Salient Findings on Host Range, Resistance Screening, and Molecular Studies on Sterility Mosaic Disease of Pigeonpea Induced by *Pigeonpea* sterility mosaic viruses (PPSMV-I and PPSMV-II)

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Two distinct emaraviruses, *Pigeonpea* sterility mosaic virus-I (PPSMV-I) and *Pigeonpea* sterility mosaic virus-II (PPSMV-II) were found to be associated with sterility mosaic disease (SMD) of pigeonpea (*Cajanus cajan* (L.) Millsp.). The host range of both these viruses and their vector are narrow, confined to *Nicotiana benthamiana* identified through mechanical transmission, and to *Phaseolus vulgaris* cvs. Top Crop, Kintoki, and Bountiful (F: Fabaceae) through mite transmission. A weed host *Chrozophora rottleri* (F: Euphorbiaceae) was also infected and tested positive for both the viruses in RT-PCR. Among the wild *Cajanus* species tested, *Cajanus platycarpus* accessions 15661, 15668, and 15671, and *Cajanus scarabaeoides* accessions 15683, 15686, and 15922 were infected by both the viruses and mite vector suggesting possible sources of SMD inoculum. Though accession 15666 of *C. platycarpus*, 15696 of *C. scarabaeoides*, and 15639 of *Cajanus lanceolatus* were infected by both the viruses, no mite infestation was observed on them. Phylogenetic analysis of nucleotide sequences of RNA-1 and RNA-2 of PPSMV-I and PPSMV-II isolates in southern India revealed significant divergence especially PPSMV-II, which is closely related to the *Fig* mosaic virus (FMV) than PPSMV-I. In multilocation testing of pigeonpea genotypes for their broad-based resistance to SMD for two consecutive years, genotypes ICPL-16086 and ICPL-16087 showed resistance reaction (<10% incidence) in all three locations studied. Overall, the present study gives a clear idea about the host range of PPSMV-I and PPSMV-II, their molecular relationship, and sources of resistance. This information is critical for the development of reliable diagnostic tools and improved disease management strategies.

Keywords: *Cajanus cajan*, SMD, PPSMV-I, PPSMV-II, host-range, host-plant resistance (HPR)
INTRODUCTION
Sterility mosaic disease (SMD) is one of the important production constraints of pigeonpea cultivation in the Indian subcontinent (Mitra, 1931; Reddy et al., 1998). SMD is caused by two distinct emaraviruses, *Pigeonpea sterility mosaic virus-I* (PPSMV-I) and *Pigeonpea sterility mosaic virus-II* (PPSMV-II; Elbeaino et al., 2014, 2015; Kumar et al., 2017; Patil et al., 2017; Sayiprathap et al., 2020) and transmitted by an eriophyid mite *Aceria cajani* (Acari: Arthropoda) in a semi-persistence manner (Kulkarni et al., 2002). SMD symptoms include yellow mosaic or chlorotic ring spots, reduced leaf size, stunting, excessive vegetative growth, and partial-to-complete cessation of flowering (sterility). Generally, the nature and severity of symptoms depend on the host genotype and stage of infection (Jones et al., 2004; Patil and Kumar, 2015).

Though SMD was first reported in 1931 from the Bihar state of India (Mitra, 1931), its etiology remains a mystery for several decades until Kumar et al. (1999; 2000; 2001a) unfolded the causal agent for SMD of pigeonpea as *Pigeonpea sterility mosaic virus* (PPSMV, later renamed as PPSMV-I), a putative RNA virus of negative orientation. Complete genome sequences of PPSMV-I was reported to contain four to five RNA segments (RNA-1 to RNA-5) and PPSMV-II with six RNA segments (RNA-1 to RNA-6) have been published recently (Elbeaino et al., 2015; Kumar et al., 2017; Patil et al., 2017). Based on the genome organization and morphological features, both the viruses were taxonomically included in the genus *Emaravirus* in the recently created family *Fimoviridae* of order *Bunyavirales* (Elbeaino et al., 2018). Preliminary field observations suggest that PPSMV-I was associated with chlorotic ring spots and line patterns, whereas PPSMV-II induces leaf mosaic, stunting, and sterility symptoms. A more severe form of SMD was shown by plants with mixed infection of both the viruses and more frequently occurs in nature (Elbeaino et al., 2015; Patil et al., 2017). The vector, eriophyid mite, is host specific with a narrow host range confined to pigeonpea and few of its wild relatives (Kumar et al., 2003). It is the sole vector responsible for the transmission of SMD in pigeonpea (Seth, 1962; Reddy et al., 1998; Kulkarni et al., 2002; Jones et al., 2004; Kumar et al., 2004; Patil and Kumar, 2015).

Though few alternative hosts of PPSMV have been reported, these hosts are not congenial for vector multiplication (Kumar et al., 2002a; Kulkarni et al., 2003a). The source of resistance to SMD was first reported in a pigeonpea landrace, “Sabour 2E” in India (Alam, 1933). Subsequently, several disease-resistant and tolerant lines were identified. Efforts on identifying the sources of resistance to SMD were initiated at ICRISAT in 1975. Over 13,015 pigeonpea accessions from the global pigeonpea germplasm collection at ICRISAT were screened for SMD, and 326 resistant and 97 tolerant lines were reported (Nene, 1995). Recently, 28 pigeonpea genotypes have been identified as resistant to SMD from a preliminary screening of 976 pigeonpea accessions evaluated at eight different geographical locations in India (Sharma et al., 2015).

