STUDIES ON PIGEONPEA STERILITY MOSAIC DISEASE; TRANSMISSION, VIRUS-VECTOR RELATIONSHIPS AND IDENTIFICATION OF RESISTANT SOURCES

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Affectionately Dedicated to my

Beloved parents, brother, sister & Sonu

Whose support and love has made it possible

DEPARTMENT OF PLANT PATHOLOGY
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CERTIFCATE

This is to certify that the thesis entitled, ***"STUDIES ON PIGEONPEA STERILITY MOSAIC DISEASE; TRANSMISSION, VIRUS-VECTOR RELATIONSHIPS AND IDENTIFICATION OF RESISTANT SOURCES"*** submitted by ***Mr. Naveenkumar Kulkarni*** for the award of the degree of ***DOCTOR OF PHILOSOPHY in PLANT PATHOLOGY*** to the University of Agricultural Sciences, GKVK, Bangalore, is a record of *bona-fide* research work done by him during the period of this study under my guidance and supervision and the this thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar studies.

Bangalore
April, 2002

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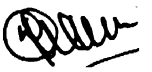

(Naveenkumar Kulkarni)

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INTRODUCTION

I. INTRODUCTION

Pigeonpea [*Cajanus cajan* (L) Millspaugh] is an important drought resistant pulse crop cultivated mainly for its protein-enriched seed in the tropics and subtropics of Asia, Africa and the Caribbean. The pigeonpea seed serves as the major dietary protein source with >30% of protein for large populations in the semi-arid tropics. Among the legumes, pigeonpea ranks fifth in area and fourth in production after beans, peas and chickpea but is used in more diverse ways than other pulse crops (Van der Maeson, 1995). India accounts for nearly 85 per cent of the world's pigeonpea production with an acreage of 3.57 m. ha and annual production of 2.36 m. tons (Muller *et al.*, 1990). Pigeonpea in India is mainly grown in the semi-arid regions of the states of Andhra Pradesh, Karnataka, Maharashtra, Madhya Pradesh, Tamil Nadu and Uttar Pradesh because of its drought resistance. Development of many high yielding short duration cultivars that can fit into diverse cropping systems, including cereal based ones, lead to increase in area of pigeonpea cultivation. The simultaneous use of pigeonpea for food, fodder and fuel, its ability to ameliorate soils and its use as a hardy crop on marginal soils fitting into many intercropping systems makes pigeonpea an important crop in the semi-arid tropics.

However, production of pigeonpea in the Indian subcontinent and other countries in Asia is severely affected by sterility mosaic disease (SMD) (Ghaneker *et al.*, 1992). SMD affected plants show mosaic symptoms on leaves and cease flowering rendering the plant sterile, but a few SMD-tolerant pigeonpea cultivars show chlorotic ring spots or mild mosaic symptoms, without significant effects on flowering (Reddy *et al.*, 1998). This disease was first reported from Pusa, Bihar state (Mitra, 1931), subsequently, from several states of India. It is currently regarded as a serious problem

in Bihar, Karnataka, Maharashtra, Uttar Pradesh and Tamil Nadu. Yield losses in susceptible genotypes due to SMD occurring early in the season can reach over 90 per cent, with an annual loss exceeding 2.05 lakh tons (valued at US$ >150 million) of grain in India alone (Kanniyan *et al.*, 1984). In nature, the causal agent of SMD is transmitted by the eriophyid mite, *Aceria cajani* ChannaBasavanna, but it is not transmitted through seed, pollen or soil (Reddy *et al.*, 1998). The mite vector is highly host-specific and, because of this, the natural host range of SMD is restricted to pigeonpea and a few of its wild relatives (Reddy *et al.*, 1998). Experimentally, SMD can be transmitted by grafting, but not by mechanical sap inoculations (Ghanekar *et al.*, 1992; Reddy *et al.*, 1994; Singh *et al.*, 1999). Despite extensive research, SMD causal agent remained unidentified. All previous studies on the SMD causal agent indicated that it is likely to be a virus or virus-like agent (Ghanekar *et al.*, 1992; Nene, 1995), but attempts to isolate and characterise the putative virus have not been successful (Reddy *et al.*, 1994; Singh *et al.*, 1999). However efforts by ICRISAT and ICAR resulted in the identification of several pigeonpea genotypes with field resistance to SMD (Ghanekar, *et al.*, 1992)). Selection of resistant genotypes was based on visible symptoms. The mechanism of resistance was not characterised. However diverse mechanisms were presumed to govern resistance to SMD. Resistance was attributed to either the pathogen or to the vector or to the both (Reddy and Nene, 1980; Muniyappa and Nangia, 1982; Sharma *et al.*, 1984; Saxena and Sharma, 1990; Reddy *et al.*, 1995). The Majority of the genotypes showed location specific resistance and this was attributed to the biodiversity among the isolates of the causal agent of SMD or to the involvement of *A. cajani* biotypes or to the occurrence of various species of *Aceria* mites (Reddy *et al.*, 1998). Further progress in development of integrated management programmes for SMD control impeded due to lack of knowledge on SMD

causal agent, absence of sensitive techniques for unambiguous disease diagnosis and factors contributing for location specific resistance.

In very recent studies, using a new purification method, SMD causal agent was identified as a novel virus, provisionally named Pigeonpea sterility mosaic virus (PPSMV) (Kumar *et al.*, 1999; 2000). Purified PPSMV preparations contain very thin, highly flexuous virus like-particles (VLPs) of c. 3-10 nm diameter and of undetermined length, a 32 kDa protein and up to 6 RNA species of 0.8 - 3.5 kb (Kumar *et al.*, 2001a). ELISA- and RT-PCR-based diagnostic tools for the precise and sensitive detection of virus in infected plants were developed (Kumar *et al.*, 2001a). The purified PPSMV preparations were not infective to plants, but PPSMV in crude plant extracts was transmitted experimentally to herbaceous hosts (*Nicotiana benthamiana* and *N. clevelandii*) by mechanical inoculations (Kumar *et al.*, 2002). In a separate study, mite populations obtained from SMD-affected plants from various SMD-endemic locations from the Indian subcontinent were analysed for biodiversity using PCR-based nuclear ribosomal DNA fingerprinting technique and morphological studies using scanning electron microscopy (Kumar *et al.*, 2001b). It was apparent that there was no other *Aceria* mite species or biotypes of *A. cajani* that differed in their ability to transmit PPSMV. It was therefore concluded that resistance breakdown at some locations is due to the existence of PPSMV strains and this was confirmed (Reddy *et al.*, 1998; Kumar *et al.*, 1999; 2001b).

In order to formulate meaningful control measures, characterisation of the causal virus, elucidation of its mode of transmission and disease epidemiology are essential. Since the vital information on SMD causal agent and tools for its detection are available, this study was undertaken to elucidate the virus-vector relationships of

PPSMV. Although some information on transmission of SMD causal agent by *A. cajani* was reported earlier (Reddy *et al.*, 1989), precise information on virus-vector relationships is lacking.

The reported natural host range of SMD is restricted to pigeonpea and a few of its wild relatives (Reddy *et al.*, 1998). Wild *Cajanus* species seldom showed clear symptoms, therefore the status of SMD infection on many wild relatives of pigeonpea and naturally occurring weed species is not known. In this study a range of plants, especially *Leguminaceous* members were tested for susceptibility to PPSMV to determine the experimental host range. Additionally, several naturally occurring weeds and other plant species in and around the SMD affected pigeonpea fields were analysed to determine the alternative virus sources.

Very few of the pigeonpea genotypes identified in the previous study were found to contain broad-based resistance to SMD. Identification of pigeonpea sources that possess broad-based multiple resistance is vital to enhance the pigeonpea production. Wild relatives of cultivated plant species have been suggested to contain useful resistant genes for diseases and pests (Remanandan, 1981). Wild relatives of pigeonpea, *C. platycarpus*, *C. cajanifolius* and *C. albicans*, *C. scaraboiedes* and several accessions of these species collected from wide eco-geographical regions are in the gene bank of ICRISAT (Remanandan, 1990). Screening of these accessions for resistant genes indicated that many of the wild *Cajanus* accessions possess multiple resistance to wilt, *Alternaria* blight, *Phytophthora* blight, pod borer and some important nematode species (Sharma *et al.*, 1987; Remanandan, 1990). Furthermore, these accessions were compatible for inter-specific hybridisation with cultivated pigeonpea and desirable genes could be transferred by conventional breeding (Reddy and Nene,

1981). In this study accessions of wild *Cajanus* species were screened for the identification of resistance to PPSMV isolates and to *A. cajani* for locating broad-based resistance to SMD, and to improve the genetic base of multiple resistance found in pigeonpea genotypes, and to assess the compatibility of resistant wild *Cajanus* accessions in breeding programmes.

Integrated management of SMD includes cultivation of resistant genotypes, changing sowing dates and vector control using pesticides. However, the later two options have limitations and cultivation of resistant sources is the most viable option available for the farmers. This study was aimed to identify broad-based durable SMD resistant sources, understand the virus-vector relationships and mode of transmission of PPSMV, and to determine the alternative inoculum sources of PPSMV, for formulating the efficient management strategies for SMD control.

The objectives of the present study are

1. Transmission of PPSMV.
2. To determine the virus-vector relationships and the mode of transmission of PPSMV.
3. To identify the broad-based durable SMD resistant sources.
4. To identify the alternative sources of PPSMV.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

2.1. Pigeonpea crop

Pigeonpea is an important food legume in the Indian sub-continent, which accounts for almost 90% of the world's crop (Nene and Sheila, 1990). It is mainly cultivated for seed, which contains nearly 30% protein and provides a vital protein diet for estimated 1.1 billion people around the world. Among legumes, it ranks fifth in area and fourth in production after beans, peas and chickpea, but it is used in more diverse ways than other pulse crops (Van der Maesen *et al.*, 1985). It is produced commercially in India, Myanmar, Kenya, Malawi, Uganda, and grown to a lesser extent in many other tropical countries of Asia and South America (Van Der Maeson *et al.*, 1985). Pigeonpea is commonly grown as an annual, intercropped with cereals, fibre crops and other legumes. It is also grown as a perennial, particularly in hedges (Nene and Sheila, 1990).

2.2. Pigeonpea diseases

More than 210 pathogens have been reported to infect pigeonpea (Nene *et al.*, 1996). These include fungi, bacteria, viruses, nematodes and phytoplasmas. However only few diseases are economically important (Kanniyan *et al.*, 1984; Reddy *et al.*, 1998). The important diseases include, *Fusarium* wilt, Sterility mosaic, *Phyotophthora* blight, yellow mosaic, witches broom, rust, *Cercospora* leaf spot, *Macrophomina* root rot, stem canker and *Alternaria* leaf blight (Nene *et al.*, 1996). Because of the wide spread occurrence and high economic losses caused, extensive research has been done on sterility mosaic, *Fusarium* wilt and *Phyotophthora* blight.

Natural infection of pigeonpea with 15 viruses, 3 virus like diseases and one viroid has been reported (Nene *et al*, 1996; Brunt *et al.*, 1996a; Reddy *et al.*, 1998). Pigeonpea is susceptible to 25 of 49 viruses tested by experimental inoculation (Brunt *et al.*, 1996b). Majority of the viruses, that infect pigeonpea cause mosaic, stunting and proliferation of vegetative growth (Reddy *et al.*, 1990).

2.3. Sterility Mosaic Disease (SMD)

Sterility mosaic disease (SMD) is the most important virus disease of pigeonpea in the Indian subcontinent cause yield loss of 205,000 tons annually in India (Kannaiyan *et al.*, 1984). SMD was first reported from Pusa, Bihar state (Mitra, 1931). It was regarded as a serious problem in Bihar, Gujrat, Uttar Pradesh, Karnataka, Tamil Nadu (Kannaiyan *et al.*, 1984), Maharashtra (Muniyappa and Chandrashekhariah, 1980, Reddy, *et al*,.1992) and Chhattisgarh (Singh *et al.*, 1999). SMD was also reported from Bangladesh, Nepal and Thailand (Nene *et al.*, 1989), Myanmar (Su, 1931) and Sri Lanka (Newton and Peiris, 1953).

SMD was first described in detail by Alam (1933). Capoor (1952) established the infectious nature of the disease by graft transmission. Seth (1962) showed that under natural conditions SMD is transmitted by an eriophyid mite vector, *Aceria cajani* ChannaBasavanna. Kumar *et al.* (1999; 2000) established that SMD is caused by a virus referred to as Pigeonpea sterility mosaic virus (PPSMV).

2.3.1. Symptoms

SMD infected plant, shows a conspicuous mosaic symptoms and become sterile. Therefore the disease is referred to as 'sterility mosaic'. SMD is characterized by bushy and pale green appearance of plants, drastic reduction in

leaf size, leaf distortion, mosaic and mottling of leaves, increased number of secondary and tertiary branches from leaf axils and complete or partial cessation of reproductive structures (Alam, 1933; Capoor, 1952; Kandaswamy and Ramakrishnan, 1960; Seth, 1962; Prasad, 1965; Nene, 1972; Reddy *et al.*, 1990). Late infected plants may not show clear symptoms, but when ratooned, the new growth shows clear mosaic symptoms and sterility (Reddy and Nene, 1980).

SMD symptoms depend on the genotype and usually are of three types; a) severe mosaic on leaves with complete sterility, b) mild mosaic with partial sterility and c) chlorotic ring spots without any sterility (Reddy *et al.*, 1998). During multilocational screening trials variation in symptom expression by some genotypes were noted (Reddy *et al.*, 1998). A variety showing resistant (no symptoms) or susceptible reaction (mosaic or chlorotic ring spots) at one place may show a different kind of reaction at another location. For example, ICP2376 at Patancheru showed chlorotic ring spots, whereas at Bangalore it showed sever mosaic symptoms. This variation in symptom expression by some genotypes and at certain locations is now attributed to existence of different virus strains (Reddy *et al.*, 1998; Kumar *et al.*, 1999a; 2001b). The virus isolates present in Bihar and Nepal cause severe reduction in internodes, shortening of the branches and leaves become filiform (Reddy, *et al.*, 1998). Pigeonpea is a cross-pollinated crop. In addition to environmental factors, genotypic variability induced as a result of cross-pollination also likely to play an important role in symptomatology. Thus, variability in the pathogen, the plant genotype, the environment, and the mixed infection with other pathogens, may all influence the symptom expression.

2.3.2. Yield losses

The extent of yield loss depends on the genotype and the age of plant at the time of infection. A susceptible genotype infected at an early stage (<45 days) of crop growth showed complete sterility and up to 100 per cent yield loss, but as the plant matured (>45 days), susceptibility to the virus decreased and such plants showed partial sterility (Muniyappa and Nangia, 1975; Reddy and Nene, 1981). Drastic reduction in pod length, pod width, number of grains per pod and 1000 grain weight (Singh and Rathi, 1994 and 1997), shoot and root weight, nodule weight and nodule number (Prameela, *et al.*, 1990) has been reported in SMD infected pigeonpea genotypes. Losses due to SMD incidence was found to be high in ratooned and perennial pigeonpea. The estimated yield losses in pigeonpea due to SMD is >205000 tons of grain, worth of Rs. 750 million in India alone (Kannaiyan *et al.*, 1984).

2.3.3. Physiological and biochemical studies

Some aspects related to the physiological and biochemical changes in SMD infected pigeonpea were studied. Decrease in total carbohydrate content, chlorophyll, carotene, xanthophyll, sugar synthesis and its translocation, chloroplast proteins, C:N ratio, peroxidase activity, organic acid, ascorbic acid contents and increased activity of chlorophyllase, catalase, nitrate reductase and proteolytic enzymes and presence of unidentified aminoacids in SMD affected pigeonpea leaves were reported (Narayanaswamy and Ramakrishnan, 1965 a, b, c & d). Reduction in reducing sugar contents, RNA and DNA levels, total nitrogen and free aminoacids; increase in respiration of diseased plants was shown to be accompanied by general reduction in organic acid content, but accumulation of citric

acid and succinic acid was noticed in the stems and roots (Nambiar and Ramakrishnan, 1968, 1969 a and b). Calcium, potassium, sodium and magnesium contents were found to be less and total nitrogen was found to be high in diseased plants (Nambiar and Ramakrishnan, 1969a, 1969b). Lower dry weight, chlorophyll content and photosynthetic rate and Hill reaction (Natarajratnam *et al.*, 1986; Singh and Mall, 1976, 1978) and presence of specific peroxidase enzymes and proteins (Rathi *et al.*, 1986) were observed in SMD infected plants. In addition to physiological and biochemical changes, reduction in leaf thickness, epidermal and palisade cell also was observed in SM infected pigeonpea plants (Prameela *et al.*, 1990).

2.3.4. The causal agent of SMD

Though SMD was reported in 1931, continuous efforts to identify the causal agent in several laboratories were unsuccessful. Based on the symptoms and mode of transmission the SMD causal agent was assumed to be a virus (Capoor, 1952). The role of mite toxaemia, fungi, bacteria, nematodes, phytoplasma and viroid in SMD was ruled out (Reddy *et al.*, 1989; Ghanekar *et al.*, 1992; Reddy *et al.*, 1994; Nene, 1995, Singh *et al.*, 1999). Ultrathin section studies revealed no virus-like particles (VLPs) or inclusion bodies in the infected tissues (Ghanekar *et al.*,1992; Reddy *et al.*, 1994).

Based on the Azure-A staining of nuclear inclusions association of a foreign ribonucleoprotein in the phloem cells of diseased leaf mid vein was reported. On this basis, it was concluded that the agent is probably a RNA containing virus (Singh and Rathi, 1996a). Recently, a breakthrough was made in identifying the causal

agent of SMD. It is shown to be caused by a virus, referred to as Pigeonpea sterility mosaic virus (PPSMV) (Kumar *et al.*, 1998; 1999a&b; 2000a&b; 2001a&b; 2002).

2.3.5. Pigeonpea sterility mosaic virus (PPSMV)

Purified PPSMV preparations contain very thin, highly flexuous virus like-particles (VLPs) of c. 3-10 nm diameter and of undetermined length, a 32 kDa protein and up to 6 RNA species of c. 1.1 – 3.5 kb. Polyclonal antiserum produced to purified PPSMV preparations detected the 32 kDa protein in sap of SMD-affected pigeonpea plants by ELISA and Western blotting (Kumar *et al.*, 2001a&b). The nucleotide sequence of some cDNA clones made to PPSMV RNA and the analysis of the virus-specific 32 kDa protein by matrix-assisted laser desorption ionisation-time of flight (MALDI-ToF), found no significant sequence matches to any known viral sequences in database searches (Kumar *et al.*, 2001a&b). Oligonucleotide primers were derived and a reverse transcription (RT)-PCR-based method for sensitive detection of PPSMV in plants was developed. The purified PPSMV preparations were not infective to plants. However, PPSMV was transmitted experimentally to *Nicotiana benthamiana* and *N. clevelandii*, by mechanical inoculation of fresh leaf sap extracts of SMD-affected pigeonpea (Kumar *et al.*, 2002).

