Madras Agric. J., 102 (10-12): 344-347, December 2015
https://doi.org/10.29321/MAJ.10.001131

Preliminary Characterization of Coimbatore Isolate of Pigeonpea Sterility Mosaic Virus

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Sterility mosaic disease (SMD), the most devastating disease of pigeonpea (*Cajanus cajan*) is caused by *Pigeonpea sterility mosaic virus* (PPSMV) transmitted by the eriophyid mite *Aceria cajani*. PPSMV is known to exist as distinct isolated in different regions of India. The Coimbatore isolate of PPSMV was purified, characterized at molecular level and compared with the type isolate of Patancheru region (Andhra Pradesh state, India). While viral nucleic acid analysis, northern hybridization and RT-PCR showed similarity among these isolates, variation in the molecular weight of virus related protein was found to be responsible for characterizing Coimbatore isolate of PPSMV as a distinct one.

Key words: *Cajanus cajan*, Sterility mosaic disease, PPSMV, Virus isolates

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is an important food legume cultivated in the semi-arid regions of the world. It is primarily grown for its protein-enriched seed that provides vital dietary protein. In India, pigeonpea is cultivated in 3.47 m ha with the production of 2.57 million M T and among the Indian states, Tamil Nadu ranks seventh in area and production (AICRP Report, 2013). Sterility mosaic disease (SMD), known as the 'green plaque' is the most economically important disease of pigeonpea in the Indian subcontinent and causes greater yield loss in India (Kumar *et al*., 2004). SMD is characterized by stunted and bushy plants, leaves of reduced size with chlorotic rings or mosaic symptoms and partial or complete cessation of flower production (sterility). The causal agent was identified as a novel virus provisionally named as the Pigeonpea Sterility Mosaic Virus (PPSMV) (Kumar *et al*., 2002). The virus is transmitted under natural condition by the eriophyid mite *Aceria cajani* Channabasavanna and experimentally by grafting (Ghanekar *et al*., 1992).

The coordinated research efforts resulted in identification of several pigeonpea genotypes possessing field resistance to SMD (Reddy *et al*., 1998). However, several of these genotypes showed location specific resistance. The variability in resistance to SMD by the different pigeonpea genotypes was assumed to be due to presence of either different biotypes of vectors or due to occurrence of different strains of causal agent (Reddy *et al*., 1998). Study on the biodiversity of the mite vector in several SMD endemic locations in the Indian subcontinent suggested that *A. cajani* was not contributing to the location specific variation in host resistance and suggested that it could be due to the involvement of various strains of the SMD pathogen (Kumar *et al*., 2001; Latha *et al*., 2007).

For efficient management of SMD and to select durable resistance sources suitable for pigeonpea farmers in Tamil Nadu, identification of pigeonpea sources that are resistant to the local strain is essential. For this purpose, it is necessary to characterize the SMD isolate prevalent in Tamil Nadu region to understand its biodiversity and to develop specific diagnostic tools for utilization in resistance screening programs. In this communication we report the characterization of PPSMV isolate prevalent in Tamil Nadu and comparison of its properties with type isolate *i.e.*, PPSMV-Patancheru (PPSMV-P), which was characterized earlier (Kumar *et al*., 2003). In recent years, the virus causing the sterility mosaic disease in pigeonpea was identified to be a virus with segmented, negative sense single stranded RNA genome. The virus is classified as a species in the genus *Emaravirus* of unassigned family (Elbeaino *et al*., 2014).

Materials and Methods

Maintenance of SMD culture

Pigeonpea sterility mosaic virus (PPSMV) culture was maintained on a susceptible pigeonpea cultivar ICP 8863 in an insect proof wire-mesh chamber at Agricultural College and Research Institute, Coimbatore, Tamil Nadu, India. The leaf stapling technique (Nene and Reddy, 1977) was used to inoculate 12 to 15 days old (two-leaf stage) healthy pigeonpea seedlings. The virus culture maintained at this location was designated as PPSMV-Coimbatore (PPSMV-C) isolate. The PPSMV cultures maintained on pigeonpea cv. ICP 8863 at ICRISAT,

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Patancheru, India (the PPSMV-P isolate) was used as reference and control for comparing PPSMV-C properties.