Studies on the host ranges of PPSMV-I and PPSMV-II help us develop better management strategies; however, there is little knowledge about the host range of PPSMV and its vector. In a previous study, when there was no information about the two distinct emaraviruses associated with SMD, an attempt was made to study the natural and experimental host range for PPSMV and concluded that a couple of *Chrozophora rotteri* weed plants tested positive for PPSMV (Kulkarni et al., 2003a). A thorough understanding of the genetic variability of emaraviruses associated with SMD of pigeonpea is essential for the development of reliable and robust diagnostic tools (Kallinen et al., 2009; Walia et al., 2009; Dong et al., 2016; Stewart, 2016). Host-plant resistance is the most viable, realistic, and cost-effective option for the management of any viral disease. However, developing stable resistant varieties of pigeonpea is complicated due to the genetic flexibility of the pathogen, which is affected by location-specific environments (Nene et al., 1989; Amin et al., 1993; Sharma and Pande, 2011; Sharma et al., 2012, 2015). By keeping the above fact in view, we studied a wide range of crop and weed species, including wild *Cajanus* accessions, to identify the host range of PPSMV. The SMD samples collected from different geographical locations in southern India were analyzed for their molecular relationship between PPSMV-I and PPSMV-II. Furthermore, several pigeonpea genotypes were screened at three distinct geographical locations for two consecutive years in order to identify their broad-based resistance to SMD.

MATERIALS AND METHODS

**Pigeonpea sterility mosaic virus Inoculum**

*Pigeonpea sterility mosaic virus* culture was maintained on susceptible pigeonpea cultivar ICP 8863 (Maruti) in a glasshouse at 27 ± 1°C with 70–80% relative humidity. The leaf stapling technique (Nene and Reddy, 1976) was used to inoculate 12- to 15-day-old healthy pigeonpea seedlings.

**Transmission of Pigeonpea sterility mosaic virus**

**Mechanical Sap Inoculation**

Sterility mosaic disease-infected young leaf tissue was ground in 0.05 M phosphate buffer (1:10 w/v) containing 0.1% β-mercaptoethanol (β-ME) using mortar and pestle on an ice bucket, filtered through a muslin cloth, and inoculated immediately onto a test plant at the two- to three-leaf stage by dusting celtie (abrasive) (Sigma-Aldrich) with the forefinger. The inoculated leaves were then slowly rinsed with distilled water and kept in a vector-proof glasshouse at 27 ± 1°C.

**Leaf-Stapling Method**

Leaf-stapling method of transmission of *PPSMV* was followed as per the protocol described by Nene and Reddy (1976). Young SMD-infected leaflets collected in a moist cloth bag were observed for mite infestation under a binocular microscope to ensure a minimum of 10 mites per leaf. The mite-infested leaflets were then stapled onto test plants at the two- to three-leaf stage in such a way that the undersurface of the diseased leaflet comes...
in contact with both surfaces of the leaf of the test plant to anchor mites for transfer and their feeding results in *PPSMV* transmission onto the test plant.

**Direct Antigen Coating-ELISA**

Polyclonal antibodies to *PPSMV* were developed at ICRISAT, Hyderabad, and were used to detect the virus in plant tissues by direct antigen coating (DAC)-ELISA as per the protocol suggested by Kumar et al. (2003). To minimize the non-specific reactions to host plant antigen, polyclonal antisera were cross-absorbed in healthy pigeonpea (cv. ICP 8863) leaf sap at 10 mg/ml in phosphate-buffered saline containing 0.2% ovalbumin and 2% PVP, at 37°C for 45 min. DAC-ELISA was performed by grinding a leaf sample in carbonate buffer, pH 9.6 (1:10, w/v), and the extract was added to wells of MaxiSorp ELISA plates (Nunc, Thermo Fisher Scientific, Denmark). The

| TABLE 1 | Synthetic oligonucleotide primers used for polymerase chain reaction (PCR) amplification. |
|-----------------|-----------------|-----------------|-----------------|
| Virus            | Segment    | Primer sequence (5′—3′) | Amplicon size  |
| Pigeonpea sterility mosaic virus I (PPSMV-I) | RNA-1 | ATCTAGGTGGTGTTTTGACA | 322 bp        |
|                  | RNA-2 | GATGGTGCTATGTAGTTTGAG | 392 bp        |
|                  | RNA-3 | ACATAATGCTATTAGTCACTAATTTAAGCA | 322 bp |
| Pigeonpea sterility mosaic virus II (PPSMV-II) | RNA-1 | ATCAATACTCCATAGTGCACCT | 332 bp |
|                  | RNA-2 | GACTTACATGATTATCTCTGTA | 384 bp        |
|                  | RNA-3 | GAGAGTAGTGAGTTGGAACCGAT | 284 bp |

| TABLE 2 | Reaction of *Nicotiana* species to *Pigeonpea sterility mosaic virus* (*PPSMV*) by sap inoculation transmission. |
|-----------------|-----------------|-----------------|-----------------|
| S. no.  | *Nicotiana* spp. | RT-PCR | PPSCMV-I | PPSCMV-II |
| 1 | *N. tabacum* cv. Xanthi | – | – | – |
| 2 | *N. tabacum* cv. Smyrna (turkish) | – | – | – |
| 3 | *N. benthamiana* | + | + | + |
| 4 | *N. clevelandii* | – | – | – |
| 5 | *N. glutinosa* | – | – | – |
| 6 | *N. rustica* | – | – | – |
| 7 | *N. sylvestris* | – | – | – |
| 8 | *N. obtusifolia* | – | – | – |
| 9 | *N. suaveolens* | – | – | – |
| 10 | *N. nudicaulis* | – | – | – |
| 11 | *N. repanda* | – | – | – |

