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Molecular diagnosis of *Papaya ringspot virus* disease in papaya seeds

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Abstract
Papaya, which originated from Central America, is one of the most widely marketed and consumed fruits worldwide. *Papaya ringspot virus* (PRSV) caused serious havoc in cultivated papaya all over the world. Papaya plants are propagated through seeds and the presence of virus in papaya seeds needs to be ascertained for better management of PRSV. Seeds were collected from PRSV infected and healthy papaya fruits assessed for the presence of PRSV. Seeds with and without sarcotesta did not produce any amplicon in RT-PCR assay using PRSV-specific primers. PRSV infected leaf sample (Positive control) showed an amplicon size of 850 Bp. Hence, the transmission of PRSV through seeds was negligible.

Keywords: *Papaya ringspot virus* (PRSV), papaya seeds, Central America

Introduction
Papaya, originated from Central America, globally marketed and consumed fruit, belongs to the family Caricaceae. Papaya is being affected by many fungal, bacterial and viral diseases. Among the viral diseases, *Papaya ringspot virus* disease, caused by *Papaya ringspot virus* belongs to the genus *Potyvirus*, (family *Potyviridae*) is a major threatening disease of papaya worldwide (Gonsalves 1998). The virions contain a single-stranded positive-sense RNA of ~10.0kb, encapsidated by coat protein with a molecular weight of 36kDa. Mosaic symptoms, chlorosis and mottling of the leaves, leaf distortion, greasy streaks on the petiole and trunk, shoestring development on leaves, C-shaped markings on fruits, reduction in fruit size and number of fruits (Purcifull, 1984) [1]. In India, PRSV in papaya was first reported during 1958 in Maharastra and subsequent spread was noted to many other states including Himachal Pradesh, Chhattisgarh, Jharkhand, Delhi, Karnataka, Uttar Pradesh, Maharashtra (Jain *et al*. 2004) [2] and Tamil Nadu (Sharma *et al*. 2005) [3] resulting in crop loss of up to 90% in some cases (Lokhande *et al*. 1992; Hussain and Varma 1994) [4, 5]. This virus is horizontally transmitted in a non-persistent manner by aphids (Ishii and Holtzmann, 1963; Deomano and Pua, 1986; Opina, 1986) [8, 6, 7], but several reports in recent years have raised the possibility of vertical transmission through seeds for some members of this genus. Bayot *et al*. 1990 [9] reported that this virus is transmitted through seeds at a very low incidence of 0.15 per cent. Hence it is essential to confirm the PRSV transmission through papaya seeds. Since transmission of plant viruses through seed can be one of the major factors contributing to long-distance dispersal through global trade of seeds and can have important ecological consequences for virus dissemination. Because seed transmissibility of plant viruses is influenced by many factors such as crop variety, virus strain, temperature, infection time, and so on (Matthews, 1970; Gibbs and Harrison, 1976) [10, 11], Hence attempts were made to study the PRSV seed transmissibility by molecular approach using RT-PCR. The findings of this study will aid in the development of adequate control measures to prevent the spread of PRSV to geographically isolated sections of the country where the disease is yet to be discovered. If the virus is found in the seed, strict seed certification and quarantine procedures can be implemented.

Materials and Methods
To assess the seed transmissibility, papaya seeds were collected from healthy and PRSV-infected ripened and unripened fruits of papaya. An infected papaya leaf sample served as a positive control. Around 20 papaya fruits with typical symptoms of papaya ring spot virus disease, such as oil-soaked ring spots on the skin of the fruit, were collected from Orchard,

TNAU, Coimbatore. Seeds were extracted from both infected and healthy fruits with sarcotesta and without sarcotesta and total RNA extraction was done as below.

Isolation of RNA

The method used by Chomczynski P and Sacchi N. (2006) [12] was adopted using TRIzol reagent. All plastic and glassware were carefully washed, dried, and treated with 0.1 per cent Diethylene pyrocarbonate (DEPC) by dipping for 24 hours before being sterilised. 0.2 g of respective samples were weighed and crushed in a pestle and mortar using liquid nitrogen. One ml of TRIzol reagent (Sigma Aldrich) was added to the powdered material and thoroughly mixed. It was transferred to a microfuge tube and centrifuged at 12000 rpm for 20 minutes at 4 °C. The supernatant was separated and added with 250 µl of chloroform and mixed, centrifuged at 12000 rpm (4 °C) for 5 minutes the upper clear aqueous phase was transferred to a new tube without disturbing the bottom layer. Added with 250 µl of ice-cold isopropanol and 250 µl of 5M NaCl and thoroughly mixed. The contents were then kept on ice for 30 minutes and centrifuged for 10 minutes at 4°C at 12000 rpm. The pellet was rinsed with 80% ethanol. The pellet was air dried and dissolved in DEPC water and kept at 4 °C for further use. RNA purity was determined using agarose gel electrophoresis.