The taxonomic relationship of PPSMV to other viruses is not clear. Morphology of particles in purified preparations and the number and sizes of its apparent nucleoprotein components has some similarities to members of the genus *Tenuivirus* and to the recently reported High Plains Virus (HPV) (Jensen *et al.*, 1996; Falk & Tsai, 1998). PPSMV and HPV are each transmitted by eriophyid mites,

and infected plants contain a virus-specific 32 kDa protein and up to 6 RNA species (Jensen *et al.*, 1996; Kumar *et al.*, 2001a&b; Mirabile *et al.*, 2001). However, the two viruses are serologically unrelated and differ in host range and in the vector mite species (Kumar *et al.*, 2001a&b; Jensen *et al.*, 1996).

Ultrastructural studies of symptom-bearing leaves of two pigeonpea cultivars, (ICP8863 and ICP2376) and *N. benthamiana* infected with PPSMV, detected quasi-spherical, membrane bound bodies (MBBs) of c. 100-150 nm and amorphous electron-dense material (EDM) (Kumar *et al.*, 2002). These structures were distributed singly or in groups, in the cytoplasm of all the cells, except those in conductive tissues. Fibrous inclusions (FIs), composed of randomly dispersed fibrils with electron lucent areas, were present in the cytoplasm of infected palisade cells (Kumar *et al.*, 2002). The MBBs and associated inclusions are similar in appearance to those reported for plants infected with the eriophyid mite-transmitted HPV and the agents of unidentified aetiology associated with rose rosette, fig mosaic, thistle mosaic, wheat spot chlorosis and yellow ringspot of budwood (Bradfute & Nault, 1969; Appiano, 1982; Roberts & Jones, 1997; Ahn *et al.*, 1996; 1998; Kumar *et al.*, 2002). *In situ* immuno-gold labelling (IGL) experiments on and PPSMV and HPV with respective antisera, indicated that MBBs may be are novel virus-like particles (Ahn *et al.*, 1998; Kumar *et al.*, 2002).

2.3.6. Transmission of the PPSMV

Graft transmission of PPSMV was first showed by Capoor (1952), subsequently confirmed in several studies (Seth, 1962; Narayanaswamy and Ramakrishna, 1965; Janarthan, 1973; Nene, 1972 and Mali *et al.*, 1977). Tissue implantation method of graft transmission was established by Ghanekar *et al.* (1992), but the transmission

rate with this technique was low (up to 12%). Seth (1962) first showed that under natural conditions PPSMV is transmitted by the eriophyid mite, *Aceria cajani* ChannaBasavana. This was further confirmed by Nene, (1972); Nene and Reddy, (1976a); Ramakrishnan and Kandaswamy, (1972) and Reddy *et al.* (1989). Report on nematode transmission of PPSMV by Narayanaswamy *et al.* (1963) was not confirmed (Mc Rae, 1932; Nene, 1972; Ramakrishnan and Kandaswamy, 1972 and Mali *et al.*, 1977; Ghanekar, *et al.,* 1992). Several experiments on PPSMV transmission suggested that the PPSMV is not transmitted through sap, seed, soil or pollen (Capoor 1952; Nene, 1972; Anon. 1979; Ghanekar, *et al* 1992 ; Reddy *et al.*, 1994) or through dodder (Seth, 1965; Nene, 1972; Reddy *et al.*, 1994).

Capoor (1952) reported the transmission of PPSMV by mechanical sap inoculation, but this report was not confirmed in subsequent studies (Ghanekar *et al.*, 1992). However in a recent study PPSMV was transmitted experimentally to *N. benthamiana* and *N. clevelandii* by mechanical inoculation of fresh leaf sap extracts of SMD-affected pigeonpea (Kumar *et al.*, 2002). This study showed that PPSMV can, with difficulty, be transmitted by mechanical inoculation of sap to *Nicotiana* species. However, in these plants symptoms and virus detection occurred after only an unusually long time (40+ days). Without the serological assay for PPSMV, such infected plants might probably have escaped detection. Attempts to transmit PPSMV from sap extracts on to pigeonpea were unsuccessful. The purified PPSMV preparations were not infective to pigeonpea or to *Nicotiana* species. This is the first reliable report of mechanical transmission of PPSMV on hosts outside *Cajanus* genus.

Three methods are being used for experimental transmission of PPSMV *viz.*, the 'leaf stapling technique' (Nene and Reddy, 1976a), 'infector-hedge technique' (Nene *et al.*, 1981a) and 'spreader row' inoculation technique (Nene *et al.*, 1981b). All these methods use mites to transmit virus from source leaf to the healthy plant.

2.4 The mite vector, *Aceria cajani*

Eriophyid mites (Arthropoda: Acari: Eriophyidae) are amongst the smallest arthropods and are obligate plant pests in all active stages of their life cycle. Several of them cause direct damage by affecting plant growth and some indirectly by acting as vectors of plant viruses (Keifer et al., 1982; Oldfield and Proeseler, 1996). *Aceria cajani* measures about 200-250 μm in length and can be seen under a stereomicroscope. These mites have short life cycle of less than 2 weeks that comprise an egg, two nymphal stages, and an adult (Janarthan, 1973 and Ramakrishnan and Kandaswamy, 1972; Oldfield *et al.*, 1981). Eggs of *A. cajani* are milky white, oval, translucent and measuring 30x40 μm. At room temperature eggs hatch in 3-5 days and the adult emerges from the final nymphal stage about a week later (Oldfield *et al.*, 1981). Like other eriophyids, *A. cajani* is highly host specific and is restricted to pigeonpea and some of its wild relatives, *Cajanus scarabaeoides* and *C. cajanifolia* (Reddy *et al.*, 1990). They feed on the lower surface of the leaf with short cheliceral stylets. The short stylets (~ 2.03μm) of these mites allow penetration of epidermal cells and the mite feeding cause no obvious damage to pigeonpea (Sheila *et al.*, 1988). On an average eriophyid chelicerae can penetrate plant tissue

up to 15-36 μm depth (Paliwal, 1980). *A. cajani* are distributed on all stages of leaves with their number more on young leaves (Reddy *et al.*, 1989). Their population density is more on SMD infected pigeonpea plants than on healthy. Even on infected plants, mite populations are concentrated towards the petiole end of young leaves and more than 90 per cent of the mites occur on the lower surface of the leaves (Dhar and Rathore, 1994). Several studies have shown that *A. cajani* populations on pigeonpea are uniform throughout the year in cooler parts of India, where as in semi-arid zones at higher temperatures mite populations decreased (Reddy and Raju, 1993; Lakshmikantha *et al.*, 1997). Dispersal and spread of mites in nature is passive and depend on wind currents. Incidence and spread of the disease depends on vector population in the field (Reddy *et al.*, 1989). Five meter/cm^2 of leaf area was correlated with SMD spread in the field and less than one mite/leaf resulted in very mild SMD incidence (Dhar *et al.*, 1998).

2.5. Studies on virus-vector relationships

About a dozen of eriophyid mites were reported to be vectors for important plant viruses and several other pathogenic agents of unknown etiology (Hiruki, 1992; Maramorosch, 1994; Oldfield and Proeseler, 1996). The relationship between eriophyid vector and transmitted agent is highly specific. The plant pathogens transmitted by eriophyid mites are not known to be transmitted by other members of any other taxa or usually by more than one species of eriophyid (Oldfield, 1970).

Studies related to virus-vector relationships have been slow due to several technical difficulties associated with handling and maintenance of eriophyid mites. More than 70 years ago black currant reversion disease agent was first recognised to be associated with mites (Amos *et al.*, 1927). Since then little progress has been

made in understanding the specifics of the transmission mechanisms of mite-born viruses. The best understood mite-virus relationship is that of wheat streak mosaic disease (WSMV) and its vector *Aceria tosichella*. The progress of such studies was impeded because of the microscopic size and delicate body of the eriophyids and their propensity to bury deep in the host tissue. Additionally, poor understanding of their anatomy, physiology and feeding habits, which are different from other arthorpods posed problems in studying virus-vector relationships (Oldfield, 1970; Paliwal, 1980). Studies using individual mites are extremely difficult due to difficulties associated in confining mites on a plant or particular plant part as these tiny creatures can escape through unnoticed openings rather than stay on a portion of leaf on which we would like them to feed (Del Rosario and Sill, 1964). For example, Staples and Alligton (1956) used a folded leaf method to study the life cycle of individual *A. tulipae* on wheat leaf. In only nine out of several hundred attempts, the complete life cycle of mites from egg to adult was followed.

2.5.1. Generation of non-viruliferous mite colony

Generation of non-viruliferous (healthy) mite colonies is essential to study the virus vector relationships. Since there is no transovarial transmission of eriophyid mite-transmitted viruses, eggs were used for generating non-viruliferous mite colonies (Slykhuis, 1965). For example; healthy mite colony of *A. tosichella* was established by transferring mite eggs on to the healthy plants (Paliwal, 1980; Mahmood and Hein, 1997). Ghanekar *et al*, (1992) described a simple method to generate non-viruliferous *A. cajani* colony using a pigeonpea cultivar, ICP8136, that supports mite multiplication, but resistant to virus. For this, SMD infected leaves carrying mites were stapled on to ICP 8136. After 30 days, leaflets carrying mites were transferred onto new batch of ICP 8136 plants to obtain virus free mite colonies.

2.5.2. Efficiency of transmission

Efficiency of eriophyid mite vectors in transmitting viruses vary from species to species, and also depends on the host and type of virus it transmits. About 30-50 per cent of *A. tosichella* population could transmit WSMV and wheat spot mosaic virus (WSpMV), and transmission efficiency of an individual *A. tosichella* ranged between 40-67 per cent (Slykhuis, 1965; Orlob, 1966). Efficiency of individual *Eriophyes insidiosus* to transmit *Peach mosaic virus* varied between 2 to 17 per cent (Wilson and Oldfield, 1966; Gispert *et al.*, 1998). Efficiency of *A cajani*, of single mite was reported to range between 20 to 60 per cent in transmitting PPSMV (Reddy *et al.*, 1989).

2.5.3. Acquisition access period (AAP)

Orlob (1966a) showed that *A. tosichella* acquired WSMV in 15 min of AAP. However the transmission rate was low (<1%). Fifty per cent of mites acquired WSMV after 16 h of AAP. *Abacarus hystrix* acquired *Ryegrass mosaic virus* (RMV) within 2h of AAP but more number of mites acquired and transmitted the virus with an increased AAP of 12 h (Mulligan, 1960; Heard and Roberts, 1975). *Aceria ficus* found to acquire fig mosaic pathogen within 5 min (Proeseler, 1972) and *Cecidophyopsis ribis* acquired *Blackcurrant reversion virus* (BRV) within 3 h of AAP (Jacob, 1976 a&b). *E. insidiosus* required 3 days to acquire PMV (Gispert, *et al,* 1998). *A. cajani* required 5 min to acquire SMV (Reddy, *et al.,* 1993).

2.5.4. Inoculation access period (IAP)

Minimum IAP for *Aceria tosichella* to transmit WSMV was 10 min. However, after 16 h of IAP, >50 per cent transmission was achieved (Orlob, 1966a). A minimum of 48

h of IAP required for *C. ribis* to inoculate BRV (Jacob, 1976 a & b). *Aceria ficus* required 5 min to inoculate fig mosaic pathogen (Proeseler, 197[illegible]). *E. insidiosus* required 6 h of IAP to inoculate PMV (Gispert, *et al*, 1998) and for *A. cajani* to inoculate SMV, >10 min IAP is required (Reddy, *et al.*, 1993).

2.5.5. Virus retention period (VRP)

Different methods were used to study the VRP in eriophyid mite vectors. Slykhuis (1955) developed a technique to study the retention of WSMV in *A. tosichella*. In this study, infective mites were transferred to an immune host and from this plant, mites were transferred on to susceptible plants at regular intervals. This study showed that, *A. tosichella* retained WSMV for at least six days. Similarly, Del Rosario and Sill, (1965) observed no loss of WSMV infectivity in *A. tosichella* for four days. Using the same technique retention period for different viruses in the eriophyid mite vectors were determined. [*A. hystrix* retained Rye grass mosaiv virus (RgMV) for at least 24 hours (Mulligan, 1960); *A. tulipae* retained WSpMV for 8 days (Nault and Stayer, 1970); retention period of BRV in *C. ribis* was 25 days (Jacob, 1976 a&b)]. Nene and Reddy, (1976) showed that *A. cajani* would not retain the PPSMV untill the death of the mite vector.

Serial transfer of eriophyid mites from one plant to another at a regular interval was also used to determine the virus retention period. Retention of WSMV in *A. tosichella* was tested by serial transfer of a large number of viruliferous mites from infected to healthy wheat seedlings. Mites were then moved to new plants after every 24 hours. This study showed that the infective mites did not replenish virus from healthy plants on which they were fed for 24 hours. However, in these experiments recovery of mites serially transferred from one plant to another was

poor and after successive serial transmission their numbers decreased drastically Transmission ability of adult mites, fed on infected plants as nymphs, gradually decreased with the age (Orlob, 1966a&b).

Eriophyid mites were maintained on an artificial medium to study the virus retention period (Del Rosario and Sill, 1965). The longevity of the Wheat spot chlorosis pathogen (WSCP) in its vector *A. tulipae* was determined by maintaining mites on a medium prepared by mixing 5 g potato dextrose agar, 5 g of charcoal and 40 ml of water. Alternatively, a wheat decoction agar was prepared with juice extracted from 500 g of wheat leaves, 20 g of dextrose, and 40 g of agar per litre. Artificial medium found to sustain adult *A. tulipae*. In these experiments WSCP retention period was found to be 18 days.

Electron microscopy was used to study virus retention period by observing the virus particles in ultrathin sections of mites. Following this method WSMV was regarded as persistent virus in *A. tosichella*. Intact virus particles were found to be distributed in the body of mite vector for 5-9 days after removal of mites from host plant (Paliwal and Slykhuis, 1967; Paliwal, 1980). Virus particles were found to be densely packed, in the posterior mid gut of the mites (Takahashi and Orlob, 1969). Orlob (1966a&b) demonstrated the persistence of virus after moulting by transferring immobile, moulting nymphs from infected plants onto healthy plants. Further more, he demonstrated virus transmission by mechanical inoculations using macerates of *A. tosichella* nymphs and adults reared on WSMV infected wheat plants.

Transovarial transmission was not observed in the vector, *A. tosichella* for WSMV and WSpMV (Slykhuis, 1955; De Rosario and Sill, 1965; Orlob, 1966a&b; Nault and Stayer, 1970); for PMV in *E. insidiousis* (Wilson *et al* 1955); for PPSMV in *A. cajani* (Ghanekar *et al.*,1992).No evidences for virus multiplication was reported in all the cases.

2.6. Detection of virus in mite vectors

For virus detection in mites, electron microscope (Paliwal and Slykhuis, 1967; Takahashi and Orlob, 1969; Stein-Margolina *et al.*, 1969 and Paliwal, 1980), and serological methods such as ELISA, immunofluorescent microscopy, Western blotting and dot-immunobinding assay were used (Sherwood, 1987). Compared to the filter paper immunobinding assay and western blotting, methods based on ELISA format were shown to be more sensitive for WSMV detection in mites. However, in general virus detection in mites was difficult to study due to the microscopic nature of eriophyid mites. WSMV in *A. tosichella* was detected using immunofluorescent microscopy and dot-immunobinding assay (Mahmood and Hein 1997).

2.7. Survival and spread of PPSMV and *A. cajani* in nature

The information relating to SMD cycle in nature is limited. The pathogen is not seed borne and spread by mites through wind (Reddy *et al.*, 1989; Ghanekar *et al.*, 1992). The vector could be carried by wind upto 35 meters (Anon. 1980) and as far as 2 km downwind (Reddy *et al.*, 1989) from the inoculum source. PPSMV and its vector could survive on off-season pigeonpea on field borders, volunteer and ratooned plants, and those grown in kitchen gardens (Reddy *et al.*, 1988, 1990, 1993a). Mites and the virus also survived on wild relatives of pigeonpea, such as *C.*

scaraboeides (Ghanekar *et al.*, 1992). The survival of SMD inoculum in areas where there are no voluntary pigeonpea plants or wild relatives of pigeonpea, is yet to be determined (Reddy *et al.*, 1989).

2.8. Alternate hosts of PPSMV and its vector

Due to lack of diagnostic tests, previously, host range of PPSMV was determined based on symptom expression on mite-inoculated plants. Natural host range of PPSMV included several accessions of cultivated pigeonpea and some of its wild relatives *viz.*, *C. scarabeoides, C. platycarpus* and *C. cajanifolia* (Reddy *et al.*, 1993a, 1998). Presence of *A. cajani* was reported on *Oxalis corniculata* (Rathi, 1983) and also on *Cannabis sativa* (Bhang) (Singh and Rathi, 1995). However, basis for *A. cajani* identification and their role in the disease cycle is not yet repoted. Very recently, PPSMV was transmitted by mechanical sap inoculation onto *Nicotiana benthamiana* and *N. clevelandii* (Kumar *et al.*, 2002).

2.9. Interaction of PPSMV with other pathogens and pests

In addition to sterility mosaic, several diseases caused by fungi, bacteria, viruses and phytoplasmas also affect pigeonpea. Infection with one pathogen either increases the severity of another disease or protects the plants. PPSMV infection protects pigeonpea from the severity of *Fusarium udum* (Anon. 1964; Chadha and Raychaudhary, 1966) and Mungbean yellow mosaic virus (Rathi, 1983), but it predisposed infected plants to powdery mildew infection and red spider mites (*Schizotetranychus cajani*) (Anon. 1979; Reddy *et al.*, 1984; Sithanantham *et al.*, 1989).

2.10. Genetics of Resistance

Information on genetics of resistance to SMD is scanty. Resistance to SMD was shown to be governed by four independent non-allelic genes (Singh *et al.*, 1987). In some lines susceptibility to SMD was dominant over resistance/tolerance, and tolerance reaction was dominant over the resistance (Sharma *et al.*, 1984). These studies suggested that the locus or loci governing resistance/tolerance were the same in all the lines tested and that the resistance was possibly controlled by multiple alleles. Recent report by Srinivas *et al.* (1997a) suggested that, the disease reaction was governed by two independent non-allelic genes with at least three multiple alleles at one loci. Monogenic inheritance of resistance was noticed in the cross between ICP 8850 X ICP 8863 (Srinivas *et al.*, 1997b) and homozygous recessive condition was found to confer resistance or tolerance to SMD (Srinivas *et al.*, 1997c).