+, positive; –, negative.

**FIGURE 1** | Experimental host range of *Pigeonpea sterility mosaic virus* (*PPSMV*). Healthy *Nicotiana benthamiana* (A), sap inoculated *PPSMV* affected *N. benthamiana* exhibiting yellow mosaic symptoms (B).
cross-absorbed polyclonal antiserum was used at 1:3,000 dilution. Alkaline phosphatase (ALP)-labeled goat anti-rabbit IgG (Sigma) was used at 1:4,000 dilution for detecting the immobilized antigen–antibody complex, and p-nitrophenylphosphate (Sigma) (0.5 mg/ml in 10% diethanolamine buffer, pH 9.8) was added as the substrate. The plate was observed for color changes and recorded as weak positive for light yellow and strongly positive for deep yellow.

**Total RNA Extraction and Reverse Transcription-Polymerase Chain Reaction**

Briefly, 100 mg of leaf tissue was ground in liquid nitrogen to a fine powder. Total RNA was extracted using the QIAGEN RNeasy plant mini kit by following the manufacturer’s instructions. The RNA quantity and quality were assessed using a spectrophotometer (NanoDrop 8000, Thermo Fisher Scientific) and stored in a refrigerator at −20°C. RT-PCR was performed as per the protocol suggested by Elbeaino et al. (2015). Total RNA (500 ng) was randomly reverse transcribed by adding 4 µl of 5× M-MuLV buffer (New England Biolabs, Ipswich, MA, United States), 0.5 µl of 10 mM dNTPs, 2 µl of 10 mM DTT, 250 ng of random primer, and 200 U of M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, United States) in a final volume of 20 µl for 1 h at 37°C followed by inactivation of the enzyme at 65°C for 20 min. Synthetic oligonucleotide primers (Table 1) were used to amplify RNA segments PPSMV-I and PPSMV-II. Random-primed cDNA (2 µl) was added to 5× Taq polymerase buffer (New England Biolabs, Ipswich, MA, United States) containing MgCl2 to a final concentration of 1 mM, 0.2 mM dNTPs, 0.2 µM of each specific primer, and 1 U of Taq DNA polymerase in a final volume of 25 µl. The PCR mixture tube was incubated by 1 cycle of denaturation at 94°C for 4 min, followed by 35 cycles at 92°C for 30 s, 45–65°C for 30 s, and 72°C for 30 s. The final extension was at 72°C for 7 min. The amplification products were resolved in a 1.2% TBE agarose gel, visualized, and documented by a gel–doc system (Major Science image analyzer).

**Determining Host Range**

Seeds of 11 *Nicotiana* species obtained from the Central Tobacco Research Institute (CTRI), Rajamahendravaram, Andhra Pradesh, India, were used in the study along with five herbaceous plants, such as two pigeonpea cultivars (ICP-8863 and ICP-2376), *Phaseolus vulgaris*, *Vigna unguiculata* c-152, and *Chenopodium album*, and were grown in 8-inch pots and inoculated mechanically with SMD-infected sap and kept inside a glasshouse at 27 ± 1°C. In another set, seeds of 12 *Cajanus* species obtained from the gene bank of ICRISAT, India, were scarified by slicing the seed coat with a scalpel blade, treated with thiram at 30 mg/10 g of seeds, and were sown in 8-inch pots along with 16 cultivated species intercrossed with pigeonpea and 46 weed species raised to the two- to three-leaf stage and inoculated by leaf stapling method and kept in the glasshouse at 27 ± 1°C with 70–80% relative humidity. The test plants were monitored regularly for symptom appearance and tested for the infection of PPSMV by DAC-ELISA. The ELISA-positive samples were tested further for the infection of PPSMV-I and/or PPSMV-II using oligonucleotide primers corresponding to the RNA-3 segment through RT-PCR.

**Molecular Variability in Pigeonpea sterility mosaic virus I and II Isolates**

**Survey and Sample Collection**

Pigeonpea leaves exhibiting typical SMD symptoms were collected during a roving survey conducted in 2017/2018 from different geographical locations covering Andhra Pradesh, Karnataka, Tamil Nadu, and Telangana states in southern India. The collected samples were placed in ziplock plastic bags and transported in cold packs to ICRISAT, Hyderabad, snap frozen in liquid nitrogen, and stored in a −80°C freezer.