Virus purification and production of polyclonal antibodies

PPSMV was purified following the procedure given by Kumar *et al*. (2003). To the purified virus particle preparations, polyclonal antiserum was produced in a New Zealand White inbred rabbit by giving four weekly intramuscular injections of 250 ìl of purified virus preparations in phosphate-buffered saline mixed with an equal volume of Freund's complete adjuvant subsequently with incomplete adjuvant. The animal was bled at weekly intervals after fourteen days of final injection.

Analysis of total protein and purified preparations of PPSMV by SDS-PAGE and Western blotting

Total proteins (TP) were extracted from the SMD-affected pigeonpea leaf samples collected from Coimbatore (Tamil Nadu state) and ICRISAT-Patancheru (Andhra Pradesh state) as per the procedure given by Kumar *et al*. (2003) and were separated in 12 per cent sodium dodecyl sulphate (SDS) – discontinuous polyacrylamide gel. Prestained protein marker (GIBCO BRL, High range; cat. # 26041-020) was used as molecular weight marker. After electrophoresis, the gel was silver stained for protein detection. Western blotting for PPSMV detection was performed as described by Kumar *et al*. (2003).

Analysis of viral nucleic acids isolated from purified PPSMV-C preparations

Purified PPSMV-C virus preparations were mixed with 10 per cent SDS to a final concentration of 1 per cent and extracted twice with phenol:chloroform (5:1 v/v) and then with choloform. RNA from the aqueous phase was treated with RNase-free DNase I (Promega) for 1 h at 37°C to remove any host DNA. This preparation was extracted with phenol: chloroform. Aqueous phase was collected and extracted with equal volumes of chloroform. RNA from the aqueous phase was isolated using the RNaid kit following the manufacturer's instructions (BIO 101, Hartfield, Middlesex, UK). Purified viral RNA was electrophoresed in one per cent agarose gel in Tris-borate EDTA buffer, pH 8.3 and viewed under a UV-transilluminator. Single stranded RNA (ssRNA) markers were used as size standards (Cat.# G3191, Promega, Southampton, UK) (Kumar *et al*., 2003).

Northern hybridization and reverse transcriptase-polymerase chain reaction

Total RNA from 100 mg of leaves from healthy and PPSMV-C and PPSMV-P infected pigeonpea plants were extracted using the Plant RNeasy mini kit (Qiagen) and electrophoresed in formaldehyde denaturing one per cent agarose gels. The gels were blotted by capillary method onto positively charged nylon membrane The membranes were processed with a digoxygenin (DIG)-labelled probe prepared by PCR from a cDNA clone (*cd1.1 clone with* 764 bp insert) corresponding to the putative PPSMV RNA-5 segment (available at ICRISAT, Patancheru; GenBank Acc. No. AJ439561) with the PCR-DIG labelling mix following the manufacturer's instructions (Cat.# 1277065, Roche, Germany). Hybridization reactions were detected chromogenically using anti-DIG ALP-antibodies and BCIP/NBT substrate following the manufacturer's protocol (Cat.# 1681451, Roche).