2.11. Resistance screening techniques

Three methods are being used for evaluating resistance to SMD. The 'leaf stapling technique' described by Nene and Reddy (1976a) is the most commonly used method under field and glasshouse conditions. This technique involves stapling of a portion of SMD infected pigeonpea leaves on to healthy pigeonpea seedlings. Mites from the stapled leaf, migrates and transmits the virus to the test plants. This technique was shown to facilitate inoculation at primary leaf stage and to rapidly express disease symptoms. The 'infector-hedge' technique was used for large-scale field inoculations (Nene *et al.*, 1981a). This consists of a hedge of pigeonpea plants infested with mites either by the leaf-stapling technique or spreading the infected twigs on 10 days old plants at the upwind border of the field. The mites and the virus multiplied on the hedge and served as a source of inoculum for disease spread

through wind onto test material sown downwind. Perennial pigeonpea was used to maintain the inoculum. This technique was modified to produce the 'spreader row' inoculation technique, where, instead of single hedge several rows of infected plants were established throughout the field to achieve more uniform disease spread (Nene *et al.*, 1981b).

2.12. Resistance sources

Alam (1931) first reported pigeonpea cultivar, Sabour 2E as resistant to SMD. Ramakrishnan and Kandaswamy (1972) reported very low incidence (<3%) in NP (WR)-15, P-1778, P-1289, P-110 and P-2621, at Coimbatore. At ICRISAT, several genotypes resistant to SMD were identified. Of nearly 15,000 germplasm accessions screened, 326 lines showed no overt symptoms were regarded as resistant and 97 lines showed only ring spot symptoms but no sterility (Nene *et al.*, 1989). ICAR-ICRISAT trials were initiated in 1976 in uniform disease nurseries to test resistant sources identified at ICRISAT, at different locations within India (Amin *et al.*, 1993a). Lines such as ICP 6997, 7035, 7197, 7234, 7867, 8094, 8862, 10976, 10977, 10979, 10996, 11049, 11204, 11206, ICPL 342, 355, 366, 8324, BSMR 235, DPPA 85-2, 85-13, 85-14, and 85-15 have been identified as resistant or tolerant across all the locations. Several other lines, ICPL 146, 269, 8327, DA 11,12, 13,14,15,51, MA-97, Sehore 367, DPPA- 84-61-3, DPPA-84-8-3, Pant A-104, 8505, 8508, Bhavanisagar-1 and NPRR-1 were tested in the All India coordinated trials and showed resistance to SMD (Reddy *et al.*, 1998). Pigeonpea cultivars/lines/accessions reported as resistant/tolerant to SMD are presented in Table-1.

2.13. Variability in PPSMV and its vector

The mechanism of SMD resistance was not clearly understood, but previous studies indicated that diverse mechanisms governed SMD resistance in pigeonpea. Genotypes were either resistant to the virus or to the vector or both (Reddy and Nene, 1980; Muniyappa and Nangia, 1982; Sharma *et al.*, 1984; Saxena and Sharma, 1990; Reddy *et al.*, 1995). Although the resistant lines have performed well under field trials at ICRISAT, Patancheru and surrounding regions, their resistance else where in India was less effective. This variability was attributed to the presence of either different *A. cajani* biotypes, or species of *Aceria* mites or due to the occurrence of different strains of the causal virus (Reddy *et al.*, 1990).

Depending on the reaction of host differentials to PPSMV at different locations, variability in PPSMV was reported to exist in India. Five distinct pathogen isolates were reported. Gwalior isolate was regarded as variant-1, Badnapur and Patancheru as variant 2; Coimbatore; Kumargunj and Pudukotti as variant 3; Bangalore and Dholi as variant 4 and Kanpur isolate as variant 5 (Reddy *et al.*, 1993). Indian isolates of PPSMV were compared with an isolate from Nepal and it was concluded to be different from all the Indian isolates (Chaurasia, 1993).

Studies were conducted to determine the involvement of different *Aceria* species in virus transmission and to understand the variation in *A. cajani* populations obtained from pigeonpea from different SMD endemic locations of India, Nepal and Myanmar. The results suggested that, no other *Aceria* species and probably no *A. cajani* biotypes existed in the Indian subcontinent. Therefore, variation in the host reaction was attributed to the variability in the causal virus (Kumar *et al.*, 2001).

2.14. Management of SMD

Various insecticides and acaricides have been used to manage SMD by controlling its vector. In addition to chemical control, cultural methods also were tried but no success has been achieved. SMD may be controlled by removing perennial and voluntary plants of pigeonpea in the vicinity of pigeonpea fields in advance of the sowing season (Seth, 1965; Anon, 1980; and Raychaudhary and Nariani, 1977). No significant difference in the disease incidence was observed with different dates of sowing (Singh and Rahti, 1996a; Lakshmikantha *et al.*, 1997). Intercropping of pigeonpea with sorghum (Bhatnagar *et al,* 1984), pearl millet (Siddappaji *et* al., 1979) or both (Zote, *et al.,*1988), border and intercropping of sorghum and sunhemp (Singh, 1992) had no effect in reducing SMD incidence.

Table: 1. Pigeonpea lines/cultivars reported to be resistant to SMD

Resistant lines	References
NP(WR) 15, P-4835, 1778, 1289, 1100 and P-2621	Ramakrishnan and Kandaswamy (1972)
L-3 and P-4785	Subramanian et al., (1973); Singh et al., (1975)
ICRISAT-3783, 6986, 6997, 7119, 1137, 2719, HY-3c, ICP-7035, and *Atylosia lineata*	Nene and Reddy, (1976a)
P-4785, L-26, ICRISAT-3784, 5449, 6497, 7035, 7119, Pant B-76, B-77 and E-41	Rathi (1977)
ICP 378, 7035, 3782, 6986, 6997, 7119, 7197, 7867, 7942 and ICP 8136	Reddy and Nene (1980)
ICP 7378, 2 S_2	Muniyappa and Nangia (1982)
ICP 3783, 6997, 7878, 7501, 7983, 8094, 8130, 8133, 8854, 8861, 8862, P-595	Sivaprakasam and Marimuthu (1983)
ICP 10976, 10984, and 7353	Samiyappa and Sivaprakasam (1985)
ICP 263	Dwivedi and Shukla (1986), Singh *et al.*, (1987)
ICPL 786, 1076, 10799	Zote and Dandanaik (1986, 1987); Gupta *et al.*, (1988); Nene *et al.*, (1989)
Pant A-8505 and 508	Pal *et al.*, (1989)
ICPLC-88046, Bahar, DA-35, K-32-1, Pusa-14, 19, Gant-9005, DA-11, 32, 33	Das and Gupta (1992); Singh *et al.*, (1995)
ICP 7035	Amin *et al.*, (1993)
ICP 8852, 11276 (Variant-1)	Srinivas and Reddy (1995)
ICPL 87119	Reddy *et al.*, (1995)
ICP 7035 and HY-3C	Rangaswamy *et al.*, (1997)

Table : 2. Transmission characteristics of eriophyid mite transmitted viruses

Disease	Vector	Virus nature	AAP[1]	IAP[1]	Mode of transmission	VRP[1]	Reference
Black currant reversion	*Cecidophyopsis ribis*	Nepovirus	3 h	48 h	Persistent	25 days	Jacob, 1976
Cherry mottle leaf	*Eriophyes inaequalis*	Closterovirus	ND[2]	ND	ND	32 Days	James and Mukerji, 1993
Peach mosaic	*Eriophyes insidiosus*	Closterovirus	3 days	6 h	Semi-persistent(?)	2 days	Gispert *et al*,1998
Fig mosaic	*Aceria ficus*	Unknown	5 min	5 min	Persistent	6-10 days	Proeseler, 1972
Pigeonpea sterility mosaic	*Aceria cajani*	Tenuivirus (?)	5 min	30 min	Persistent (?)	Unknown	Reddy *et al*, 1989
Rose rosette	*Phyllocoptes fructiphilus*	Unknown	ND	ND	ND	ND	Epistin and Hill, 1994
Prunus latent mosaic	*Vastes fockeu*	Rymovirus	ND	ND	ND	ND	Hiruki, 1992
Wheat streak mosaic	*A. tosichella*	Rymovirus	15 min	15 min	Persistent	4- 9 days	Slykhuis, 1955; Paliwal and Slykhuis, 1967 and Paliwal, 1980
Wheat spot mosaic	*A. tulipae*	Unknown	ND	ND	Persistent	8 days	Nault and Styer, 1970
Ryegrass mosaic Virus	*Abacarus hystrix*	Rymovirus	2 h	2 h	Semi-persistent	24 h	Mulligan, 1960; Heard and Roberts, 1975
High plains disease	*Aceria tosichella*	High plains virus	ND	ND	ND	ND	Jenson *et al*., 1996
Shallot mite borne virus	*Aceria tulipae*	Rymovirus (?)	ND	ND	ND	ND	Van Dijk *et al*., 1991
Agropyron Mosaic Virus	*Abacarus hystrix*	Rymovirus	ND	ND	ND	ND	Slykhuis, 1969
Onion mosaic	*A tulipae*	Rymovirus(?)	ND	ND	ND	ND	Van Dijk *et al*., 1991
Garlic mosaic	*A tulipae*	Rymovirus	30 min	30 min	Persistent	8 days	Ahmed and Benigno, 1985

1. AAP –Acquisition access period, IAP – Inoculation access period, VRP – Virus retention period, 2. ND – Not determined

MATERIAL AND METHODS

III. MATERIALS AND METHODS

3.1. SMD culture

Pigeonpea Sterility Mosaic Virus culture was maintained on a pigeonpea cultivar, ICP8863 in a growth chamber at 28 °C for 14 h day time and 20 °C for 10 h night time, with 70 to 80% relative humidity. The leaf stapling technique (Nene and Reddy, 1976) was used to inoculate 12-15 days old healthy pigeonpea seedlings.

3.2. Transmission of Pigeonpea Sterility Mosaic Virus (PPSMV)

3.2.1. Mechanical sap inoculation

Different inoculation buffers (Phosphate buffer, Citrate buffer and Tris-$MgSo_4$ (TM) buffer) (Appendix) with different pH (7.0, 8.0 and 9.0) and molarity (0.1 and 0.5) were tested. The young leaves showing severe mosaic symptoms from SMD-affected pigeonpea plants were ground in cold inoculation buffer (1:10 w/v), each of them was incorporated with α-Monothioglycerol (0.75%) (Sigma Chemicals Company, USA) and the extract was rubbed onto the carborundum (600 mesh) dusted leaves of the test plants, *Chenopodium amaranticolor, C. quinoa, C. album, C. murale, Nicotiana benthamiana, N. glutinosa, N. rustica N. tabacum* var. Samsun,, *N. tabacum* var. Xanthi, *N. tabacum* var. Turkish, *Phaseolus vulgaris* var. Pinto, *P. vulgaris* var. Topcrop, *P. vulgaris* var. Kintoki, *P. vulgaris* var. Bountiful, *Vigna unguiculata* cv. Early ramshorn and *Cajanus cajan* cv. ICP8863. Inoculated leaves were rinsed with tap water and the plants were maintained in growth cabinets. All the inoculated plants were tested for PPSMV by DAS-ELISA (see section 3.4.1) and also observed for external symptoms. If the plants tested positive by ELISA the presence of virus was also confirmed by RT-PCR (see section 3.4.3).

3.2.2. Graft transmission

In order to establish an efficient method to transmit PPSMV by grafting, wedge grafting, chip grafting and petiole grafting were tested. For all grafting experiments SMD-affected pigeonpea *cv.* ICP8863 was used as the scion. Tissue from virus source plant was treated with a contact acaricide, Dicofol (Indofil Chemicals Company Ltd., Mumbai, India) to kill mites. A clean razor blade was used to make an incision or to cut the tissue and a Scotch tape (Scotch Mark, USA) was used to seal the grafted region. Soon after grafting, plants were covered with a polythene bag for a week to maintain under high humidity. Grafted plants were maintained in a growth chambers.

3.2.2.1. Wedge grafting

Using a scalpel blade, a vertical slit was made to the stem of a 25-30 days old plant. Stem of SMD plant was sliced to fit the slit of stock plant and then sealed with a tape.

3.2.2.2. Chip grafting

An incision was made on the stem, below the growing bud of a healthy plant. A piece of tissue (chip) from SMD-affected plant was inserted into the slit and the flap was folded and the grafted area was sealed with a tape.

3.2.2.3. Petiole grafting

Twelve to 15 days old healthy pigeonpea plants were used for petiole grafting. A vertical slit was made to top of the healthy plant. Petiole with a leaflet from an SMD-affected plant was sharpened at both ends and was inserted into the slit made in to the healthy plant. The grafted area was sealed with a tape

3.2.3. Transmission by dodder

Dodder (*Cuscuta* spp) was established on SMD-affected plants inoculated by grafting. Following the establishment of dodder on SMD affected plants, its stem was placed on the leaf axils of the 15-20 days old healthy pigeonpea plants and allowed to colonize. Donor (SMD-affected plants), recipient plants (healthy plants) and the dodder (*Cuscuta* spp) were tested for PPSMV presence by DAS-ELISA.

3.3. Virus-Vector relationships

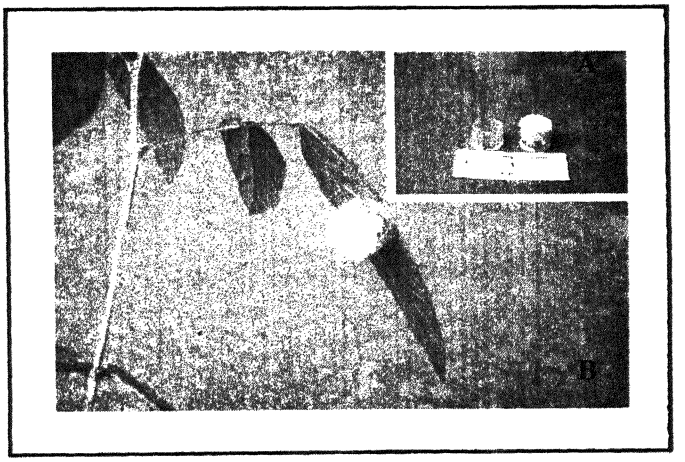
3.3.1. Handling *A. cajani* and maintenance of mite inoculated plants

All mite manipulations were done under a binocular microscope (40x magnification, Leica, Wild M3C). A human eyelash affixed to a 6 cm long wooden toothpick was used for transferring mites from the source plant/leaf on to test plants. All plants inoculated with mites were covered with mite-proof cages (mpc) and maintained in growth chambers.

3.3.2. Confining individual mites

For experiments involving serial transfer of individual mites, confining them to a selected area on the leaf surface was essential. For this purpose, a polypropylene micro-cage (5x10 mm) was devised from bottom half of the 1 ml micropipette tips (Finntip, Cat no. 9401030) and covering one end with a muslin cloth (Fig. 1). Micro-cage was affixed to the pigeonpea leaf using synthetic adhesive (Vami gum, Vam Organic Chemicals Ltd., New Delhi). This adhesive did not cause any damage to the leaf and was not toxic to mites. Further more the cages could be removed easily without damaging the leaf surface. A single mite was placed on the leaf and covered with a micro-cage.

Fig. 1. A. Polypropylene micro-cage used for confining a single *Aceria cajani* on a single leaflet. B. Micro-cage affixed to the pigeonpea seedling by an adhesive.



3.3.3. Generation of non-viruliferous mites

A new method, the 'float-leaf technique,' was developed to generate non-viruliferous mite colonies. A young healthy trifoliate pigeonpea leaf was floated on sterile distilled water surface in a Petridish. Mites from infected leaves were manually transferred onto the floated leaf. After two days exposure, mites were transferred to another healthy leaflet floated on water surface. Approximately 50 mites were collected from this leaf and transferred to pigeonpea cv ICP8863, to determine if the mites become non-viruliferous. Plants colonized by mites and the float-leaf on, which mites were reared were assayed for the presence of virus by DAS-ELISA (see Section 3.4.1). To avoid contamination, all mite-inoculated plants were covered individually with a mite proof cage and were maintained in growth chamber.

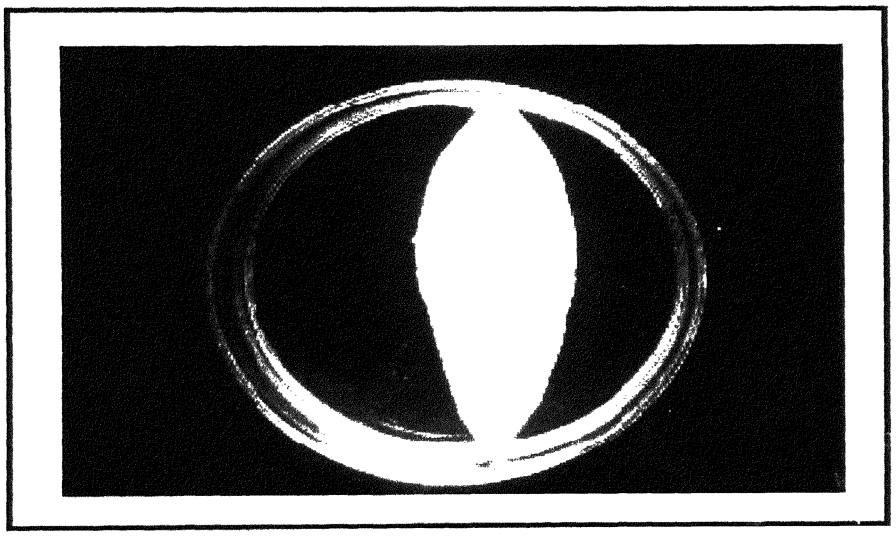
3.3.4. Efficiency of PPSMV transmission by *A. cajani*

To determine the number of mites required to obtain 100% virus transmission, viruliferous *A. cajani* obtained from PPSMV infected pigeonpea plants were transferred onto healthy pigeonpea seedlings at two leaf stage using one, 2, 3, 4, 5, 10 and 20 mites per plant. Plants were covered with mpc and the inoculated plants were assayed by DAS-ELISA after three weeks pi. This experiment was repeated three times.

3.3.5. Acquisition access period (AAP)

A young SMD-affected leaflet showing prominent mosaic symptoms from graft-inoculated (mite-free) plants were floated on to water surface in a Petridish to use it as a virus source. Non-viruliferous mites were transferred and allowed to feed on these infected leaflets for 5 min, 10 min, 15 min, 30 min, 2 h, 5 h, 10 h, 15, 24 h and 36 hrs. After the stipulated AAP, either asingle or a group of 10 mites were transferred onto

Figure: 2. Float leaf technique used to generate non-viruliferous *Aceria cajani*



healthy pigeonpea plants and were covered with mpc. The exposed plants were assayed for PPSMV by DAS-ELISA after three weeks pi.