**Total RNA Extraction, Reverse Transcription-Polymerase Chain Reaction, Sequencing, and Phylogenetic Analysis**

Total RNA was extracted using the QIAGEN RNeasy plant mini kit by following the manufacturer’s
### TABLE 3 | Reaction of different crop and weed species to mite inoculation transmission of PPSMV.

| S. no. | Plant species          | Mites/leaf$^a$ | ELISA | RT-PCR | PPSMV I | PPSMV II |
|--------|------------------------|----------------|-------|--------|---------|----------|
| Crop species |                        |                |       |        |         |          |
| 1      | Arachis hypogaea       | -              | -     | -      | *       | *        |
| 2      | Capsicum annuum        | -              | -     | -      | *       | *        |
| 3      | Cicer arietinum        | -              | -     | -      | *       | *        |
| 4      | Dolichos lablab        | -              | -     | -      | *       | *        |
| 5      | Eleusine coracana subsp. Coracana | -      | -     | -      | *       | *        |
| 6      | Glycine max            | -              | -     | -      | *       | *        |
| 7      | Gossypium hirsutum     | -              | -     | -      | *       | *        |
| 8      | Macrotyloma uniflorum  | -              | -     | -      | *       | *        |
| 9      | Pennisetum glaucum     | -              | -     | -      | *       | *        |
| 10     | Phaseolus vulgaris cv. Bountiful | -      | +     | +      | +       | +        |
| 11     | Phaseolus vulgaris cv. Kintoki | 1      | +     | +      | +       | +        |
| 12     | Phaseolus vulgaris cv. Top Crop | 2      | +     | +      | +       | +        |
| 13     | Solarium lycopersicum  | -              | -     | -      | *       | *        |
| 14     | Sorghum bicolor        | -              | -     | -      | *       | *        |
| 15     | Vigna unguiculata c-152 | -        | -     | -      | *       | *        |
| 16     | Zea mays               | -              | -     | -      | *       | *        |

| Weed species | Mites/leaf$^a$ | ELISA | RT-PCR | PPSMV I | PPSMV II |
|--------------|----------------|-------|--------|---------|----------|
| 1            | Abelmoschus ficulneus | -     | -      | *       | *        |
| 2            | Abutilon indicum     | -     | -      | *       | *        |
| 3            | Acanthospermum hispidum | -      | -     | *       | *        |
| 4            | Achyranthes aspera   | -     | -      | *       | *        |
| 5            | Ageratum conyzoides  | -     | -      | *       | *        |
| 6            | Alternanthera pungens | -     | -      | *       | *        |
| 7            | Amananthus vivids    | -     | -      | *       | *        |
| 8            | Argeone mexicana     | -     | -      | *       | *        |
| 9            | Bidens biternata     | -     | -      | *       | *        |
| 10           | Cardiospermum helicacabum | -     | -      | *       | *        |
| 11           | Cassia tora          | -     | -      | *       | *        |
| 12           | Chenopodium amaranthicolor | -     | -      | *       | *        |
| 13           | Chenopodium album     | -     | -      | *       | *        |
| 14           | Chloris barbata      | -     | -      | *       | *        |
| 15           | Chromolaena odorata  | -     | -      | *       | *        |
| 16           | Chrozophora rotteri  | -     | +      | +       | +        |
| 17           | Crotonia juncea      | -     | -      | *       | *        |
| 18           | Cyperus rotundus     | -     | -      | *       | *        |
| 19           | Cynodon dactylon     | -     | -      | *       | *        |

| S. no. | Plant species          | Mites/leaf$^a$ | ELISA | RT-PCR | PPSMV I | PPSMV II |
|--------|------------------------|----------------|-------|--------|---------|----------|
| 20     | Datura stramonium      | -              | -     | -      | *       | *        |
| 21     | Eleusine coracana subsp. Africana | -     | -     | -      | *       | *        |
| 22     | Euphorbia heterophylla | -              | -     | -      | *       | *        |
| 23     | Euphorbia hirta        | -              | -     | -      | *       | *        |
| 24     | Hibiscus pandoformis   | -              | -     | -      | *       | *        |
| 25     | Lantana camara        | -              | -     | -      | *       | *        |
| 26     | Macroptilium atropurpureum | 2     | -     | -      | *       | *        |
| 27     | Malvastrum coromandelanum | -    | -     | -      | *       | *        |
| 28     | Mimosa pudica         | -              | -     | -      | *       | *        |

(Continued)
### TABLE 3 (Continued)

| S. no. | Plant species               | Mites/leaf<sup>a</sup> | ELISA | RT-PCR |
|--------|----------------------------|------------------------|-------|--------|
|        |                            |                        |       | PPSMV I | PPSMV II |
| 29     | *Parthenium hysterophorus* | −                      | −     | +      | +        |
| 30     | *Phyllanthus niruri*       | −                      | −     | +      | +        |
| 31     | *Physalis floridana*       | −                      | −     | *      | *        |
| 32     | *Portulaca oleracea*       | −                      | −     | *      | *        |
| 33     | *Solanum xanthocarpum*     | −                      | −     | *      | *        |
| 34     | *Solanum nigrum*           | −                      | −     | *      | *        |
| 35     | *Synedrella nodiflora*     | −                      | −     | *      | *        |
| 36     | *Tridax procumbens*        | −                      | −     | *      | *        |
| 37     | *Xanthium strumarium*      | −                      | −     | *      | *        |

<sup>a</sup>Average mites from three leaves. +, positive; −, negative; *, not tested.

