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 isolates 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 m² ha with the production of 2.57 million MT 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,
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 μl 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 chloroform. 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 digoxigenin (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).

**Western blot analysis of total and purified protein**

Purified proteins extracted from SMD-affected leaf...
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 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
isolates are similar. The probe which corresponded suggested that genomic RNA profiles of P and C 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.

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