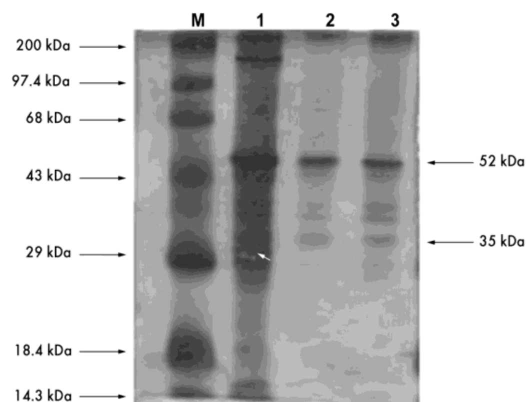
Using oligonucleotide primers, SM-1 (5'ACA TAG TTC AAT CCT TGA GTG CG3') and SM-2 (5'ATA TTT TAA TAC ACT GAT AGG A3') derived from the putative RNA-5 sequence of PPSMV-P (obtained from ICRISAT) PPSMV specific 321-bp product was amplified from purified RNA of PPSMV-C as per the procedure of Kumar *et al*. (2003). The PCR products were analysed in one per cent agarose gel using one kb DNA ladder (GeneRuler™, cat. #SM0313) as DNA marker. The gel was stained with ethidium bromide (0.5 µg/ml) and viewed under UV transilluminator.

Results and Discussion

Analysis of viral proteins by SDS-PAGE

Analysis of purified preparations of PPSMV-P and PPSMV-C in PAGE gel revealed that the c. 32 kDa protein commonly associated with SMD samples collected from central India (Kumar *et al*., 2003) was absent in the samples collected from the Coimbatore region. However, c.35 kDa protein was found associated with the SMD-affected plants collected from Coimbatore region (Fig. 1). This protein was apparently absent in the healthy controls (Results not shown).

Fig. 1. PAGE analysis of PPSMV purified protein of Coimbatore and Patancheru locations (12% gel, silver stained)

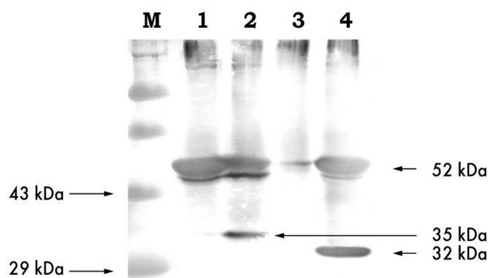


Lanes M – Marker (GIBCO, High range); 1 – PPSMV-P Purified protein ; 2&3 – PPSMV-C Purified protein

Western blot analysis of total and purified protein

Purified proteins extracted from SMD-affected leaf

Fig. 2. Western immune blot analysis of purified proteins of Coimbatore and Patancheru locations probed with PPSMV-P antiserum



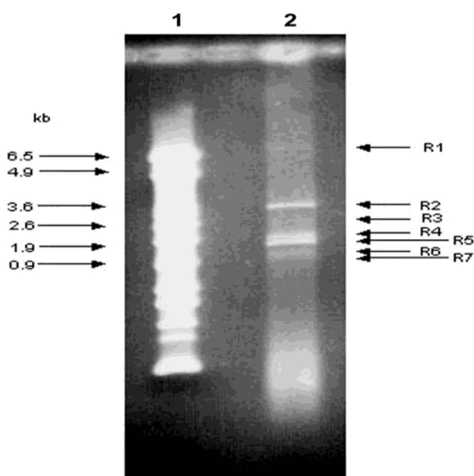
Lanes M – Marker (GIBCO, High range); 1 - Crude protein (Healthy leaf) ; 2- PPSMV-C purified protein ; 3 - Crude protein (Healthy leaf); 4- PPSMV-P purified protein

samples of ICRISAT, Patancheru and Coimbatore were analyzed by Western blot using PPSMV-P polyclonal antibodies. The polyclonal antibody detected two major proteins. One corresponding to the PPSMV specific c. 32 kDa protein in total virus preparations of samples from Patancheru (PPSMV-P). Whereas this protein was absent in purified virus preparations from SMD infected samples collected from Coimbatore. However, PPSMV-P antiserum reacted faintly with a c. 35 kDa protein in purified preparations of samples from Coimbatore region. The second major protein detected by the PPSMV-P antiserum is of c. 52 kDa host protein, which is common in all pigeonpea samples (Fig. 2).