### TABLE 4 | Reaction of wild *Cajanus* species to PPSMV by mite inoculation transmission.

| S. No. | Wild *Cajanus* spp. | Accession no. | No. of mites<sup>a</sup> | Type of symptom<sup>b</sup> | ELISA | RT-PCR |
|--------|---------------------|---------------|---------------------------|-------------------------------|-------|--------|
|        |                     |               |                           |                               |       | PPSMV-I | PPSMV-II |
| 1      | *C. platycarpus*    | 15666         | −                         | NS                            | +     | +      |
|        |                     | 15668         | 2                         | NS                            | −     | −      |
|        |                     | 15671         | 1                         | NS                            | −     | −      |
|        |                     | 15661         | 3                         | MM                            | +     | +      |
|        |                     | 15664         | −                         | NS                            | −     | −      |
| 2      | *C. scarabaeoides*  | 15696         | −                         | NS                            | −     | −      |
|        |                     | 15922         | 12                        | MM                            | +     | +      |
|        |                     | 15683         | 2                         | SM                            | +     | +      |
|        |                     | 15686         | 10                        | MM                            | +     | +      |
|        |                     | 15711         | −                         | NS                            | −     | −      |
| 3      | *C. seuriceus*      | 15760         | −                         | NS                            | −     | −      |
|        |                     | 15762         | −                         | NS                            | −     | −      |
| 4      | *C. acutifolius*    | 15603         | −                         | NS                            | −     | −      |
|        |                     | 15611         | −                         | NS                            | −     | −      |
| 5      | *C.abicans*         | 15614         | 3                         | NS                            | −     | −      |
|        |                     | 15620         | 2                         | NS                            | −     | −      |
| 6      | *C. mollis*         | 15658         | −                         | NS                            | −     | −      |
| 7      | *C. crassus*        | 15767         | −                         | NS                            | −     | −      |
|        |                     | 15768         | −                         | NS                            | −     | −      |
| 8      | *C. conflertiflorus*| 15674         | −                         | NS                            | −     | −      |
| 9      | *C. lanceolatus*    | 15639         | −                         | NS                            | +     | +      |
| 10     | *C. marmoratus*     | 15651         | −                         | NS                            | −     | −      |
| 11     | *C. cnenius*        | 15874         | 12                        | NS                            | −     | −      |
| 12     | *C. reticulatus*    | 15675         | −                         | NS                            | +     | +      |
| 13     | *C. cajan*          | ICP-8863      | 34                        | SM                            | +     | +      |

<sup>a</sup>Mean count from three leaves/plant.
<sup>b</sup>NS, no symptom; MM, mild mosaic; SM, severe mosaic. +, positive; −, negative.

Instructions. RT-PCR was performed using oligonucleotide primers (Table 1) to amplify partial RNA-1 and RNA-2 of PPSMV-I and PPSMV-II. Amplicons were purified and sequenced by Sanger’s dideoxy chain-termination method [ABI 3730 (48 capillaries) electrophoresis]. Later, the nucleotide homology searches were done with the BLASTN sequence analysis of the NCBI<sup>1</sup>. Multiple alignments were performed using MUSCLE (Edgar, 2004), and the phylogenetic tree was constructed by MEGA X (Kumar et al., 2018) employing maximum-likelihood (ML) criterion using the neighbor-joining method,  

<sup>1</sup>https://blast.ncbi.nlm.nih.gov/Blast.cgi
to examine the molecular relationship between and among the isolates of PPSMV-I and PPSMV-II in southern India.

**Multi-Environment Evaluation of Pigeonpea Genotypes for Their Reaction to Sterility Mosaic Disease**

Twenty pigeonpea advanced breeding lines along with SMD-susceptible genotype, ICP 8863 (Maruti), were obtained from the Pigeonpea Breeding Unit, ICRISAT, Patancheru, India. All these genotypes were evaluated for their reaction to SMD at three geographical locations, such as Bengaluru (13°04’48”N 77°34’14”E, altitude-914 m), Coimbatore (11°01’24”N 76°55’45”E, altitude-431 m), and Patancheru (17°30’35”N 78°16’31”E, altitude-547 m), in southern India for two consecutive years (rainy season 2017/2018 and 2018/2019). Plants were raised to the two- to three-leaf stage and inoculated by following the leaf stapling method as described earlier.

**Data Collection and Analysis**

The test genotypes were regularly monitored for the symptom expression, SMD incidence was recorded, and percent disease incidence was calculated using the formula:

\[
\text{% SMD incidence} = \frac{\text{Number of SMD infected plants}}{\text{Total number of plants}} \times 100
\]

Based on the SMD incidence, test genotypes were categorized as resistant (<10.0% incidence), moderately resistant (10.1–20.0% incidence), susceptible (20.1–40.0% incidence), and highly susceptible (>40.0% incidence) (Sharma et al., 2015).