Quantification of RNA

Nanodrop was used to evaluate the quality and quantity of extracted RNA (Bio drop 7141 V1.0.4). The optimal concentration of RNA was around 1500µg. The A260/A280 ratio was used to assess RNA quality.

cDNA conversion

The RNA extracted was converted into cDNA using Prime Script™ 1st strand cDNA Synthesis Kit. Oligo dT Primer -1 µl, dNTP Mixture- 1 µl, Template RNA (our sample RNA)-3 µl, RNase Free dH2O-5 µl (Total 10 µl) were added and mixed. The mixture was heated at 65 °C for 5 minutes and cooled immediately on ice. Again added with 5X Prime Script Buffer- 4 µl, RNase Inhibitor -0.5 µl, Prime Script RTase -1.0 µl, RNase Free dH2O -4.5 µl (Total 20 µl) and thoroughly mixed. The mixture was kept in heat at 42^0 C for 30 minutes at 42^0 C and then at 70 °C for 15 minutes in a thermocycler and cooled immediately on ice. For future usage, the converted cDNA was stored at -20 °C

Polymerase Chain Reaction (PCR) amplification using specific primer

The reaction mixture was prepared for 10 µl containing 1 µl of template cDNA, 5 µl of master mix, 1 µl each of the forward and reverse primer and 2 µl of nuclease-free water.

Table 1: Polymerase Chain Reaction (PCR) amplification using specific primer

Primer type	Primer sequence	Target
Forward (HRP 52)	5'- TCC AA (A/G) AAT GAA GCT GTG GAT GCT - 3'	CP gene
Reverse (RKJ3)	5'- GTT GCG CAT ACC CAG GAG AG -3'	CP gene

Table 2: PCR conditions for PRSV

Steps	Temperature (°C)	Time
Denaturation (initial)	94 °C	5 min
Denaturation	94 °C	1 min
Annealing	46 °C	2 min
Extension	72 °C	60 min

Agarose Gel electrophoresis

Using agarose solution, agarose gel electrophoresis was done.0.6 g of agarose was weighed and melted in 50 ml of 1X Tris borate EDTA buffer to make a 1.2 per cent agarose solution. The agarose solution was made to lukewarm stage, added with 4µl of ethidium bromide and thoroughly mixed. At one end of the gel tray, an appropriate size of the comb was placed, and the agarose solution was poured into the tray and allowed to settle. After solidification, the comb was carefully removed without disturbing the gel and placed in an electrophoresis tank containing 1X TAE buffer. The PCR product (10 µl) was carefully loaded into the wells, and electrophoresis at 80 volts was used. The banding pattern on the gel was examined using a UV transilluminator. The sizes of the PCR products were calculated by comparing them to a standard 1 kb molecular marker.

Gel documentation

The PCR products got separated with 1.2 per cent agarose gel and photographed using Alpha imager TM1200 documentation and analysis system Alpha Innotech Corporation, San Leandro, California. The PCR products were separated with 1.2 per cent agarose gel and documented using the Alpha imager TM1200 documentation and analysis system (Alpha Innotech Corporation, San Leadro, California).

Grow out test

Twenty seeds each from PRSV-infected fruits and healthy fruits were collected and sown in 40 polybags in PL- 480 glasshouse in Orchard, TNAU, Coimbatore. Seeds of healthy fruit from plants maintained under an insect-proof cage and sprayed with neem oil 3% at the fortnight interval served as negative control. Plants were observed up to 3 leaf stage leaf samples were collected.

Reverse Transcriptase-PCR was performed as above to confirm the presence of the virus in the leaves collected from the plants at 45th Days after sowing during the grow-out test.

Results and Discussion

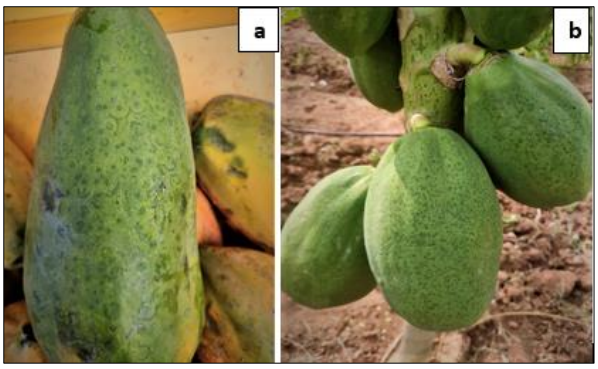

Plate 1: a) Mature papaya fruit with oil-soaked ringspot
b) Immature papaya fruit with water-soaked ringspot

To test for the presence of PRSV, RNA was isolated from the seeds with sarcotesta, seeds without sarcotesta and the sarcotesta alone from ripened PRSV infected fruit. In the

unripened fruit, it was difficult to separate the sarcotesta from the seeds, so the whole seeds as such was used for the isolation of RNA. Molecular studies using RT-PCR revealed that PRSV was not transmitted by seeds. None of the samples yielded the amplicon of 850 Bp size except positive control. The infected leaf sample produced an amplicon of 850bp. In grow-out studies the samples were collected and analyzed for the presence of PRSV. The plants produced from infected seeds were not showing any symptoms and proved negative in RT- PCR assay. The positive control collected from the field yielded an amplicon of 850 Bp.