RNA from PPSMV-C purified preparations

The RNA prepared from the purified PPSMV-C preparations migrated as 5 to 7 discrete species in the agarose gel (Fig. 3). Some RNA segments were present in low concentration (for example R1, R3). Individual RNA segments from PPSMV-C preparations varied in molar proportion. The sizes of the RNA segments are in the range of R1 (6.8 kb), R2 (2.7 kb), R3 (2.1 kb), R4 (1.6 kb), R5 (1.4 kb), R6 (1.2 kb) and R7 (1.1 kb).

Fig. 3. Resolution of PPSMV-C RNA in 1% TBE-Agarose gel

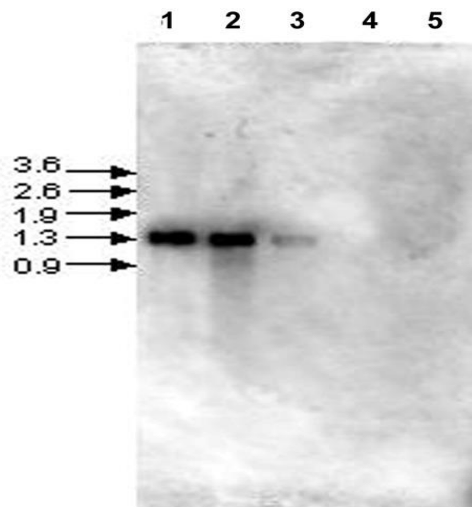


Lanes 1 – Single stranded RNA marker
2 – PPSMV-C RNA

Northern hybridization and RT-PCR

The DIG-labelled cDNA probe (cd1.1) corresponding to the putative PPSMV RNA-5, hybridized with a segment of molecular weight c. 1.4 kb band present in the total RNA preparations of PPSMV-C and PPSMV-P infected pigeonpea and French bean samples but, not with the healthy controls (Fig. 4). The oligonucleotide primers SM-1 and SM-2 derived from the nucleotide sequence of PPSMV RNA-5 specifically detected a 321 bp product from total RNA extracted from SMD-affected

Fig. 4. Northern blot hybridization using DIG-labelled cd1.1 insert as probe

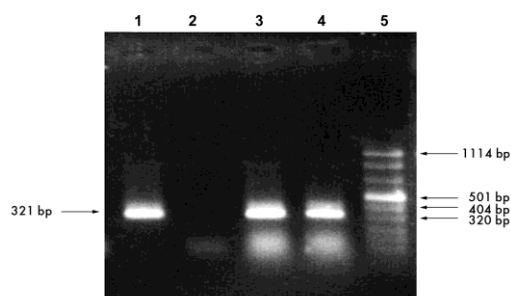


Lanes 1 – Total RNA from PPSMV-P infected pigeonpea; 2 - Total RNA from PPSMV-C infected pigeonpea ; 3 - Total RNA from PPSMV-C infected French bean ; 4 - Total RNA from healthy pigeonpea ; 5 - Total RNA from healthy pigeonpea

pigeonpea leaf samples collected from Coimbatore region. The size of RT-PCR product was similar in both PPSMV-P and PPSMV-C infected samples. No product resulted in reactions containing RNA extracts from healthy pigeonpea samples (Fig. 5).

This is the first report on the characterization of PPSMV-C, one of the pigeonpea sterility mosaic virus isolates in south India. Earlier works on Patancheru isolate of this virus greatly helped in this regard (Kumar *et al.*, 2003). Sterility mosaic disease in Tamil Nadu was first reported from Coimbatore in the year 1938. Emphasis was given to the identification of host plant resistance for disease management and thus extensive screening work was carried at Coimbatore (Nene *et al.*, 1981). However, most of the resistant lines developed at other locations and tested at Coimbatore region were found to be susceptible to disease. Subsequent studies using a set of differential pigeonpea genotypes have shown that at least five variants of SMD were prevalent in India (Reddy *et al.*, 1993), three of which are prevalent in the peninsular Indian states: variant 2 (Patancheru isolate or P isolate), variant 3 (Coimbatore isolate or C isolate) and variant 4 (Bangalore isolate or B isolate). The SMD strain at the Coimbatore region

Fig. 5 Resolution of RT-PCR products in 1% agarose gel



1 – PPSMV-P infected pigeonpea ; 2 – Healthy pigeonpea
3 – PPSMV-C infected pigeonpea ; 4 - PPSMV-C infected pigeonpea
5 – DNA marker

is a distinct isolate and the resistance sources identified at other locations are not effective in reducing the risk of SMD incidence. Additionally, no data is available on the diversity of the SMD pathogen within the Tamil Nadu state.