**RESULTS**

**Host Range of Pigeonpea sterility mosaic virus**

Of the 11 Nicotiana species (Table 2) and five herbaceous plants tested by mechanical sap inoculation, only Nicotiana benthamiana was found positive for PPSMV infection. Symptoms appeared after 35–40 days of post-inoculation (dpi) as chlorotic spots, deformation of leaves, and stunted growth, while in the advanced stage (65–70 dpi), the symptoms were systemic and appeared on young leaves as yellow mosaic and crinkled leaves (Figure 1). However, the PPSMV was not mechanically transmitted onto the pigeonpea. The ELISA-positive samples when tested in RT-PCR were found infected with both the viruses (PPSMV-I and PPSMV-II) (Figure 2).

When 16 cultivated crop species and 46 weed species were tested (Table 3) by leaf stapling method inoculation, Phaseolus vulgaris cvs. Top Crop, Bountiful, and Kintoki (F: Fabaceae) were infected with PPSMV but not supported for mite multiplication. The symptoms appeared as stunting, reduced leaf size, and mild crinkling. A weed host, Chrozophora rottleri (F: Euphorbiaceae) was also tested positive for PPSMV infection; however, there were no visual symptoms of virus infection or mites. A member of Fabaceae, Macroptilium atropurpureum, was found infested with a few mites but tested negative for PPSMV. Similar to the Nicotiana species, all the ELISA-positive samples when tested in RT-PCR were found infected with both the viruses (Figure 2). Though P. vulgaris cvs. Top Crop, Bountiful, Kintoki, and C. rottleri were found infected with the virus, they have not supported mite multiplication, and therefore, were not considered to be a potential source of inoculum for SMD.

Among the 24 accessions of 12 wild Cajanus species tested for PPSMV infection and mite infestation (Table 4), the accessions 15661, 15668, and 15671 of Cajanus platycarpus and 15683, 15686, and 15922 of C. scarabaeoides were found positive for both the virus and mite vector. So, in nature, these can act as potential sources of SMD inoculum multiplication, whereas accession 15666 of C. platycarpus, 15696 of C. scarabaeoides, and 15639 of C. lanceolatus were infected with the virus though no mites were observed on them. While accessions 15614 and 15620 of Candida albicans and 15874 of Cajanus cinereus supported mite multiplication, no infection of PPSMV was found. The rest of the Cajanus spp. accessions were neither supportive of mite multiplication nor PPSMV infection (Figure 3). The accessions that are tested positive in ELISA were confirmed for the infection of both the viruses in RT-PCR (Figure 4).

**Phylogenetic Analysis of Pigeonpea sterility mosaic virus I and II Nucleotide Sequences**

The oligonucleotide primers targeted for RNA-1 and RNA-2 of PPSMV-I and PPSMV-II resulted in amplicons of distinct sizes (Figure 5). The nucleotide sequences of RNA-1 when subjected to phylogenetic analysis along with available corresponding sequences, the isolates of PPSMV-I and PPSMV-II, formed two separate and distinct clusters (Figure 6). The isolates Coimbatore-A BRS1117, Coimbatore-B BRS1117, Bengaluru BRS1117, Raichur BRS1117, Patancheru BRS1117, and Chevella BRS1117 of PPSMV-II clustered into a distinct subcluster, and the isolate Tandur BRS1117 distinctly separated. The RNA-1 sequence of Fig mosaic virus (FMV) also clustered with PPSMV-II isolates, whereas the RNA-1 of PPSMV-I isolates Gulbarga BRS1117 and Bengaluru BRS1117 clustered together, and the isolates Tirupati BRS1117, Vamban BRS1117, Patancheru BRS1117, and Tandur BRS1117 clustered in another subcluster. Similarly, distinct clusters were formed when the RNA-2 sequences of PPSMV-I and PPSMV-II were subjected to the phylogenetic analysis in which the RNA-2 sequence of PPSMV-I isolate Coimbatore BRS1117 and PPSMV-II isolate Gulbarga BRS1117 were distinctly separated from the rest of the isolates. The RNA-2 sequence of FMV is also clustered with PPSMV-II isolates. The RNA-1 and RNA-2 sequences of PPSMV-I and PPSMV-II isolates were phylogenetically closer. However, RNA-1 and RNA-2 sequences of PPSMV-II isolates exhibited a close relationship with FMV than the PPSMV-I.
FIGURE 3 | Typical sterility mosaic disease (SMD) symptoms on pigeonpea as yellow mosaic (A), chlorotic spots (B), mite-inoculated PPSMV infected wild Cajanus accessions exhibiting SMD symptoms (C–F).

FIGURE 4 | RT-PCR amplified product of RNA-3 segment of PPSMV-I (A) and PPSMV-II (B) from wild Cajanus accessions in 1.2% agarose gels. Standard 100-bp DNA marker (lane M), Cajanus cajan (lane 1), C. cinereus 15874 (lane 2), C. lanceolatus 15639 (lane 3), C. marmoratus 15651 (lane 4), C. scarabaeoides 15696 (lane 5), C. scarabaeoides 15711 (lane 6), C. scarabaeoides 15922 (lane 7), C. scarabaeoides 15683 (lane 8), C. scarabaeoides 15686 (lane 9), C. platycarpus 15668 (lane 10), C. platycarpus 15668 (lane 11), C. platycarpus 15671 (lane 12), C. platycarpus 15664 (lane 13), C. platycarpus 15661 (lane 14), C. sericeus 15760 (lane 15), C. sericeus 15762 (lane 16), C. mollis 15658 (lane 17), C. reticulatus 15675 (lane 18), C. acutifolius 15600 (lane 19), C. acutifolius 15611 (lane 20), C. albicans 15614 (lane 21), C. albicans 15620 (lane 22), C. crassus 15767 (lane 23), C. crassus 15768 (lane 24), and C. confertiflorus 15674 (lane 25).
Broad-Based Resistance to Sterility Mosaic Disease in Pigeonpea Genotypes