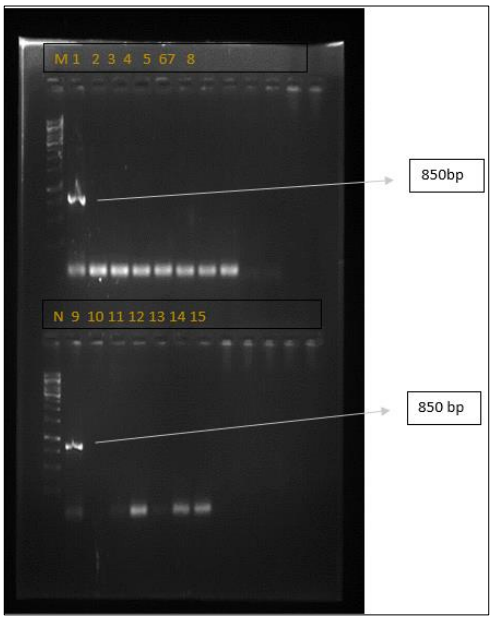

Fig 2: Gel electrophoresis image of PCR products amplified using PRSV-specific primer

Lane M: 1kb ladder; 1: Positive control (leaf); 2: Unripe healthy seeds; 3: Unripe diseased seeds; 4: Healthy ripe seeds (whole) 5: Healthy ripe seeds; 6: Healthy ripe sarcotesta 7: Healthy ripe seeds only; 8: Healthy ripe sarcotesta.

Lane N: 1kb ladder; 9: Positive Control; 10: Diseased ripe seeds (whole) 11: diseased ripe seeds only; 12: diseased ripe sarcotesta 13: Diseased ripe seeds (whole) 14: Diseased ripe seeds only; 15: Diseased ripe Sarcotesta.

The grow-out test was conducted on the seeds from infected Papaya fruits. The plants were grown in an insect-proof cage and precautionary measures were taken to control aphid infestation. The plants showed no Typical PRSV symptoms (Plate 2) and were similar in morphological features to the healthy plants maintained as control.

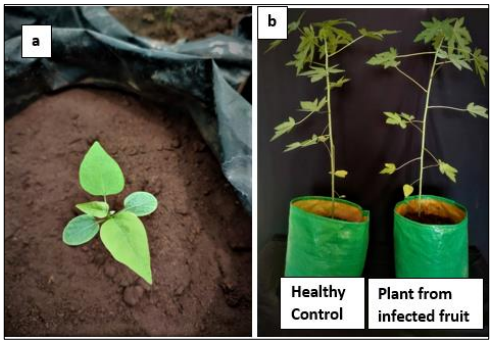

Plate 2: a) Grow-out test of seeds from the infected fruits

The leaves from the plants in the grow-out test were collected and RT-PCR was performed. Except for the positive control, none of the samples had an amplicon of ~860 Bp (Fig.2). The PRSV-specific primers used has an amplification region of 860 Bp.

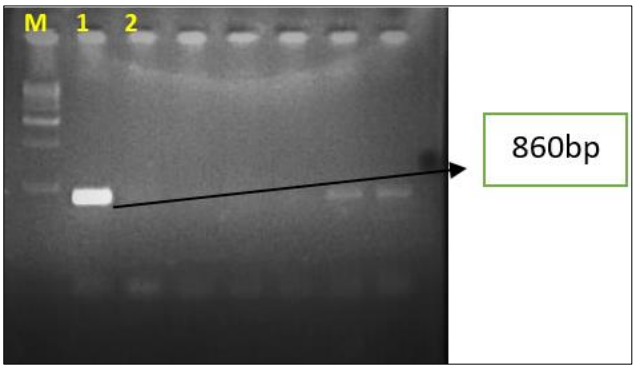

Fig 2: Gel electrophoresis image of PCR products amplified using PRSV-specific primer Lane M: 1kb ladder; 1: Positive Control; 2: plant from grow-out test

Despite a report from the Philippines (Bayot *et al*. 1990) [9] stating that PRSV is seed transmitted at a very low rate of 0.15 per cent, this study found no virus in the seed or sarcotesta.

Seed transmission plays an important role in the rapid spread of viruses from one part of the world to another. Seeds can also serve as a source of inoculum in adverse conditions, and the virus can survive for a longer period of time inside the seed. As a result, confirmation of seed transmission of PRSV is extremely important. Virus infection may disrupt the normal relations of stamens and pistils to such an extent as to cause sterility. Duggar (1930) [13] proposed that the inactivating action of some "specific protein or other specific material" in the seeds could prevent the seed transmission of those highly infectious viruses, which are almost ubiquitous in their distribution within a plant. Bennett (1936) [14] also proposed that the lack of plasmodesmata connection between the embryo and the parent plant prevents seed transmission of even the most infectious virus diseases.

Conclusion

This study established that PRSV is not seed-transmitted. Furthermore, PRSV vertical transmission by seeds also does not occur. More research and studies have to be made giving emphasis on the other modes of transmission of the virus since seed transmission of the virus is negligible. Vector transmission of PRSV aphids is still a serious concern. And adequate control measures have to be taken as once the field is affected, it leads to the entire papaya plant dwarfing and sterility. It causes huge losses to the farmers.

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