3.3.6. Inoculation Access Period (IAP)

To determine IAP, natural viruliferous *A. cajani* collected from PPSMV infected plant were used. Ten viruliferous mites were transferred onto each healthy pigeonpea seedlings and were allowed 30 min, 1 h, 1.5 h, 2 h, 3 h, 5 h, 10 h, 15 h, and 24 hrs of IAP. Feeding of mites was terminated by spraying plants with a contact acaricide, Dicofol (Indofil Chemicals Company Ltd., Mumbai, India). Exposed plants were covered with mpc and maintained growth chamber and assayed for PPSMV by DAS-ELISA after three weeks pi.

3.3.7. Latent period and retention of PPSMV in the vector

To determine the possible latent period, non-viruliferous mites were exposed to minimum AAP of 45 min. The individual mites were transferred serially to each of the pigeonpea plant by allowing 2 h IAP at each transfer to ascertain that the mite was given opportunity to feed. Each mite was transferred to another healthy pigeonpea seedling and then allowed 2 h IAP. Five serial transfers in this manner were done using single mite. Plants were covered with mpc and assayed for PPSMV after three weeks.

To determine the retention period of PPSMV in its vector, *A. cajani,* healthy mites exposed to optimum AAP of one day were used. A single mite was serially transferred onto healthy pigeonpea plants allowing different IAP at each transfer. Different IAP tested were, 1 h, 2 h, 5 h, 6 h, 10 h, 15 h and 24 h in separate experiments. After each IAP, individual mites were transferred to another set of plants.

Serial transfers in this manner were done until the mite died. All the inoculated were covered with mpc and then assayed for PPSMV three weeks pi.

3.3.8. Effect of mite starvation on transmission of PPSMV

To determine the effect of starvation on the retention of PPSMV, non-viruliferous mites were given 24 h AAP and then starved on dried healthy pigeonpea leaflets enclosed in a dry Petridish. Starvation periods tested were 1h, 3h, 5h, 6h, 9h and 13h. Ten mites per plant were transferred to individual healthy pigeonpea seedlings.

To determine the effect of starvation of mites prior to AAP, non-viruliferous mites were starved for 3-4 h and then allowed AAP of 5 min, 10 min, 15 min, 30 min, 1h, 2h and 5 h. Exposed mites transferred to healthy pigeonpea seedlings. All the tests were done using individual mites.

Similarly, to determine the effect of post-AAP starvation on inoculation of PPSMV, healthy mites generated by float-leaf technique were allowed AAP of 45 min by feeding them on SMD-affected leaflets and then starved for at least 4 h. Individual mites were transferred serially onto healthy pigeonpea plants and were given IAP for 30 min, 1h and 2 h in separate experiments. After each IAP individual mites were transferred to another set of plants. Three serial transfers in this manner were done using single mite. Plants were covered with mpc and assayed for PPSMV after three weeks.

3.3.9. Trans ovarial transmission studies

Eggs of *A. cajani* were picked from the SMD affected pigeonpea leaves using fine needle. Ten eggs were transferred to each plant. The plants were covered with mpc

and maintained in a growth chamber for about one and a half months. Plants were assayed for PPSMV using DAS-ELISA.

3.3.10. Survival of mites on healthy and SMD affected plants

Over 200 non-viruliferous mites were transferred onto three healthy and SMD affected (graft inoculated, of 35 days old) pigeonpea plants. Preference of mites was determined by counting the mites on all the leaf lets of each plant two months pi.

3.3.11. Distribution of PPSMV and *A. cajani* on the infected plant

Three, 70 days old SMD affected pigeonpea plants, inoculated at two leaf stage, were used for this study. Ten trifoliate leaves from each of these plants were collected starting from the oldest to youngest leaf and number of mites was counted under a binocular microscope. PPSMV concentration was assayed in the same leaves by DAS-ELISA. The number of mites present per trifoliate was correlated with the virus concentration using correlation analysis.

Distribution of PPSMV alone in the infected plant was also tested using DAS-ELISA. Two months old pigeonpea plants collected form a glass house, were used for this study in three replications. The virus concentration was checked separately in the roots, root nodules, stem, leaves and growing buds.

3.4. Detection of PPSMV in plants and *A. cajani*

3.4.1. DAS-ELISA

DAS-ELISA was performed as described by Kumar *et al.* (2001). PPSMV-polyclonal antibodies (150 µl) diluted in a coating buffer (Appendix) was added to each of the well of an ELISA plates (Nunc, Denmark) and incubated at 37 ^{0}C for 1 h or 4 ^{0}C over night.

Plates were washed three times with PBS-T (Appendix). The leaf material was macerated in antigen extraction buffer (Appendix) and 150 µl of the extract was added to the wells of ELISA plates and incubated at 37 °C for 1 h. Plates were washed three times with PBS-T. IgG extracted from PPSMV antiserum were conjugated with Penicillinase enzyme (Appendix) and they were cross absorbed with extracts from healthy pigeonpea leaves (Appendix) and then 150 µl PNC-conjugated, cross absorbed antibodies diluted in antibody buffer (Appendix) was added to each of the wells. Plates were incubated at 37 °C for 1 h and washed three times with distilled water-tween (0.05%). Substrate (Penicillin + bromothymol blue) (Appendix) was added and incubated at room temperature. The absorbance of yellow colour of the reacting substrate was read at 620 nm in a Multiscan ™ Plus (Labsystems) ELISA plate reader after 30 min and again at 1 h. Dilutions of antiserum, antigen and PNC-conjugate were optimized by trying various dilutions of each of them. Results were considered positive if the difference in absorbance value is thrice to that of healthy.

For detection of PPSMV in mites, individual, 5, 10, 25, 50 and 100 viruliferous mites were used. Mites from infected plant were transferred to eppendorf tubes containing 50µl of antigen extraction buffer (Appendix). Tubes were centrifuged at 12,000 rpm/2 min to sediment them to the bottom of the tube. These were macerated using an eppendorf homogenizer and the extract was transferred to wells of ELISA plate (Maxi Sorp) pre-coated with the PPSMV polyclonal antibodies (1:10,000 dilution) and incubated at 37 ^{0}C for 2h. Subsequent steps are as described in section 3.4.1.

3.4.2. Dot immunobinding assay

Single, 5, 10, 25, 50 and 100 viruliferous mites were transferred to 1.5 ml eppendorf tubes containing 50 µl TBS (Appendix) and centrifuged at 12,000 rpm/2 min to sediment mites at the bottom. Forty µl of TBS was taken out without disturbing the pelleted mites and the mites were macerated using an eppendorf homogenizer. Non-viruliferous mites were used as a control. A 5 x 5 cm nitrocellulose (NC) membrane [Bio-Rad Laboratories] was pretreated with TBS buffer, pH 7.5 for 10 min. Membrane was dried on filter paper for 10 min. Five µl of the mite extract was dotted on to the NC membrane and air dried for 10 min. NC membrane was blocked by soaking in a blocking solution (Appendix) for 2 hours at room temperature. The membrane was then soaked in PPSMV polyclonal antiserum (1:1000 dilution; cross-absorbed with healthy pigeonpea tissue) and incubated for 2h and then washed 3 times with TBS-T containing milk powder, allowing 5 min at each wash. Membranes were incubated in alkaline phosphatase-labelled goat anti-rabbit IgG [Sigma Chemicals, USA] diluted to 1:500, for 2 h. Membranes were washed 3 times with TBS-T containing and then placed in a substrate solution prepared by mixing a single tablets of Fastred TR/Naphthol AS-MX in 10 ml of Tris buffer [Sigma Chemicals, USA] until the colour development was complete (15-20 min).

3.4.3. RT-PCR

For PPSMV detection in plants by RT-PCR, procedure described by Kumar *et al.* (2001b) was followed, using the oligonucleotide primers SM-1 (5'ACA TAG TTC AAT CCT TGA GTG CG 3') and SM-2 (5' ATA TTT TAA TAC ACT GAT AGG A3') derived from the nucleotide sequence of PPSMV RNA-5 (Kumar *et al.*, 2001b).

Total RNA from about 100 mg leaf material from the test plants was isolated using RNeasy kit™ (Qiagen) as per the manufacturer recommended protocol. RNA was eluted into 30 µl of RNase free water. One to 4 µl of this RNA was used for RT-PCR reaction.

One hundred and 200 viruliferous mites were transferred to 1.5 ml eppendorf tubes containing 50 µl 5x MMLV RT buffer and centrifuged at 12,000 rpm for 2 min to collect mites at the bottom. Forty µl of TBS was taken out without disturbing the pelleted mites. Mites were macerated using an eppendorf homogenizer. Entire content was used for RT-PCR reaction. Non-viruliferous mites were used as a control.

RT reaction mixture:

The following components were added into a sterile 0.2 ml tube

5x MMLV buffer	4 µl
25 mM Mg Cl_2	2 µl
0.1 mM DTT	2 µl
10 mM dNTP mix	1 µl
Rnasin	10 U
MMLV-RT	100 U
Primer-1 (upstream)	0.5 µl (10ng)
Primer-2 (down stream)	0.5 µl (10ng)
Sterile distilled water	to 20 µl

The reaction mixture was incubated at 42 °C for 45 min. Following the RT step, PCR reaction mixture was added to the same tube and continued the reaction.

PCR- reaction mixture:

10x PCR buffer	5 µl
25 mM Mg Cl_2	3 µl
10 mM dNTPs	0.5 µl
Primer-1	0.5 µl
Primer-2	0.5 µl
Taq-polymerase	2 U
Sterile distilled water	to 30µl.

The PCR programme used for the amplification of the first strand cDNA in a thermal cycler was initial denaturation at 94 °C for 5 min, followed by 35 cycles of amplification by denaturation at 94 °C for 45 sec, primer annealing at 55 °C for 45 sec. and polymerization at 72 °C for 1 min and finally at 72 ° C for 5 min for extension.

3.4.4. Analysis of RT-PCR products

The entire product of PCR reaction was mixed with 5 µl of gel loading dye (Appendix) and electrophoresed in a 1% agarose gel using TBE buffer system, pH 8 (Sambrook *et al.*, 1989). Gels were stained with ethidium bromide and viewed under a UV trans-illuminator (Spectroline TR-312 A, Spectronic corporation, Westbury, USA). DNA ladder [Boehringer] was used as molecular weight marker.

3.5. Screening of pigeonpea genotypes for SMD resistance

3.5.1. Screening Techniques

3.5.1.1. Leaf stapling technique

This technique was used for screening pigeonpea genotypes in growth chambers. Infected leaves from SMD-affected plant containing mites were stapled on to the leaves of healthy pigeonpea plants at two leaf stage as per the Nene and Reddy (1976a).

3.5.2. Screening of ICRISAT pigeonpea genotypes

Thirty late maturing pigeonpea germplasm accessions obtained from Genetic Resource Division (GRD), ICRISAT were screened for SMD resistance. These accessions were planted in SMD screening nursery during 2000-2001 growing season both at ICRISAT, Patancheru and at UAS, Hebbal, Bangalore. Each entry was planted in a single row of five meters length and replicated twice. Susceptible check, ICP8863 was planted after every two-test entries and was inoculated at two leaf stage by leaf stapling technique to provide inoculum for the test entries and for its uniform spread. ICP7035 was used as resistant check. Observations on disease incidence, symptom type and flowering were recorded. The entries were graded as resistant, tolerant or susceptible based on per cent disease incidence. Mite count were taken on younger leaves (trifoliate) of five randomly selected plants for each genotype.

3.5.3. Testing of host differentials at different locations

Variability in the reaction of SMD to selected genotype at different locations was reported (Reddy *et al.*, 1993). Ten host differentials of pigeonpea, ICP2376, C-11, ICP 11164, ICP 8862, Purple-1, ICP 7035, ICP 10976, LRG- 30, ICP 8863, BDN-1 were planted at ICRISAT, Patancheru and at UAS, Hebbal, Bangalore to evaluate their

response to SMD at theses locations. Each entry was planted in two replications, of five meters length. Susceptible check was planted after every two test rows and was inoculated at two leaf stage by leaf stapling. Observations on disease incidence, symptom type and flowering were recorded. Mite counts were taken on younger leaves (trifoliate) of five randomly selected plants for each host.

3.5.4. Screening of wild *Cajanus* accessions

Sixty-one accessions of wild relatives of pigeonpea obtained from GRD, ICRISAT were tested for SMD resistance under glass house conditions. Seeds of wild species were scarified by slicing the seed coat and treated with a soil fungicide, Thiram 75% WP (Sudama Chemtech P. Ltd, Gujrat, India), to protect from soil borne fungal pathogens. They were sown in 8 inches pots in four replications. All the plants were inoculated at two-leaf stage by leaf stapling technique. Observations on symptom type, number of days taken for symptom expression, number of mites per trifoliate and percent disease incidence were recorded. All the plants were checked for the presence of virus by ELISA. To identify the type of resistance offered by the wild accessions, that were identified as resistant to SMD by staple inoculation method were tested by petiole grafting (see section 3.2.2.3). Observations were recorded on symptom type, number of days for symptom expression and disease incidence. All plants were assayed for virus by ELISA two months post inoculation. Mite count was taken on younger leaves (trifoliate) of five randomly selected plants for each accession.

3.5.6. Studies on Inheritance of Resistance

F_2 seeds (118 in number) obtained from the cross made between *C. scaraboeides* (resistant to SMD) and Pant A2 (a cultivated pigeonpea variety, with good agronomic traits, but susceptible to SMD) were obtained from GRD, ICRISAT. They were tested

for SMD resistance along with its parents. F_2 seeds were sown in eight inches pots in a glass house. Individual plants were labelled separately and then inoculated at two-leaf stage by leaf stapling technique. One-month post inoculation, all the plants were tested by ELISA and the disease incidence was calculated. The F_2 plants susceptible to SMD were discarded. The resistant ones were advanced to next (F_3) generation. F_3 seeds obtained were screened for SMD resistance as described. The susceptible F_3 plants were discarded. The resistant ones were advanced to the next (F_4) generation. Data from this study was used to understand the inheritance of resistance with the advancement of generation.

3.6. Experiments for identification of alternate sources of PPSMV infection

Thirty-three weed species commonly present in SMD infected pigeonpea field, belonging to the families Amaranthaceae, Asteraceae, Boraginaceae, Convolulaceae, Euphorbiaceae, Laminaceae, Leguminaceae, Sapindaceae, Solanaceae and Tiliaceae were tested for natural infection of PPSMV using DAS-ELISA (Table 16). The plants were also observed for the presence of *A. cajani*, under stereo binocular microscope.

In a glass house experiment, twenty-three cultivated crop species of economic importance, six *Nicotiana* species and twenty-nine commonly available weed species were sown in eight inch pots in four replications (Table 17). Plants were inoculated at seedling stage following leaf stapling technique (See section 3.5.1.1). All the inoculated plants were tested for PPSMV by DAS-ELISA after 20, 40 and 60 days post inoculation (pi). Plants were also observed for *A. cajani* under stereo binocular microscope. The ELISA positive plants were also tested by RT-PCR.

The plant species, which were found positive to ELISA and RT-PCR were also tested by back inoculation studies (using them as a virus source to acquire for healthy mites) to determine its epidemiological importance for the spread of SMD in nature. In this experiment, non-viruliferous mites were exposed to young leaves of PPSMV positive plants for 3-4 h. Five such exposed mites were transferred to 12-15 day old healthy pigeonpea cv ICP8863. All the plants were assayed for PPSMV by ELISA 3 weeks pi.

3.6.1. Behaviour of *A. cajani* on pigeonpea and non-host species

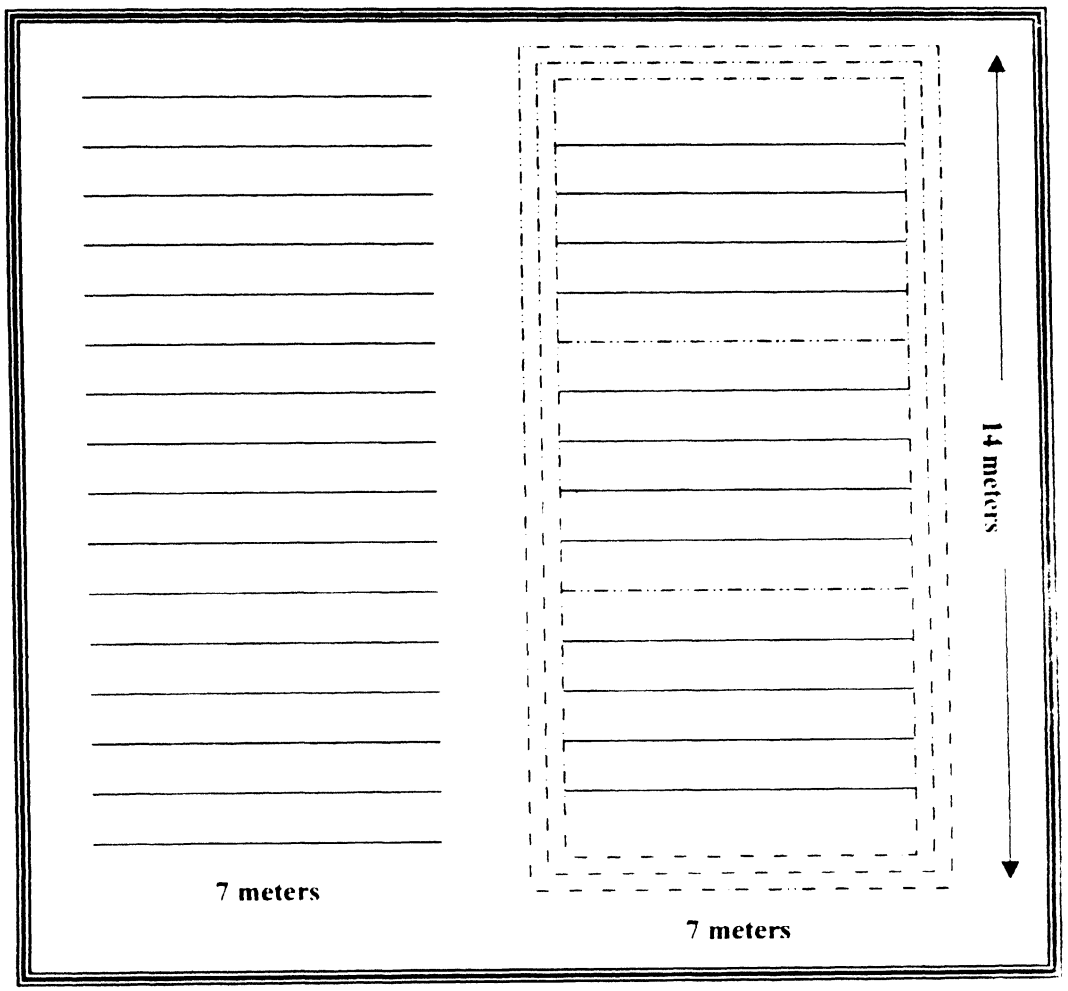
The behaviour of *A. cajani* was studied on its natural host, pigeonpea [cv. ICP7035, ICP8136 (SMD resistant) and ICP8863 (susceptible to SMD)] and a non-host like sorghum and Groundnut. Mites from SMD infected pigeonpea plants were transferred on to the test plant leaf and then placed in a Petridish. Moist cotton was placed to maintain humidity. The behavior of mites was studied at regular intervals by observing them under a stereo binocular microscope.