Screening of pigeonpea genotypes to SMD in different geographic locations revealed considerable variations in response to SMD infection (Table 5). Susceptible check (ICP 8863) showed a highly susceptible reaction (>40% incidence) in all three locations. The susceptible test genotypes exhibited typical SMD symptoms between 15 and 18 days after postinoculation (dpi). Among the test entries, ICPL-16086 and ICPL-16087 showed resistance reactions (<10% incidence) in all three locations, whereas genotypes ICPL-16078 and ICPL-16079 showed resistance reaction at the Bengaluru and Coimbatore locations, while at the Patancheru location showed moderate (10.1–20.0% incidence) and susceptible (20.1–40% incidence) reactions, respectively. The genotypes ICPL-16072, ICPL-16077, and ICPL-16083 expressed resistant reaction at the Coimbatore and Patancheru locations, but highly susceptible reaction at the Bengaluru location. ICPL-16050 and ICPL-16052 exhibited resistant reaction in Coimbatore, while they exhibited highly susceptible reaction at both the Bengaluru and Coimbatore locations.

DISCUSSION

Our study described the host range of PPSMV-I and PPSMV-II, the molecular relationship between them, and the source of resistance to SMD. The host range of PPSMV is narrow and confined to Nicotiana benthamiana through sap inoculation. The evidence of susceptibility of N. benthamiana to a wide range of plant viruses has been provided by Yang et al. (2004) as it has been linked to a naturally occurring mutation in an RNA-dependent RNA polymerase (RdRp) gene in the genome. The present study followed Kumar et al. (2002a; 2002b) and Manjunatha et al. (2018) who successfully transmitted PPSMV onto N. benthamiana and Nicotiana clevelandii by sap inoculation, but not on to the pigeonpea or any herbaceous hosts. Through mite inoculation, both the viruses can be successfully transmitted onto Phaseolus vulgaris cvs. Top Crop, Bountiful, Kintoki (F: Fabaceae) and Chrozophora rotteri (F: Euphorbiaceae), and was confirmed by the reports of Kulkarni et al. (2003a) and Kumar et al. (2004). However, there were contradicting observations of mite infestation on Hibiscus panduriformis, as in our study, neither the mite infestation nor PPSMV infection was found in both the field-collected samples as well as in artificially inoculated plants of H. panduriformis. Among the 24 accessions of 12 wild Cajanus species tested for the PPSMV infection, the accessions 15661, 15668, and 15671 of C. platycarpus and 15683, 15686, and 15922 of C. scarabaeoides were positive for both the viruses and supported mite multiplication, confirming earlier reports of Kulkarni et al. (2003b) and Kumar et al. (2005) that they can harbor the virus and vector and act as potential sources of inoculum in the field.

The study of the diversity of PPSMV-I and PPSMV-II associated with SMD of pigeonpea showed that these two eamarviruses are widespread across southern India. Analysis of sequence identity among the isolates of PPSMV-I and PPSMV-II indicated the presence of significant sequence variability. The RNA-1 and RNA-2 sequences of PPSMV-II isolates exhibited
a close relationship with FMV than with PPSMV-I, and it is convincing and in agreement with the previous reports of Elbeaino et al. (2015); Kumar et al. (2017), Patil et al. (2017), and Sayiprathap et al. (2020) suggesting that these two emaraviruses infecting pigeonpea have followed two independent evolutionary paths. Patil et al. (2017) reported the prevalence of the PPSMV-II in the Coimbatore and Bengaluru locations. However, when we analyzed samples from different geographical locations in

![FIGURE 6](image-url)
TABLE 5 | Screening of pigeonpea advanced breeding lines for their reaction to sterility mosaic disease (SMD) at different geographic locations during the rainy seasons of 2017/2018 and 2018/2019.