Characterization of PPSMV-P isolate provided a scope to further compare virus isolates at biochemical level to identify differentiating properties. Purified PPSMV-C was compared with P isolate in polyacrylamide gel electrophoresis. This revealed that the c. 32 kDa commonly detected in P isolate was absent in C isolate. However, a 35 kDa virus related protein was detected consistently in preparations of C isolate and this protein was absent in the purified healthy pigeonpea preparations, thus suggesting its apparent specific association with PPSMV-C. The 35 kDa found with C isolate may be homologous to 32 kDa protein detected with P isolate and the difference in molecular weights could be due to difference in derivative post-translational modification. However, further studies are required to assess the nature and role of 35 kDa protein. In western blot analysis, the PPSMV-P polyclonal antiserum did not detect the c. 32 kDa protein in total protein and purified preparation of Coimbatore. But in Patancheru samples, the antiserum detected the c. 32 kDa protein. In Coimbatore sample, the antiserum reacted weakly to the 35 kDa protein. This suggests that the C isolate is distinguishable from P isolate based on this protein. However, in ELISA assay differences in serological reactivity was not observed and antiserum detected isolates of Coimbatore, Vamban and Patancheru regions with equal efficiency and in DAS-ELISA (data not shown).

The RNA isolated from purified PPSMV-C migrated as 5 to 7 discrete species in agarose gel. The RNA profile of PPSMV-C was similar to the PPSMV-P (Kumar *et al*., 2003). Variation in molar proportion of individual RNA segments from PPSMV preparations were observed in the earlier studies also (Kumar *et al*., 2003). This comparative account suggested that genomic RNA profiles of P and C isolates are similar. The probe which corresponded to the PPSMV-P RNA-5 hybridized with C isolate indicating that they apparently have similar sequences.

Serological interaction and Northern blot analysis using immune and nucleoprobe of PPSMV-P clearly indicated that the PPSMV-C isolate though related to PPSMV-P could be a very distinct variant. The oligoprobe of RNA5 segment hybridized with RNA extracted from PPSMV-C infected plants. A detailed study revealed that the ribonucleic acid - nucleocapsid protein complex are organized within a spherical membrane bound bodies of (100-150 nm). The genome of PPSMV-P isolate has been resolved by NGS technology (Elbeaino *et al*., 2014). The genome consists of RNA 1 (7.0 kb) encoding RdRp gene, RNA 2 (2.2 kb) coding for glycoprotein, RNA 3 (1.4 KB) encoding nucleocapsid proein and RNA 4 (1.5 kb) for movement protein. The RNA 5 segment is 1659 bp long and encodes for one protein (P5) the function of which is uncertain (Patil and Kumar, 2015). Positive hybridization with RNA 5 probe clearly reveals that PPSMV-C genome may share more than 60% identity in the nucleotide sequence.

In the context of our recent understanding on PPSMV-P genome, characterization of PPSMV-C genome could be reasonably easy. The distinct difference in the pathogenicity studies may be clearly understood if genome of both PPSMV-P and PPSMV-C isolates are compared.

Acknowledgements

First author is grateful to the Council of Scientific Industrial Research, New Delhi, India for providing financial assistance in the form of Senior Research Fellowship. The work presented in this paper is financially supported by the United Kingdom Department for International Development (DFID) Project R8205. Views expressed are not necessarily those of DFID.

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Received after revision: December 05, 2015; Accepted: December 10, 2015