3.7. Effect of barrier crop on the incidence of PSMD

A field experiment was conducted at ICRISAT, Patancheru to study the influence of a barrier crop to PSMD. Pigeonpea cv. ICP8136, resistant to PPSMV but support mite multiplication, was used as a barrier between the PPSMV inoculum source and the susceptible pigeonpea cv ICP 8863 was sown wind ward. The virus inoculum was established by planting three rows of susceptible pigeonpea cv ICP3863 all along the border of the test plot. These plants were staple-inoculated at two leaf stage and allowed inoculum to build up. Then barrier plants, ICP8136 were planted in three rows all along the border (7 x 14 meters) and a single row of 7 m length within the border leaving a gap of four empty rows. One month after planting the barrier

plants, susceptible cv. ICP8863 was planted in the four empty rows of 7 m length in three replications to test if the difference in the heights of canopies between the susceptible and barrier rows of pigeonpea contribute to reduction in SMD. ICP8863 planted under similar conditions without any barrier served as a check (Fig. 3). Percent disease incidence and mite population per trifoliate on five randomly selected plants from all the four rows were recorded at fifteen days interval.

Figure 3: Field plan for testing the influence of a barrier crop on SMD incidence



EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The results of the experiments conducted on virus-vector relationships, identification of resistant sources against Pigeonpea sterility mosaic disease and identification of alternate sources of PPSMV infection are presented here

4. 1. Pigeonpea Sterility Mosaic Culture

Seeds of pigeonpea cv ICP8863 germinated in 8 to 10 days and the plants inoculated staple leaf technique, developed SMD symptoms 10-15 days post inoculation (pi) Typical SMD symptoms were apparent in two weeks after inoculation This includes over all stunting, characteristic mosaic symptoms with distorted leaves and drastic reduction in leaf size and sterility of plants (Fig 4)

4. 2. Transmission of Pigeonpea Sterility Mosaic Virus

4. 2. 1. Sap transmission of PPSMV

Of the thirteen herbaceous host species tested by mechanical sap inoculation transmission of PPSMV was achieved only onto *Phaseolus vulgaris cv* Topcrop in two of the four experiments conducted (Table 3) Of the three buffers tested with different pHs and molarity transmission was achieved only with 0 1 M Phosphate buffer (pH 7 0) Infection occurred three weeks after inoculation Symptoms appeared as over all stunting of plants with reduction in size of leaf Flower and pod development was affected Younger leaves showed mosaic symptoms and crinkling All symptomatic plants were tested positive by ELISA and RT-PCR (Fig 6). Inoculated leaves were found to be symptom less and were found ELISA negative Transmission from infected *Phaseolus* to *Phaseolus* and to pigeonpea could not be achieved

Figure: 4. Sterility mosaic disease infected pigeonpea plant

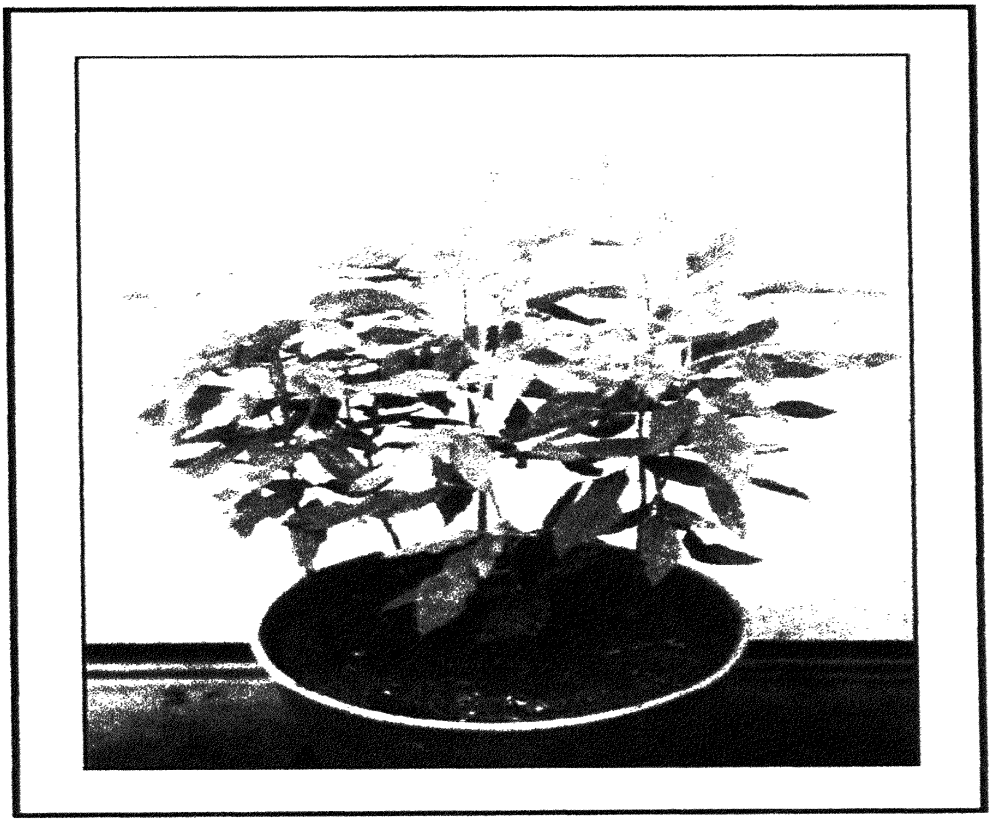
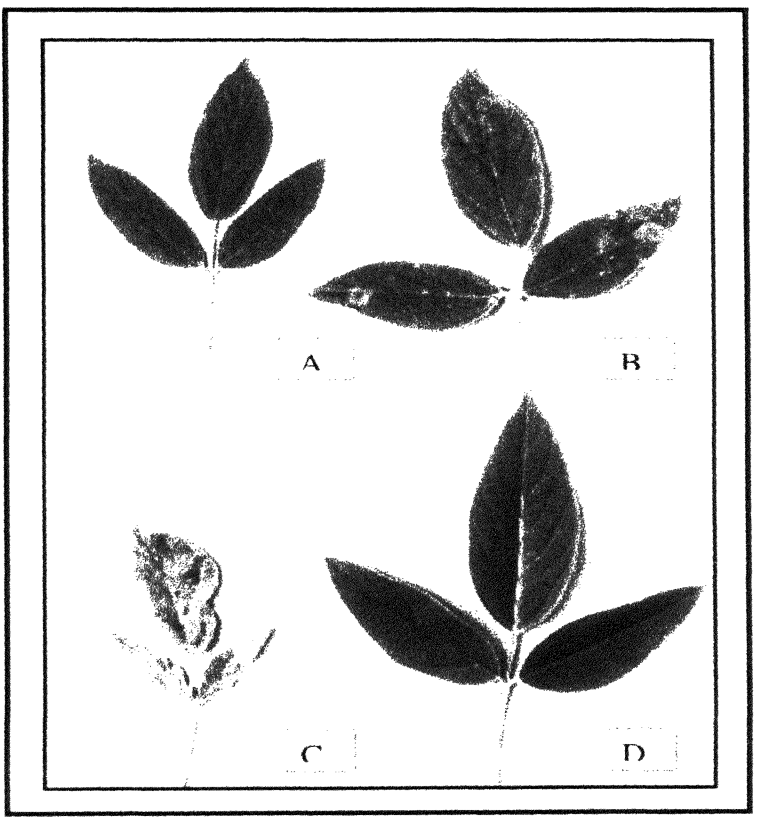


Figure: 5. Different types of symptoms produced due to PPSMV infection on pigeonpea



A. Mild mosaic; B. Ring spot; C. Severe mosaic; D. Healthy

Table 3. Sap transmission of PPSMV[a]

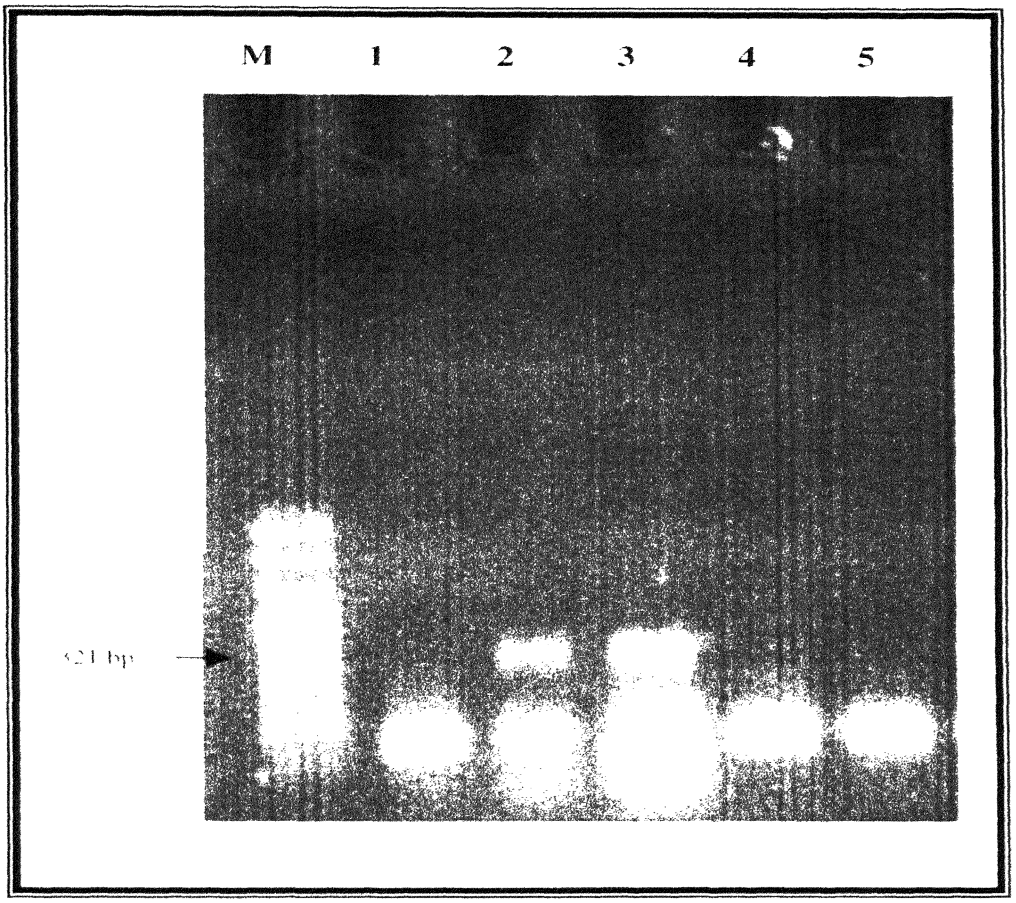
Sl. No.	Host	No. of plants infected/tested [b]			
		Trial I	Trial II	Trial III	Trial IV
1	*Chenopodium amaranticolor*	0/14	0/9	NT	NT
2	*C. murale*	0/16	0/12	NT	NT
3	*Nicotiana glutinosa*	0/9	0/8	NT	NT
4	*N. rustica*	0/11	0/9	NT	NT
5	*N. tobaccum var Xanthi*	0/12	0/14	NT	NT
6	*N. tobacum var. Samson*	0/18	0/10	NT	NT
7	*Phaseolus vulgaris*	0/18	0/12	0/13	NT
8	*Phaseolus vulgaris* cv Bountiful	0/15	0/10	0/8	NT
9	*Phaseolus vulgaris* cv Kintoki	0/14	0/9	NT	NT
10	*Phaseolus vulgaris* cv Pinto	0/16	0/11	0/13	NT
11	*Phaseolus vulgaris* cv Top crop	1/6 [b]	1/16 [b]	0/14	0/13
12	*Vigna sinensis*	0/11	0/9	0/12	NT
13	*Cajanus cajan* cv ICP8863	0/11	0/13	0/18	0/8

[a] Extracts were prepared in 0.1M Phosphate buffer (pH 7.0)

[b] Confirmed by ELISA and RT-PCR

NT – Not tested

Figure: 6. Detection of PPSMV in sap-inoculated *Phseolus vulgaris* var topcrop plant by RT-PCR



M-Molecular weight marker, Lane-**1**, Control, **2**-Sap inoculated *Phaseolus* plant, **3**-SMD infected pigeonpea, **4,5**-Healthy pigeonpea and *Phaseolus* plants respectively

4. 2. 2. Graft transmission of PPSMV

In an experiment conducted to establish an efficient grafting method for PPSMV transmission in pigeonpea, three grafting methods *viz*., wedge grafting, chip grafting and petiole grafting were tried. Of the three grafting methods, maximum virus infection (86.6%) occurred with petiole grafting. Virus transmission by chip (13.3%) and wedge (23.52) grafting was comparatively low (Table 4). Symptoms on grafted plants appeared in 12-15 days.

4. 2. 3. Dodder transmission of PPSMV

There was no transmission of PPSMV by dodder from infected to healthy pigeonpea plants. The inoculated plants and the dodder were found PPSMV negative when tested by ELISA.

4. 3. Virus -Vector relationships

4. 3. 1. Generation of non-viruliferous *A. cajani* colony

Pigeonpea cv. ICP8863 seedlings inoculated with *A. cajani*, after feeding on healthy ICP8136 floated leaves did not develop SMD symptoms. These plants tested negative to PPSMV in DAS-ELISA indicating that the mite colonies raised by this method are non-viruliferous. The floated pigeonpea leaves on which mites were allowed to feed were also tested PPSMV negative in ELISA. Whereas the control ICP8863 plants inoculated with viruliferous *A. cajani* produced clear mosaic symptoms and tested virus positive in DAS-ELISA (results not shown). The method described facilitated generation of non-viruliferous *A. cajani* colony in 3 days period.

Table 4. Efficiency of different grafting methods on PPSMV transmission

Sl. No	Grafting method	No. of plants infected/ plants tested [a]	% Infection
1	Chip grafting	2/15	13
2	Wedge grafting	4/17	24
3	Petiole grafting	13/15	87

[a] PPSMV infection was confirmed by DAS-ELISA

4. 3. 2. Efficiency of *A. cajani* in transmission of PPSMV

In three separate experiments, 35-45% (mean 40%) of the plants were infected when individual mites were used. Significant increase in transmission frequency was noted when plants were inoculated with more than two viruliferous mites. Near 100% virus transmission occurred when plants were exposed to more than five mites and twenty viruliferous mites always resulted in 100% transmission (Table 5). SMD symptoms appeared within one-week when test plants were inoculated with more than ten mites. When one to five mites were used, plants took about 10-14 days to show symptoms (Table 5).

4. 3. 3. Acquisition access period

Single *A. cajani* acquired PPSMV after a minimum AAP of 15 min and transmitted the virus to 13% of the plants. Transmission frequency was 50 to 83% (mean 64%) when ten mites were used (Table 6). Increase in AAP resulted increase in virus transmission in all the trials conducted using single as well as 10 mites. In tests using single mites, a maximum of 53% of the plants were infected. PPSMV could not be transmitted if AAP was given less than 15 minutes (Table 6).

4. 3. 4. Inoculation access period

None of the plants exposed to *A. cajani*, which were allowed an IAP of 1 h or less were infected with PPSMV. Six percent of the plants were infected after 1.5 h IAP. Increase in virus transmission occurred with increased IAP. Ten hour or more IAP resulted in 100% transmission (Table 7). Therefore, *A. cajani* required a minimum of 1.5 h IAP to transmit PPSMV, and an IAP more than 5 h is resulted in 100% transmission (Table 7).

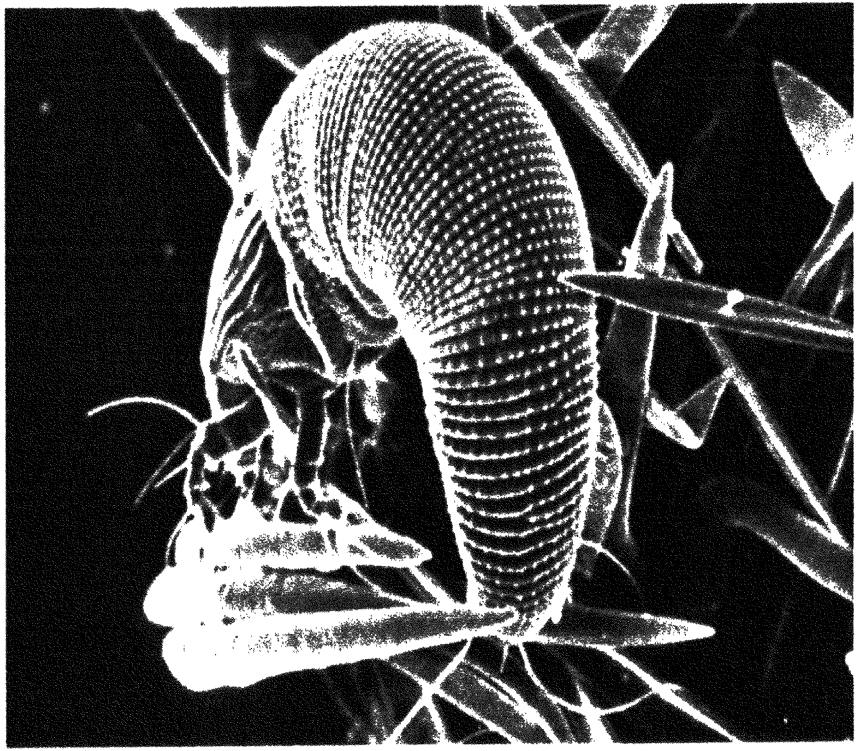


Figure:7 . Scanning electron micrograph of *Aceria caiani*

Table 5. Efficiency of *Aceria cajani* to transmit PPSMV

No. of mites/plant	No. of plants infected/plants tested [a] (% Transmission)			Mean % transmission
	Trial I	Trial II	Trial III	
1	8/20 (40)	7/20 (35)	9/20 (45)	40
2	7/15 (47)	10/15 (67)	7/15 (47)	53
3	11/15 (73)	9/15 (60)	8/10 (80)	71
4	7/10 (70)	8/10 (80)	6/10 (60)	70
5	10/10 (100)	8/10 (80)	8/10 (80)	87
10	9/10 (90)	10/10 (100)	10/10 (100)	97
20	10/10 (100)	10/10 (100)	10/10 (100)	100
Control [b]	0/10 (0.0)	0/10 (0)	0/10 (0)	0