| S. no. | Genotype   | Days to symptom initiation | Bengaluru Avg. c per cent disease incidence (PDI) | Coimbatore Avg. PDI | Patancheru Avg. PDI | Overall avg. PDI | Overall reaction |
|-------|------------|-----------------------------|-----------------------------------------------|---------------------|---------------------|------------------|-----------------|
| 1     | ICPL-16050 | 16–18                       | 55.06                                        | HS                  | 0.00                | R                | 40.54           | HS              | 31.87           | S               |
| 2     | ICPL-16052 | 16–18                       | 52.74                                        | HS                  | 8.33                | R                | 51.23           | HS              | 37.43           | S               |
| 3     | ICPL-16053 | 16–18                       | 14.17                                        | MR                  | 19.62               | MR               | 26.18           | S               | 19.99           | MR              |
| 4     | ICPL-16054 | 17–19                       | 17.65                                        | MR                  | 59.52               | HS               | 61.32           | HS              | 46.16           | HS              |
| 5     | ICPL-16058 | 17–19                       | 42.86                                        | HS                  | 23.04               | S                | 67.63           | HS              | 44.51           | HS              |
| 6     | ICPL-16059 | 16–18                       | 3.13                                         | R                   | 68.24               | HS               | 43.06           | HS              | 38.14           | S               |
| 7     | ICPL-16061 | 17–19                       | 17.50                                        | MR                  | 12.14               | MR               | 38.89           | S               | 22.84           | S               |
| 8     | ICPL-16065 | 17–19                       | 45.54                                        | HS                  | 64.38               | HS               | 25.00           | S               | 44.97           | HS              |
| 9     | ICPL-16067 | 15–17                       | 56.25                                        | HS                  | 0.00                | R                | 33.52           | S               | 29.92           | S               |
| 10    | ICPL-16068 | 16–18                       | 17.65                                        | MR                  | 0.00                | R                | 59.79           | HS              | 25.81           | S               |
| 11    | ICPL-16072 | 17–19                       | 41.90                                        | HS                  | 2.94                | R                | 2.38            | R               | 15.74           | MR              |
| 12    | ICPL-16077 | 17–19                       | 46.05                                        | HS                  | 0.00                | R                | 0.00            | R               | 15.35           | MR              |
| 13    | ICPL-16078 | 16–18                       | 4.17                                         | R                   | 0.00                | R                | 18.18           | MR              | 7.45            | R               |
| 14    | ICPL-16079 | 18–20                       | 0.00                                         | R                   | 0.00                | R                | 30.30           | S               | 10.10           | MR              |
| 15    | ICPL-16081 | 17–19                       | 35.63                                        | S                   | 33.82               | S                | 30.56           | S               | 33.34           | S               |
| 16    | ICPL-16083 | 17–19                       | 42.61                                        | HS                  | 0.00                | R                | 5.26            | R               | 15.96           | MR              |
| 17    | ICPL-16085 | 18–20                       | 40.63                                        | HS                  | 10.83               | MR               | 0.00            | R               | 17.15           | MR              |
| 18    | ICPL-16086 | 18–20                       | 0.00                                         | R                   | 0.00                | R                | 2.94            | R               | 0.98            | R               |
| 19    | ICPL-16087 | 18–20                       | 0.00                                         | R                   | 2.78                | R                | 0.00            | R               | 0.93            | R               |
| 20    | ICPL-16088 | 14–16                       | 86.15                                        | HS                  | 18.89               | MR               | 0.00            | R               | 35.01           | S               |
| 21    | ICP-8863*  | 13–15                       | 91.67                                        | HS                  | 85.19               | HS               | 96.30           | HS              | 91.05           | HS              |

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aAverage percent disease incidence of SMD in 2017/2018 and 2018/2019.
bR, resistant (≤10.00%); MR, moderately resistant (10.1–20.0%); S, susceptible (20.1–40.0%); HS = highly susceptible (>40.00%). *Indicate Highly susceptible genotype/check. Bold values indicated that, genotypes exhibited resistance reaction to SMD in all 3 locations tested.

In southern India, there is an existence of both the viruses in the Coimbatore and Bengaluru locations. This development of mixed infection over the years is possibly due to the spread of the virus to these locations by its mite vector, *A. cajani*. In nature, mites are the only means of transfer of SMD causal agent to pigeonpea and not through seed, pollen, or soil (Seth, 1962; Reddy et al., 1998; Kulkarni et al., 2002; Jones et al., 2004; Kumar et al., 2004; Patil and Kumar, 2015).

Host-plant resistance is the most viable and cost-effective option for the management of any viral disease. Though several researchers identified resistant sources to SMD in the past, most of their studies involved evaluation in one location with one isolate/strain (Nene, 1995). In contrast, our efforts led to the identification of two resistant genotypes such as ICPL-16086 and ICPL-16087 with broad-based resistance to SMD. In the present study, we also found high susceptibility of pigeonpea genotypes at the Bengaluru location, so the Bengaluru isolate could be considered as the severe strain in causing SMD in pigeonpea, and this was confirmed in an earlier report too (Nene et al., 1989). The variation in the disease reaction in different locations, attributed to different eriophyid mite vectors, was ruled out previously as Kumar et al. (2001b) reported that there is only one biotype present in India, which is transferring SMD to pigeonpea. So, the possible variation in our study is mainly due to virus variants, as Reddy et al. (1993) identified five distinct virus variants in India with different levels of virulence. There are conflicting reports about the genetics of resistance to SMD claiming both resistance and susceptibility to being dominant. Nevertheless, in most cases, susceptibility was shown to be dominant, and resistance is controlled by recessive genes (Singh and Vishwadhar, 2003). The resistance to SMD has been reported to be controlled by a single recessive gene (Srinivas et al., 1997) with oligogenic nature (Sharma et al., 1984; Gnanesh et al., 2011).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/online.

AUTHOR CONTRIBUTIONS

BS conceptualized the work in discussion with AP and HS, executed the work in the field and glasshouse, and wrote the initial draft of the manuscript. VP, KJ, KS, and MS provided necessary inputs in designing the work plan. HR, ER, and LK provided all necessary support in the execution of the work in various locations. All authors contributed to the article and approved the submitted version.
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