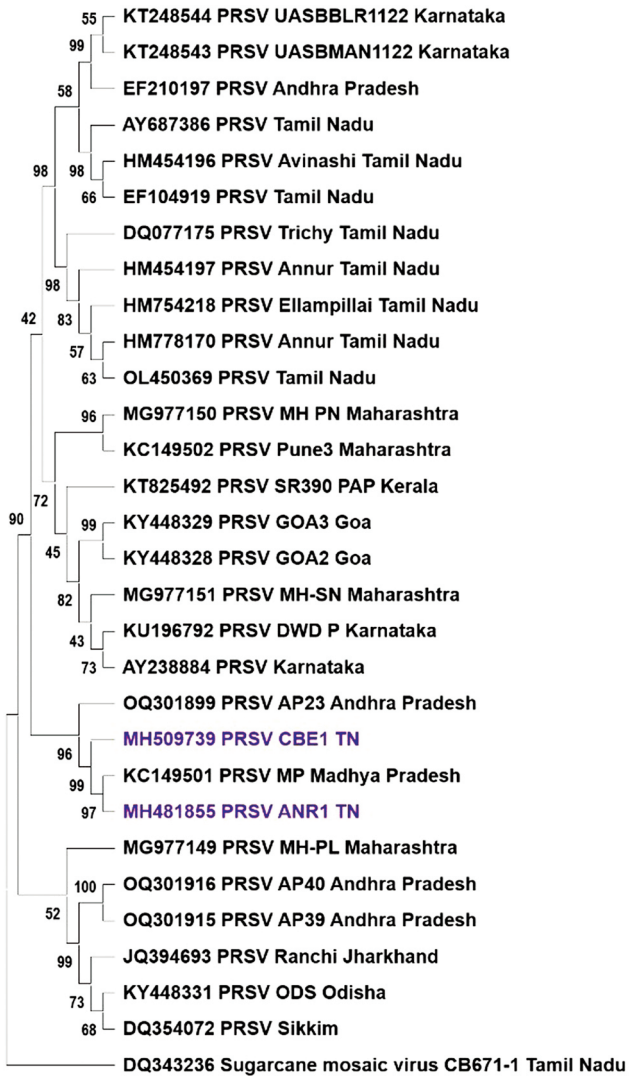


Fig. 3 Phylogenetic tree of *CP* gene sequences of two isolates (CBE1-TN, ANR1-TN) based on MSA using MEGA11.

12 Brazilian strains with 27 strains from outside Brazil employing a homology tree. Molecular analysis, through *CP* gene-specific primers, has consistently detected the presence of PRSV in papaya plants, and sequence comparisons have revealed genetic variations among different PRSV isolates. In India, researchers sequenced the *CP* gene of 14 PRSV-P isolates found in different papaya growing areas in the western Indian states of Maharashtra and Gujarat. They compared these sequences to those of other Indian isolates that shared between 81 and 98% and 80 and 100% of their nucleotide sequence identities, respectively (Gorane *et al.* 2021).

In conclusion, PRSV has emerged as a severe threat, capable of causing devastating losses of up to 100% in papaya production. The present study of PRSV and its impact on papaya cultivation in Tamil Nadu underscores the importance of early and accurate detection, and disease management strategies to ensure the sustainability of the papaya industry, particularly in regions where the virus poses a significant threat.

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