[a] PPSMV infection was rated by visual symptoms and confirmed by DAS-ELISA

[b] uninoculated plants

Table 6. Effect of different Acquisition Access Periods (AAP) on transmission of PPSMV

AAP [a]	No. of plants infected/plants tested (% transmission) [b]			
	10 mites/plant			Single mite/plant
	I	II	III	
5 min	0/7 (0.0)	0/5 (0.0)	0/5 (0.0)	0/15 (0.0)
10 min	0/6 (0.0)	0/8 (0.0)	0/6 (0.0)	0/15 (0.0)
15 min	5/6 (83)	3/5 (60)	3/6 (50)	2/15 (13)
30 min.	4/6 (67)	5/6 (83)	4/5 (80)	2/15 (13)
2 h	6/7 (86)	5/5 (100)	4/6 (67)	2/15 (13)
5 h	5/6 (83)	6/7 (86)	5/5 (100)	4/15 (27)
10 h	5/6 (83)	5/5 (100)	7/7 (100)	4/13 (31)
15 h	6/6 (100)	6/7 (86)	6/6 (100)	5/15 (33)
24 h	6/6 (100)	6/6 (100)	7/7 (100)	8/15 (53)
36 h	7/7 (100)	NT*	NT*	8/15 (53)
Continuously fed mites	5/5 (100)	6/6 (100)	5/5 (100)	7/15 (47)
Control [c]	0/5 (0.0)	0/6 (0.0)	0/5 (0.0)	0/10 (0.0)
Un-inoculated plants	0/10 (0.0)	0/10 (0.0)	0/5 (0.0)	(0 .0)

[a] Duration of *Aceria cajani* exposed to PPSMV source leaf. Mite feeding was not terminated after transferring onto test plants

[b] PPSMV infection was rated by visual symptoms and confirmed by DAS-ELISA

[c] Inoculated with non-viruliferous mites generated by float leaf technique

* NT - Not tested

Table 7. Effect of different Inoculation Access Periods (IAP) on transmission of PPSMV

IAP [a] (hrs)	Plants infected/plants tested [b] (% Transmission)			Mean % transmission
	Trial I	Trial II	Trial III	
0.5	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)	0.00
1	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)	0.00
1.5	1/10 (10)	0/10 (0.0)	1/10 (0.0)	7
2	2/10 (20)	2/10 (20)	1/10 (10)	17
3	3/10 (30)	2/10 (20)	2/10 (20)	23
5	8/10 (80)	8/10 (80)	6/10 (60)	73
10	9/10 (90)	10/10 (100)	10/10 (100)	97
15	10/10 (100)	10/10 (100)	NT*	100
24	10/10 (100)	10/10 (100)	NT	100
Control[c]	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)	0.0

[a] Pigeonpea cv. ICP8863 seedlings were exposed to ten viruliferous *A. cajani*. At the end of stipulated IAP, mite feeding was terminated by spraying test plants with a contact acaricide

[b] PPSMV infection was rated by visual symptoms and confirmed by DAS-ELISA

[c] uninoculated plants

* NT – Not tested

4. 3. 5. Latent and retention periods of PPSMV in the vector

Individual mites, which were allowed 2 h IAP soon after AAP of 45 min, transmitted PPSMV only during first serial transfer. Mites that transmitted virus during first transfer failed to transmit the virus during the second transfer. This suggests that *A. cajani,* which requires minimum 15 min AAP, and 90 min IAP (see Tables 6 and 7), can transmit PPSMV soon after virus acquisition without any latency.

During serial transmissions using single *A. cajani* which were allowed an AAP of one day, transmission of PPSMV occurred only during the first serial transfer when mites were allowed 10 h, 15 h and 24 h of IAP at each of the serial transfers. However, serial transfers at IAP of 2h, 5h and 6 h resulted in virus transmission during first as well as second serial transfers. It is apparent that the vector did not retain the virus after first or second serial transfers. Serial transfers at 1 h IAP resulted in no PPSMV infection (Table 8).

4.3.6. Effect of pre and post acquisition starvation of *A. cajani* on PPSMV transmission

Aceria cajani starved on dried pigeonpea leaves survived without feeding up to 13 hours at room temperature and retained PPSMV in its infective state even after 13 h of starvation however, transmission efficiency was low (Table 9a). Movement of starved mites was sluggish, they became reddish-brown in colour and size was reduced considerably.

Transmission experiments using starved mites indicated that starvation influenced AAP and IAP. Non-viruliferous *A. cajani* starved for 3 to 4 h prior to virus acquisition, resulted in acquisition of PPSMV in 10 min (Table 9b). Similarly,

Table 8. Effect of serial transfer of single *Aceria cajani*[a] at different IAP on retention of PPSMV

IAP[b] (hrs)	No. of plants infected/plants tested in each transfer[c]									
	I	II	III	IV	V	VI	VII	VIII	IX	X
1	0/15	0/15	0/15	0/14	0/14	0/12	0/12	NT	NT	NT
2	7/15	3/15	0/15	NT	NT	NT	NT	NT	NT	NT
5	2/14	3/14	0/14	NT	NT	NT	NT	NT	NT	NT
6	2/15	2/15	0/15	NT	NT	NT	NT	NT	NT	NT
10	3/15	0/15	0/15	NT	NT	NT	NT	NT	NT	NT
15	2/11	0/11	0/11	NT	NT	NT	NT	NT	NT	NT
24	2/12	0/10	0/9	0/9	0/7	0/7	0/7	0/5	0/4	0/4

[a] Mites exposed to AAP of one day were used

[b] Duration of feeding period on test plant before transferring *A. cajani* to another healthy pigeonpea cv. ICP8863 seedling.

[c] PPSMV infection was rated by visual symptoms and confirmed by DAS-ELISA

* NT – Not tested

Table 9a. Effect of starvation of *Aceria cajani* on retention of PPSMV

Starvation period [a] (hrs.)	No. of plants infected/ tested (% transmission) [b]	
	I trial	II trial
1	3/12 (25)	4/16 (25)
3	4/15 (27)	3/16 (19)
4	2/13 (15)	2/15 (13)
6	2/11 (18)	2/14 (14)
9	2/9 (22)	3/11 (27)
13	2/11 (18)	2/7 (29)
Control[e]	0/10 (0.0)	0/10 (0.0)

Table 9b. Effect of Pre-AAP starvation of *Aceria cajani* on acquisition of PPMSV

AAP [c]	No. of plants infected/ tested (% Transmission) [b]		
	I trial	II trial	III trial
5 min	0/10 (0.0)	0/12 (0.0)	0/10 (0.0)
10 min	2/12 (17)	3/11 (27)	1/8 (13)
15 min	3/11 (27)	1/10 (10)	2/11 (18)
30 min	2/13 (15)	3/10 (30)	2/10 (20)
1 h	3/9 (33)	2/8 (25)	3/9 (33)
3 h	2/8 (25)	3/10 (30)	3/10 (30)
5 h	3/11 (27)	3/10 (30)	2/8 (25)
Control[e]	0/10 (0.0)	0/10 (0.0)	0/10 (0.0)

Table 9c. Effect of Post-AAP starvation of *Aceria cajani* on inoculation of PPSMV

IAP [d]	No. of plants infected/plants tested in each transfer [b] (% Transmission)		
	I transfer	II transfer	III transfer
30 min	0/9 (0.0)	0/7 (0.0)	0/7 (0.0)
1 h	5/9 (56)	3/8 (38)	1/7 (14)
2 h	3/12 (25)	1/11 (9)	2/9 (22)

[a] Starvation periods of *A. cajani* on dried healthy pigeonpea leaflet.

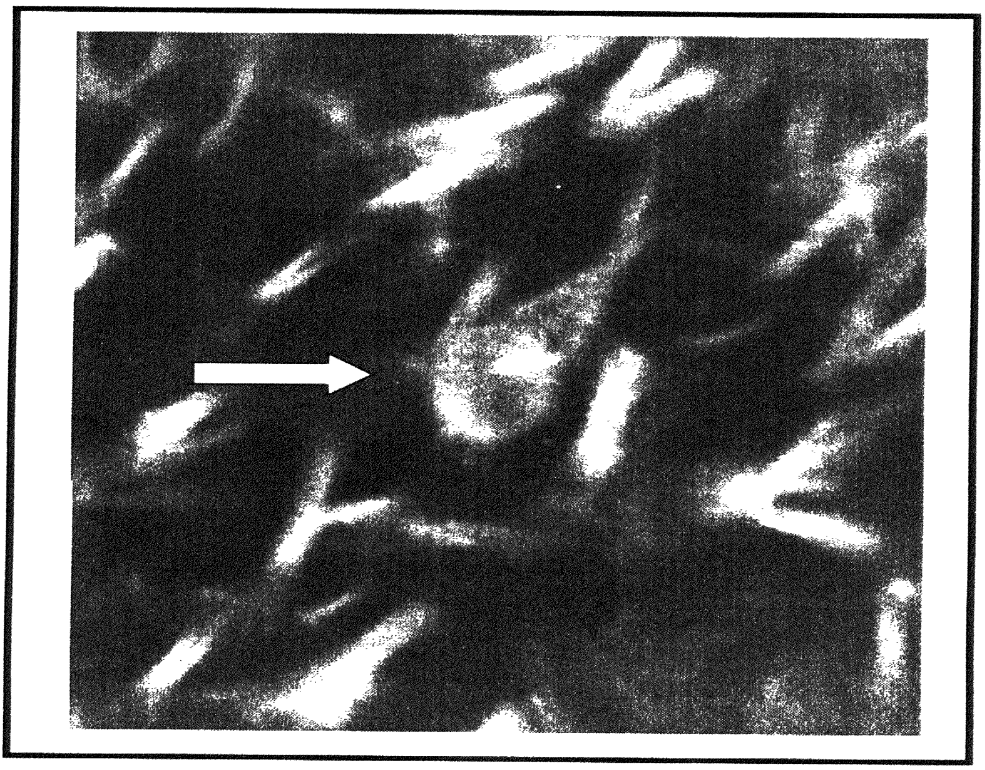
[b] PPSMV infection was rated by visual symptoms and confirmed by DAS-ELISA

[c] Duration of virus acquisition access period (AAP) on PPSMV source leaf.

[d] Duration of inoculation access period (IAP) on healthy pigeonpea seedlings (ICP8863). Mites after stipulated feeding period transferred serially.

[e] uninoculated plants

Figure: 8. Egg of *Aceria cajani*



viruliferous mites starved for 3-4 hours before exposing them to healthy pigeonpea transmitted PPSMV following 1 h IAP (Table 9c).

4. 3. 7. Transovarial transmission of PPSMV

Larvae hatched from the eggs laid by viruliferous mites did not transmit virus in three independent experiments. This indicates that there is no transovarial transmission of PPSMV by *A. cajani*.

4. 3. 8. Survival of *A. cajani* on healthy and PPSMV infected pigeonpea

Observations recorded on PPSMV infected and healthy pigeonpea plants two months pi revealed fewer mites (7-8 per leaflet) on healthy plants. However, more than 50 mites per leaflet were observed on SMD infected plant (data not shown), indicating that *A. cajani* multiplied and survived better on SMD affected plants than those maintained on uninfected plants.

4. 3. 9. Distribution of PPSMV and *A. cajani* on the SMD infected pigeonpea plant

PPSMV concentration was determined in 70 days old SMD infected pigeonpea plants by DAS-ELISA. High concentration of virus was recorded in young leaflets than in matured or old leaflets (Table 10). Younger leaves supported higher mite numbers (>50 mites/leaflet) compared to older leaflets. Interestingly, the mite numbers were positively correlated with the virus concentration (Table 10).

4. 4. Detection of PPSMV

4. 4. 1. Detection of PPSMV in SMD-affected pigeonpea plants by ELISA

PPSMV was consistently detected in SMD infected pigeonpea leaves and none of the healthy leaflets showed the presence of virus. Antigen dilution of 1:10, antiserum

Table 10. Distribution of PPSMV and its vector, *Aceria cajani* on SMD infected pigeonpea plant (cv ICP8863)

Leaf [a]	Virus concentration [b] (OD values at 620 nm)	No. of mites/leaf [c]
1	0.267	3
2	0.201	1
3	0.233	5
4	0.278	10
5	0.224	14
6	0.205	9
7	0.226	43
8	0.173	63
9	0.173	56
10	0.200	27
Healthy Control	1.982	NT*

[a] Pigeonpea leaflets analyzed for PPSMV and *A. cajani* populations. Leaf sample 1 to 10 represents age of the test leaf, with sample-1 representing the oldest leaflet on the test plant, and sample-10 the youngest leaflet. Leaf samples analyzed were collected from similar position, from three different

Virus concentration determined by analyzing 100 mg leaf by double antibody sandwich-enzyme-linked immunosorbent assay. Absorbance values are mean from three tests from three different PPSMV infected plants

[c] Mean mite populations per leaflet from three different PPSMV infected

* Not tested

Correlation analysis

	Virus concentration	Leaf	Mite population
Virus concentration	1.000	-	-
Leaf	- 0.664*	1.000	-
Mite population	- 0.648	0.786 *	1.000

*Highly significant

dilution of 1:15,000 and for PNC-IgG conjugate, at 1:1500 dilutions were found to be the optimum for PPSMV detection in pigeonpea.

4. 4. 2. Detection of PPSMV in *A. cajani* by ELISA

Extracts from single, 5, 10, 25, 50 and 100 *A. cajani* exposed to SMD infected plants were used for the detection of PPSMV. Strong positive ELISA reaction was observed with 100 mites and weak positive reaction with 50 mites but no reaction occurred with single, 5, 10 or 25 mites. The results show that PPSMV could be detected successfully in *A. cajani* by ELISA.

4. 4. 3. Detection of PPSMV in *A. cajani* by Dot immunobinding assay (DIBA)

PPSMV was detected by spotting of 5µl aliquot of homogenate prepared by grinding 5, 10, 25, 50 or 100 viruliferous *A. cajani*. However, virus could not be detected in a single mite. No back-ground reaction was observed when extracts from non-viruliferous mites were similarly assayed (Fig. 9). This suggest, at least a group of five are required for detection of PPSMV by DIBA.

4. 4. 4. Detection of PPSMV by RT-PCR

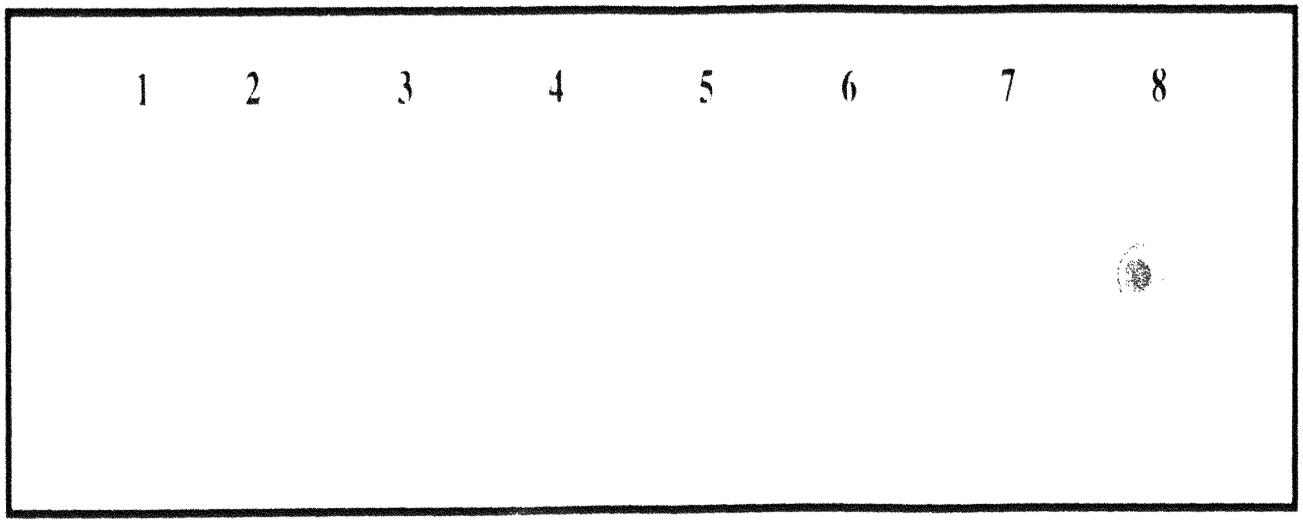
Detection of PPSMV in SMD infected pigeonpea cv. ICP8863 was done by RT-PCR using the oligonucleotide primers SM-1 and SM-2 derived from the nucleotide sequence of PPSMV RNA-5. The primers amplified specifically a 321 bp product from total RNA extracts from SMD-affected pigeonpea, when the RT-PCR product was analyzed in 1% agarose gel. Results indicated the presence of PPSMV. No amplification was observed from total RNA isolated from healthy pigeonpea leaves (Fig. 10).

Table: 11. Detection of PPSMV in healthy and SMD infected pigeonpea leaves by DAS-ELISA

Antiserum dilutions	Antigen dilution									Enzyme – IgG conjugate dilutions
	Healthy				Infected				Buffer	
	1:10	1:100	1:1000	1:10000	1:10	1:100	1:1000	1:10000		
1 : 7500	1.751	1.768	1.801	1.806	0.134	0.226	1.557	1.760	1.836	1 : 1500
	1.877	1.832	1.910	1.875	0.524	0.757	1.729	1.815	1.831	1 : 2500
1 : 15000	1.734	1.836	1.828	1.736	0.245	0.357	1.588	1.747	1.803	1 : 1500
	1.750	1.826	1.804	1.823	0.770	0.954	1.698	1.813	1.886	1 : 2500

Note : Readings taken 30 min after adding PNC substrate at 620 nm. OD values represent average from two replications

Figure: 9. Detection of PPSMV in *Aeria cajani* by Dot Immuno-binding Assay (DIBA)



1 to 6 represents, Single, 5, 10, 25, 50 and 100 mites respectively, **7.** Healthy mites, **8.** SMD infected pigeonpea leaf

4. 4. 5. Detection of PPSMV in *A. cajani* by RT-PCR

Detection of PPSMV in *A. cajani* using RT-PCR resulted in no amplification from the macerates obtained from 100 and 200 virulifeorus mites when the RT-PCR product was analyzed in 1% agarose gel. However amplification occurred when RNA from SMD-affected pigeonpea leaves were used.

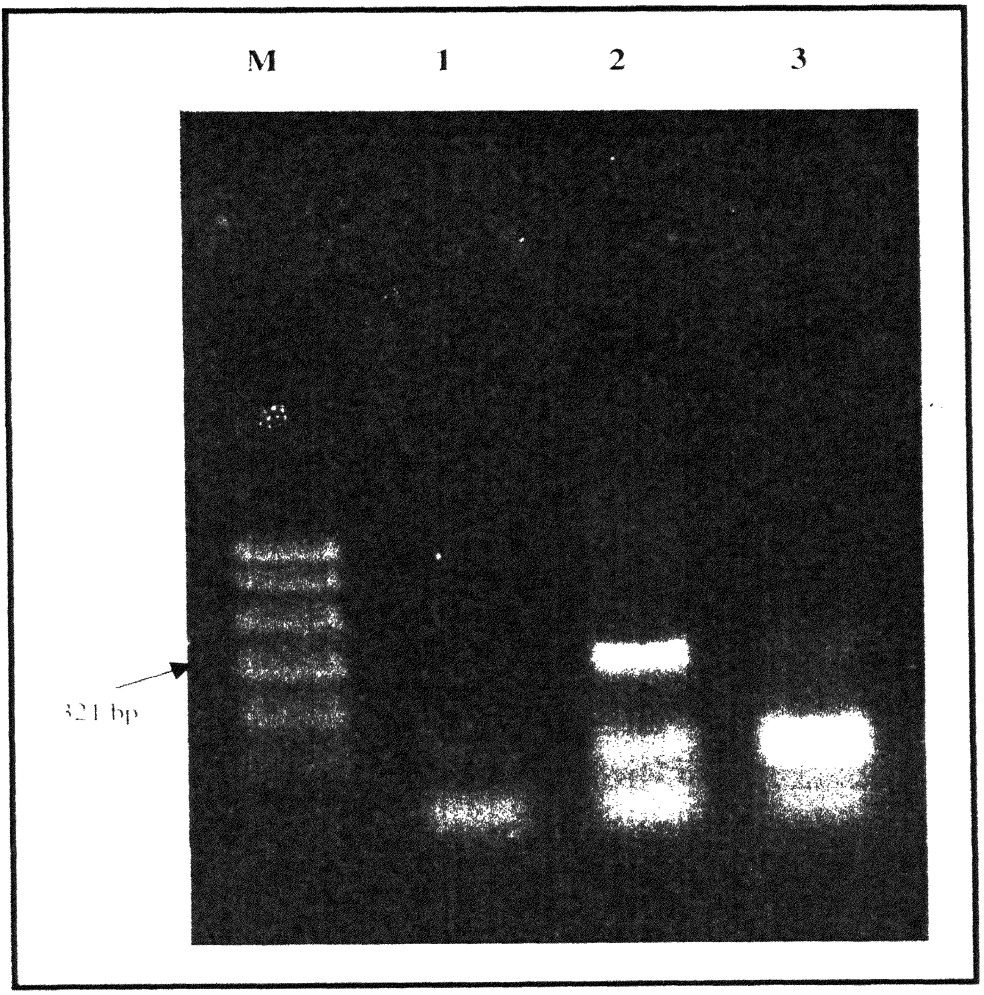
4. 5. Screening of pigeonpea genotypes for SMD resistance

4. 5. 1. Evaluation of pigeonpea breeding lines

In a field experiment, 28 advanced pigeonpea breeding lines were screened for SMD resistance at ICRISAT, Patancheru and at UAS, Bangalore following "infector row technique". Disease appeared on all the test lines at ICRISAT, Patancheru with percent incidence ranging from 1% on ICPL99044 and 60% for ICPL87119. No disease was observed on ICP7035 and over 95 percent incidence was recorded in susceptible checks ICP8863, C-11 and BDN-1. The following genotypes, ICPL93001, ICPL96048, ICPL96053, ICPL96061, ICPL99044, ICPL99046, ICPL99051 and ICPL99087 showed ring spot (RS) symptoms and rest of the genotypes showed severe mosaic (SM) symptoms. Flowering occurred on all the genotypes that showed RS symptoms indicating tolerance to SMD. Mites were found on all the lines except on ICP7035 (resistant check). Among the breeding lines tested, ICPL93003 supported the lowest number of mites (3 mites/leaf) and ICPL96053 supported the maximum numbers (89 mites/leaf). Over 100 mites/leaf were observed on susceptible check C-11 (Table 12).

At UAS Bangalore, percent disease incidence ranged between 15 to 100 with minimum infection on ICPL96061 (15.34%) and maximum on ICPL87051, ICPL96047 and ICPL99098 (100%). ICP7035 was found to be free from disease with no mite infestation. With the exception of genotypes, ICPL96061, ICPL97087 and ICPL99092

Figure: 10. Detection of PPSMV in SMD infected pigeonpea by RT - PCR



M-DNA molecular weight marker, Lane-**1**, Control; **2**-SMD infected pigeonpea; **3**-Healthy pigeonpea

Table 12. Evaluation of advanced pigeonpea breeding lines for SMD resistance at ICRISAT, Patancheru and at Bangalore during kharif 2000-2001

Sl. No.	Genotype	ICRISAT, Patancheru					Bangalore				
		No. of plants infected/tested		Mean % Incidence	Symptom Type	No. of ** mites/leaf	No. of plants infected/tested		Mean % Incidence	Symptom Type	No. of ** mites/leaf
		R1	R2				R1	R2			
1	ICPL 93001	7/63	17/70	18	RS	46	32/32	7/9	89	SM	37
2	ICPL 93003	3/48	6/65	8	RS+SM	3	14/15	5/6	88	SM	6
3	ICPL 87051	3/31	5/43	9	RS+SM	17	4/4	1/1	100	SM	26
4	ICPL 96047	3/56	18/66	16	RS+SM	79	27/27	1/1	100	SM	4
5	ICPL 96048	0/63	14/68	10	RS*	48	11/13	12/13	88	SM*	6
6	ICPL 99044	0/72	1/76	1	RS*	22	13/15	11/11	93	SM	36
7	ICPL 96053	3/41	9/82	9	RS*	89	9/11	6/6	91	SM	41
8	ICPL 96061	0/80	7/63	5	RS*	27	5/21	2/29	15	MM*	5
9	ICPL 99046	1/91	4/80	3	RS*	12	17/26	27/27	83	SM	8
10	ICPL 99047	3/42	6/68	8	RS+SM	31	4/27	16/16	57	SM	28
11	ICPL 99048	1/63	11/88	7	RS+SM	10	8/10	28/33	82	SM	41
12	ICPL 99049	1/71	8/64	7	RS+SM	10	5/7	21/25	78	SM	77
13	ICPL 99050	4/76	3/64	5	RS+SM	10	5/8	24/24	81	SM	21
14	ICPL 99051	0/86	4/75	3	RS*	8	4/13	33/33	64	SM	16
15	ICPL 99054	3/69	6/64	7	RS+SM	9	3/4	22/25	82	SM	48
16	ICPL 99055	4/65	18/73	6	RS+SM	6	5/6	11/12	88	SM	39
17	ICPL 96058	15/57	37/86	35	RS+SM	27	3/8	37/37	69	SM	82
18	ICPL 87119	22/68	61/69	60	RS+SM	84	18/24	18/18	88	SM	7
19	ICPL 99086	6/58	13/67	15	RS+SM	6	19/29	28/31	78	RS+SM*	23
20	ICPL 99087	2/50	10/57	11	RS*	48	11/27	18/24	68	MM+SM*	14

21	ICPL 99092	7/70	5/55	10	RS-SM	50	15/18	14/32	63	MM*	17
22	ICPL 99093	0/72	7/68	5	RS+SM	36	17/34	36/38	72	SM	5
23	ICPL 99096	5/55	14/87	13	RS+SM	10	35/35	39/44	94	SM	24
24	ICPL 99097	3/73	6/65	7	RS+SM	4	28/28	31/31	98	SM	17
25	ICPL 99098	5/68	9/82	9	RS+SM	70	38/38	42/42	100	SM	4
26	ICPL 99100	3/55	4/64	6	RS+SM	2	23/28	48/48	91	SM	8
27	ICPL 99101	2/66	12/70	10	RS+SM	2	23/24	36/46	87	SM	10
28	ICPL 99102	4/55	14/58	16	RS+SM	11	12/27	10/35	37	SM	27
29	BDN 1	63/65	56/59	96	SM	79	NT	NT	-	-	-
30	C-11	43/43	22/23	98	SM	91	NT	NT	-	-	-
31	ICP 8863	50/54	58/60	95	SM	96	34/34	38/38	100	SM	67
32	TTB-7	NT	NT	-	-	-	51/51	38/38	100.0	SM	69
33	ICP 7035	0/25	0/27	0.0	-	0.0	0/11	0/16	0.00	-	0.0

RS-Ring spot. **MM**-Mild Mosaic, **SM**-Severe Mosaic. **NT**-Not Tested

* Flowering observed. ** Mean of five replications

which showed mild mosaic, all the other showed severe mosaic symptoms. Ring spot type of symptoms appeared on ICPL99086. Mite population found to be low on all the lines at UAS, Bangalore when compared to Patancheru. ICPLI96058 supported the maximum number of mites (81 mites/leaf) and ICPL96047 and ICPL99098 supported the lowest number (about 4 mites/leaf). Flowering occurred only on ICPL96061, ICPL99086, ICPL99087 and ICPL99092 (Table 12).

4. 5. 2. Response of pigeonpea host differentials

Ten pigeonpea host differentials were tested at ICRISAT, Patancheru and UAS, Bangalore for SMD reaction using infector-row technique. Only ICP7035 found to be free from the disease at both the locations with no mite infestation. Disease incidence was found to be relatively high at Bangalore than at ICRISAT on all the genotypes. ICP2376 produced ring spots at ICRISAT (93%) and severe mosaic at Banglore (100%). ICP11164 and ICP10976 produced ring spots and severe mosaic respectively at ICRISAT and they showed severe mosaic at Bangalore. ICP8862 did not show any overt symptoms at ICRISAT. It showed mild mosaic (MM) symptoms at Bangalore (75%). C-11, Purple-1, LRG-30, ICP8863 and BDN-1 showed severe mosaic symptoms at both the locations. Unlike percent disease incidence, over all mite population was found to be less at Bangalore than in ICRISAT, Patancheru (Table 13).

4. 5. 3. Screening of wild relatives of pigeonpea for SMD resistance

Sixty-two wild relatives of pigeonpea were screened at ICRISAT Patancheru and at UAS, Bangalore for SMD resistance, using leaf-stapling technique under glasshouse conditions. Of 62 wild species accessions tested, ICP15614, ICP15697, ICP15700, ICP15701, ICP15702, ICP15708, ICP15709, ICP15712, ICP15726, ICP15728, ICP15734 and ICP15743 were found free from SMD. Although, ICP15650, ICP15685,

Table 13. Reaction of pigeonpea host differentials to PPSMV at ICRISAT, Patancheru and Bangalore

Sl. No.	Genotype	ICRISAT, Patancheru					Bangalore			
		No. of plants infected/tested		Mean % Incidence	Symptom Type	No. of mites/leaf*	No. of plants infected/tested	% Incidence	Symptom Type	No. of mites/leaf*
		R1	R2							
1	ICP2376	3/3	6/7	93	RS	40	15/15	100	SM	23
2	C-11	24/31	13/40	55	SM	91	22/25	88	SM	36
3	ICP11164	2/38	3/37	7	RS + SM	72	29/33	88	SM	46
4	ICP8862	0/22	0/24	0.0	NS	9	6/8	75	MM	2
5	Purple-1	1/31	9/36	14	SM	28	7/8	88	SM	23
6	ICP7035	0/25	0/27	0.0	NS	0	0/11	0.0	NS	0
7	ICP10976	3/40	7/34	14	RS + SM	59	12/29	41	MM	6
8	LRG 30	36/42	45/45	93	SM	57	33/39	85	SM	32
9	ICP8863	33/36	45/45	98	SM	96	13/14	93	SM	49
10	BDN-1	30/30	35/40	94	SM	79	12/12	100	SM	28

RS - Ring spot, **MM** - Mild Mosaic, **SM** - Severe Mosaic, **NS** - No Symptoms. * Mean of five replications

Table 14. Screening of wild genotypes of pigeonpea at ICRISAT and at Bangalore for SMD resistance

Sl .no	Genotype	ICRISAT, Patancheru			UAS, Bangalore		
		Plants infected/tested [a]	Symptom Type	No. of mites/Leaf [b]	Plants infected/tested [a]	Symptom Type	No. of mites/Leaf [b]
1	ICP15614	0/14 (0.0)	NS	2	0/6 (0)	NS	0
2	ICP15650	16/24 (67)	SM	0	NT	-	-
3	ICP15683	16/16 (100)	MM-SM	3	0/3 (0)	NS	0
4	ICP15684	1/28 (4)	MM	2	0/24 (0)	NS	0
5	ICP15685	14/26 (54)	MM	0	0/14 (0)	NS	0
6	ICP15686	25/27 (93)	MM-SM	4	2/26 (8)	SM	3
7	ICP15687	8/20 (40)	MM-SM	3	2/16 (13)	SM	0
8	ICP15688	1/33 (3)	MM	0	1/9 (11)	SM	2
9	ICP15689	16/25 (64)	MM-SM	2	7/34 (21)	SM	2
10	ICP15690	15/26 (58)	MM-SM	15	9/27 (33)	SM	1
11	ICP15691	14/24 (58)	MM-SM	2	5/20 (25)	SM	2
12	ICP15692	3/20 (15)	MM-SM	2	1/22 (5)	SM	2
13	ICP15693	22/34 (65)	SM	3	2/22 (9)	SM	1
14	ICP15694	10/27 (37)	MM-SM	2	4/22 (18)	SM	1
15	ICP15695	1/21 (5)	SM	0	1/37 (3)	SM	0
16	ICP15696	9/26 (35)	MM	0	4/33 (12)	SM	2
17	ICP15697	0/21 (0)	NS	0	2/14 (14)	MM	3
18	ICP15698	9/21 (43)	SM	4	9/21 (43)	SM	1
19	ICP15699	3/26 (12)	SM	2	2/15 (13)	SM	1
20	ICP15700	0/16 (0)	NS	0	0/17 (0)	NS	0
21	ICP15701	0/22 (0)	NS	0	0/18 (0)	NS	0
22	ICP15702	0/21 (0)	NS	0	2/24 (8)	SM	0
23	ICP15703	2/25 (8)	MM-SM	0	0/7 (0)	NS	0
24	ICP15704	4/29 (14)	SM	0	3/19 (16)	SM	0
25	ICP15705	3/19 (16)	MM-SM	1	1/19 (5)	SM	0
26	ICP15706	6/29 (21)	SM	3	2/23 (9)	SM	1
27	ICP15707	1/22 (5)	MM	0	0/23 (0)	NS	0
28	ICP15708	0/23 (0)	NS	0	6/23 (26)	MM	0
29	ICP15709	0/15 (0)	NS	0	4/12 (33)	MM	0
30	ICP15710	26/30 (87)	SM	5	4/15 (27)	SM	2

31	ICP15711	16/27 (59)	MM	0	1/20 (5)	SM	0
32	ICP15712	0/10 (0)	NS	0	0/17 (0)	NS	0
33	ICP15713	2/16 (13)	MM-SM	2	5/24 (21)	SM	4
34	ICP15716	8/10 (80)	MM	9	0/32 (0)	NS	0
35	ICP15717	2/15 (13)	MM	0	4/21 (19)	SM	1
36	ICP15718	21/26 (81)	SM	18	4/19 (21)	SM	2
37	ICP15719	10/12 (83)	MM-SM	6	3/10 (30)	SM	2
38	ICP15720	10/11 (91)	SM	14	4/18 (22)	SM	3
39	ICP15721	15/18 (83)	MM-SM	2	2/32 (6)	MM-SM	3
40	ICP15722	18/19 (95)	MM	0	1/33 (3)	SM	0
41	ICP15723	31/39 (79)	SM	14	10/26 (39)	SM	4
42	ICP15724	34/41 (83)	MM	4	0/27 (0)	NS	0
43	ICP15725	1/20 (5)	MM	0	0/16 (0)	NS	0
44	ICP15726	0/24 (0)	NS	0	1/26 (4)	MM	0
45	ICP15727	22/32 (69)	MM-SM	7	4/34 (12)	SM	2
46	ICP15728	0/20 (0)	NS	0	0/25 (0)	NS	0
47	ICP15729	7/26 (27)	MM-SM	8	1/36 (3)	SM	0
48	ICP15730	2/17 (12)	SM	3	6/20 (30)	SM	2
49	ICP15731	20/31 (65)	SM	3	5/12 (42)	SM	3
50	ICP15732	6/26 (23)	MM	1	3/9 (33)	SM	2
51	ICP15733	19/28 (68)	MM-SM	20	1/14 (7)	MM	2
52	ICP15734	0/23 (0)	NS	0	0/10 (0)	NS	0
53	ICP15735	14/14 (100)	MM-SM	0	2/14 (14)	SM	0
54	ICP15736	1/26 (4)	MM	2	0/7 (0)	NS	0
55	ICP15737	2/35 (6)	MM	0	1/11 (9)	MM	0
56	ICP15738	34/41 (83)	SM	9	3/9 (33)	SM	1
57	ICP15739	1/20 (5)	MM	0	0/12 (0)	NS	0
58	ICP15740	1/21 (5)	MM	0	0/15 (0)	NS	0
59	ICP15741	1/25 (4)	MM	0	1/12 (8)	MM	0
60	ICP15742	2/22 (9)	MM	0	12/16 (75)	SM	1
61	ICP15743	0/23 (0)	NS	0	4/15 (27)	MM	0
62	ICP15744	4/22 (18)	MM	1	6/13 (46)	SM	0
63	ICP8863	47/48 (98)	SM	18	19/21 (90)	SM	11
64	TTB 7	NT	-	-	15/16 (94)	SM	6

RS - Ring spot, **MM** - Mild Mosaic, **SM** - Severe Mosaic, **NS** - No Symptoms, [a] All the plants tested by DAS-ELISA, [b] Mean of five replications, NT Not tested

ICP15696, ICP15703, ICP15704, ICP15711, ICP15717, ICP15735 were found infected with SMD, but did not support mite multiplication (Table 14). However rest of the susceptible accessions supported mites (1 to 18 mites/leaf) and SMD resistant accessions did not.

At Bangalore, the following genotypes, ICP15614, ICP15683, ICP15684, ICP15685, ICP15700, ICP15701, ICP15703, ICP15707, ICP15712, ICP15716, ICP15724, ICP15725, ICP15728, ICP15734, ICP15736, ICP15739, and ICP15740 were found to be resistant. Mite population was found to be relatively low (1-4 mites/leaf) on all the susceptible accessions at Bangalore. ICP15614, ICP15700, ICP15701, ICP15712, ICP15728 and ICP15734 genotypes were found to be resistant at both the locations with out mite infestation. Symptoms observed are listed in table 14.

The accessions, which showed resistance at ICRISAT and at Bangalore (ICP15614, ICP15684, ICP15688, ICP15700, ICP15701, ICP15725, ICP15736, ICP15737, ICP15740), were tested by graft inoculation. Initially, RS symptoms were recorded on ICP15614 (only one of thirteen plants tested) which later disappeared with advancement of the age of the plant and rest of the accessions showed MM type of symptoms indicating tolerance to PPSMV (Table 15).

4. 5. 4. Studies on inheritance of resistance

F_2 plants obtained from the crosses made between *C. scaraboeides* (resistant to SMD) and Pant A_2 (a cultivated pigeonpea variety, with a good agronomic traits, but susceptible to SMD) were tested by ELISA. Of 118 F_2 plants tested, 92 (78%) plants were found to be infected with PPSMV and 26 (22%) plants were free from infection

Table 15. Effect of graft inoculation of cultivated and wild accessions (resistant to staple inoculation) of pigeonpea on PPSMV transmission

Sl. No.	Genotype	No. of plants infected/tested (% infection) *	Symptom type
1	ICP15614	1/13 (8)	RS
2	ICP15684	5/15 (33)	MM
3	ICP15688	6/13 (46)	MM
4	ICP15700	2/31 (6)	MM
5	ICP15701	4/24 (17)	MM
6	ICP15725	1/10 (10)	MM
7	ICP15736	5/21 (24)	MM
8	ICP15737	7/28 (25)	MM
9	ICP15740	7/24 (29)	MM
10	ICP7035	1/25 (4)	MM
11	TTB 7	14/16 (86)	SM
12	ICP8863	14/17 (82)	SM

RS-Ring spot, MM-Mild mosaic, SM-Severe mosaic

* Tested by DAS-ELISA

(data not shown). The susceptible plants were discarded and the resistant ones were advanced to F_3 generation. Of 260 F_3 plants tested by ELISA, only 12 plants were found to be ELISA positive, and the rest of 248 plants were found to be free from the virus (Data not shown). The susceptible plants were discarded and resistant ones were advanced to F_4 generation.

4. 6. Experiments on identification of alternate sources of PPSMV infection

In an experiment to find out natural infection of PPSMV to other hosts, weeds present in SMD affected pigeonpea field were tested by DAS-ELISA. Of 30 weeds tested, only one weed, *Chrozophora rottleri* (Family-Euphorbiaceae) was found to be infected with PPSMV as detected by ELISA tests (2 of 12 plants). No mosaic symptoms occurred on ELISA positive plants and there was no mite infestation. None of the weeds tested for virus presence supported *A. cajani* with the exception of *Hibiscus penduliformis* (Family – Malvaceae) where, 9 mites/leaf were observed (Table 16). However, *H. penduliformis* was not infected by PPSMV. Back inoculations of mites from the leaves of such plants on to a susceptible pigeonpea cv. ICP8863 resulted in PPSMV infection within two weeks and theses plants were found to be ELISA positive (Data not shown). This result indicates that mite did not feed on *H. penduliformis*, hence retained the virus.

In a glasshouse experiment, 23 cultivated crop species, 6 *Nicotiana* species and 29 commonly available weeds were tested for PPSMV infection by leaf stapling technique. Of the crop species tested, PPSMV infection occurred only on *Phaseolus vulgaris* (36%), *P. vulgaris* var. Bountiful (32%), *P. vulgaris* var Kintoki (46%) and *P. vulgaris* var Topcrop (60%) (Table 17). Symptoms appeared 20 days pi. PPSMV infection on these host species resulted in stunting of plant. Reduction in the size of

Table 16. Testing of weeds found in SMD infected pigeonpea field for natural infection of PPSMV and its vector *Aceria cajani*

Sl. No	Type	Family	ELISA reaction	No. of* mites/leaf
1	*Abelmoschus ficulneus*	Malvaceae	-	0
2	*Abutilon indicun*	Malvaceae	-	0
3	*Ageratum conyzoides*	Asteraceae	-	0
4	*Alternanthera pugens*	Amaranthaceae	-	0
5	*Amaranthus tricolor*	Amaranthaceae	-	0
6	*Amaranthus viridis*	Amaranthaceae	-	0
7	*Cardiospermum helicacabum*	Sapindaceae	-	0
8	*Cassia tora*	Leguminosae	-	0
9	*Celotia argentea*	Amaranthaceae	-	0
10	*Chrozophora rottleri*	Euphorbiaceae	+	0
11	*Corchorus trilocularis*	Tiliaceae	-	0
12	*Corchrus aestuans*	Tiliaceae	-	0
13	*Datura stramonium*	Solanaceae	-	0
14	*Desmodiumdichotomum*	Leguminosae	-	0
15	*Digera muricata*	Amaranthaceae	-	0
16	*Euphorbia heterlphylla*	Euphorbiaceae	-	0
17	*Euphorbia hirta*	Euphorbiaceae	-	0
18	*Euphorbia hirta*	Euphorbiaceae	-	0
19	*Hibiscus panduriformis*	Malvaceae	-	9
20	*Ipomea hispida*	Convalulaceae	-	0
21	*Lagasca mollis*	Asteraceae	-	0
22	*Lantana camara*	Laminaceae	-	0
23	*Leucana leucacephala*	Leguminosae	-	0
24	*Macroptilum atropurpureum cv sirato*	Leguminosae	-	0
25	*Merremia gangetica*	Convolvulaceae	-	0
26	*Ocimum canum*	Laminaceae	-	0
27	*Parthenium histerophorus*	Asteraceae	-	0
28	*Phyllanthus maderaspatenis*	Euphorbiaceae	-	0
29	*Trichodesma zelanicum*	Boraginaceae	-	0
30	*Tridax procumbans*	Asteraceae	-	0
31	*Cajanus cajan*	Leguminosae	+	14
32	Infected control (ICP8863)	-	+	-
33	Healthy control	-	-	-

* Mite population was recorded during February 2001 (Mean of five replications)

Table 17. Cultivated Crop species and weeds tested for SMD in glass house

Sl. No.	Type[a]	Family	No. of plants infected/ Tested [b]	No. of mites/leaf [c]
1	*Arachis hypogaea*	Leguminosae	0/32	0
2	*Canavalia ensiformis*	Leguminosae	0/22	0
3	*Capsicum annuum*	Sonalanceae	0/9	0
4	*Cicer arietinum*	Leguminosae	0/16	0
5	*Dolichos lablab*	Leguminosae	0/13	0
6	*Glycine max*	Leguminosae	0/30	0
7	*Gossypium arboreum*	Malvaceae	0/15	0
8	*Helianthus anuus*	Compositae	0/10	0
9	*Lycopersicon esculentum*	Solanaceae	0/17	0
10	*Medicago sativa*	Fabaceae	0/21	0
12	*N. rustica*	Sonalanceae	0/13	0
13	*N. tabacum* var. turkish	Sonalanceae	0/7	0
14	*N. tabacum* var. xanthi	Sonalanceae	0/12	0
15	*N. tabacum* var.samsun	Sonalanceae	0/16	0
11	*Nicotiana benthamiana*	Sonalanceae	0/10	0
16	*Nicotiana glutinosa*	Sonalanceae	0/14	0
17	*Phaseolus aconitifolius*	Leguminosae	0/17	0
18	*Phaseolus aureus*	Leguminosae	0/16	0
19	*Phaseolus mungo*	Leguminosae	0/22	0
20	*Phaseolus vulgaris*	Leguminosae	9/25	0
21	*Phaseolus vulgaris* cv. Pinto	Leguminosae	0/17	0
22	*Phaseolus vulgaris* cv.Bauntiful	Leguminosae	8/25	0
23	*Phaseolus vulgaris* cv.Kintoki	Leguminosae	12/26	0
24	*Phaseolus vulgaris* cv.Top crop	Leguminosae	12/20	0
25	*Pisum sativum*	Leguminosae	0/16	0
26	*Trigonella foenum-graecum*	Fabaceae	0/12	0
27	*Vicia faba*	Leguminosae	0/13	0
28	*Vigna sinensis*	Leguminosae	0/29	0
29	*Vigna unguiculata* cv.Early Ramshorn	Leguminosae	0/32	0

Table 17 continued...

Sl. No.	Type	Family	No. of plants infected/tested	No. of mites/leaf
30	*Acanthospermum hispidium*	Asteraceae	0/18	0
31	*Achyranthes aspera*	Amarathaceae	0/21	0
32	*Ageratum conyzoides*	Asteraceae	0/17	0
33	*Alternanthera echinata*	Amarathaceae	0/16	0
34	*Alternanthera pungens*	Amarathaceae	0/15	0
35	*Amaranthus viridis*	Amarathaceae	0/29	0
36	*Argemone mexicana*	Papaveraceae	0/5	0
37	*Bidens biternata*	Asteraceae	0/22	0
38	*Cassia ceresea*	Leguminosae	0/22	0
39	*Cassia taurica*	Leguminosae	0/19	0
40	*Chenopodium amaranticolor*	Chenopodiaceae	0/29	0
41	*Chenopodium murale*	Chenopodiaceae	0/22	0
42	*Chenopodium. album*	Chenopodiaceae	0/20	0
43	*Chenopodium. quinoa*	Chenopodiaceae	0/27	0
44	*Corchorus trilocularis*	Tiliaceae	0/25	0
45	*Datura metel*	Solanaceae	0/9	0
46	*Datura stramonium*	Solanaceae	0/22	0
47	*Euphorbia hirta*	Euphorbiaceae	0/10	0
48	*Gomphrena celosioides*	Amaranthaceae	0/12	0
49	*Hibiscus penduliformis*	Malvaceae	0/15	0
50	*Ipomea acquaticus*	Convalulaceae	0/15	0
51	*Lagasca mollis*	Asteraceae	0/11	0
52	*Malvastrum coromandelianum*	Malvaceae	0/16	0
53	*Ocimum canum*	Laminaceae	0/9	0
54	*Physalis floridum*	Solanaceae	0/17	0
55	*Physalis minima*	Solanaceae	0/12	0
56	*Solanum xanthocarpum*	Solanaceae	0/13	0
57	*Syndrella nodiflora*	Asteraceae	0/18	0
58	*Tridax procumbans*	Asteraceae	0/9	0
59	*Cajanus cajan* cv. ICP8863	Leguminosae	30/30	35

[a] Plants were inoculated by leaf stapling technique

[b] PPSMV infection was rated by visual symptoms and confirmed by DAS-ELISA

[c] Mean of five replications

Figure: 12. Comparison of PPSMV infected and Healthy

Phseolus vulgaris var topcrop plants

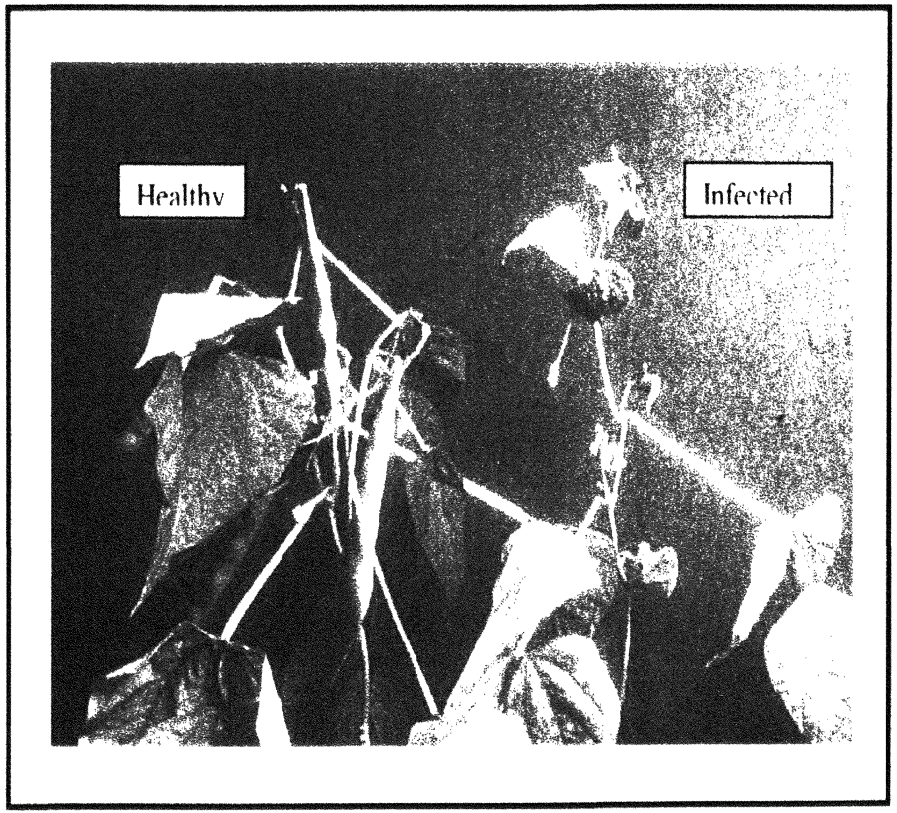
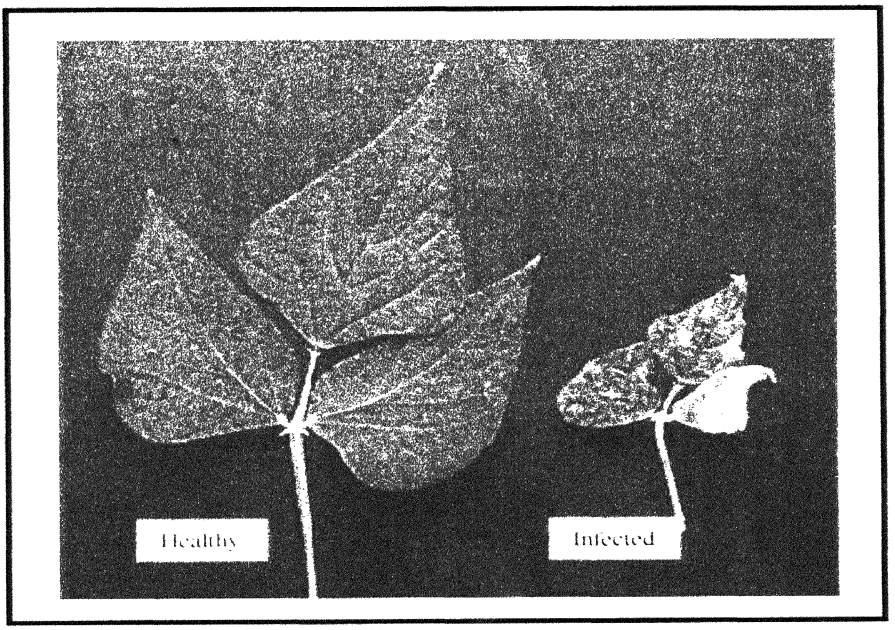


Figure: 13. Comparison of healthy and SMD infected *Phseolus vulgaris* var topcrop leaves



leaves, flower and pods was also observed. Young leaves showed mosaic and crinkling type of symptoms (Fig. 13). Presence of PPSMV in theses plants was confirmed by ELISA and by RT-PCR (Fig.14). Virus was not recorded in inoculated leaves.

Mites exposed to young leaves of PPSMV infected *Phseolus vulgaris* var topcrop leaves for 3-4 h resulted in PPSMV transmission (on 2 of 7 plants tested) to pigeonpea plants. This result suggested that, *Phaseolus vulgaris* may serve as a source of PPSMV under natural conditions.

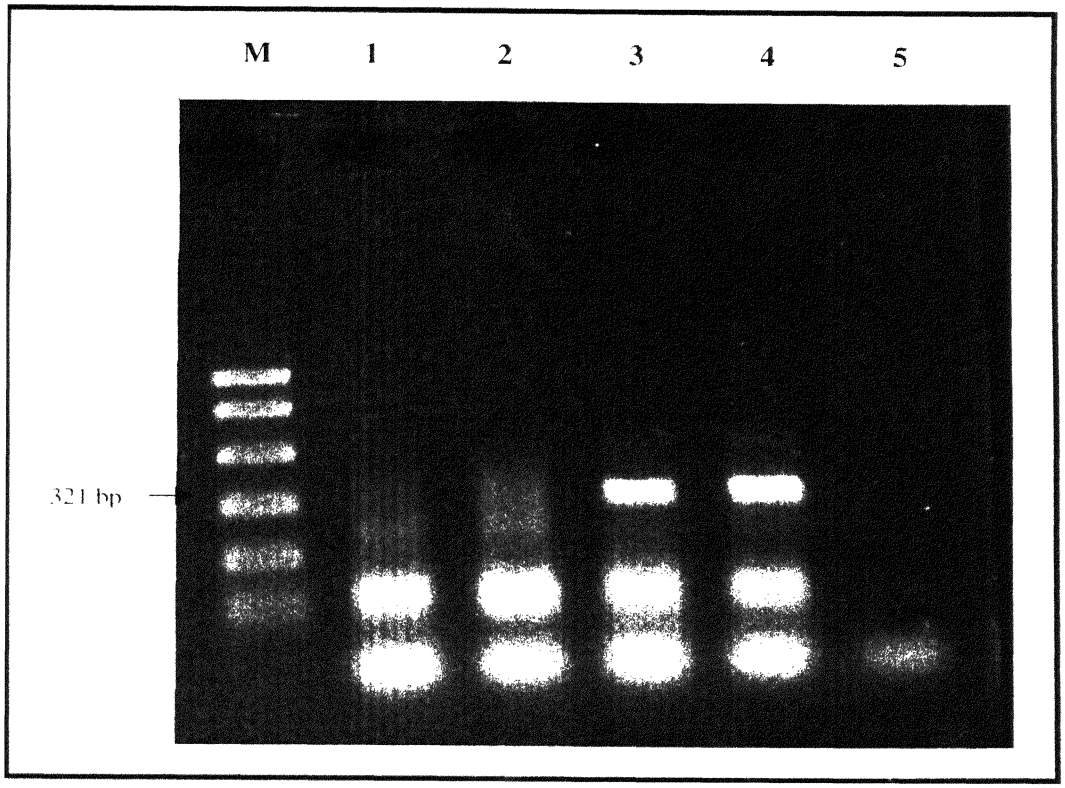
4.6.1. Behavior of *A. cajani* on pigeonpea and non-hosts species

Behavior of *A. cajani* was determined by transferring them on to the leaves of its natural host, pigeonpea and non-hosts like sorghum and groundnut. *Aceria cajani*, soon after its transfer on to pigeonpea moved randomly for some time (about five minutes) and then started to feed in case of susceptible as well as resistant pigeonpea genotypes. Where as on a non-host, sorghum, after moving randomly for few minutes (about 5 min), it moved towards the leaf blade (leaf edge) and then stood erect with the help of its caudal setae presumably to facilitate dispersal by wind. Out of the ten mites transferred, at least four mites showed such behavior on sorghum leaf. No such behavior was noticed on either pigeonpea or groundnut, which is non-host

4. 7. Effect of barrier crop on the incidence of SMD

In a field experiment conducted on SMD management, pigeonpea cv. ICP8136 (resistant to PPSMV but supports mites) was used as a barrier between the SMD infected hedge and the susceptible pigeonpea cv. ICP8863 by planting three rows of ICP8136 all along the border. Planting of susceptible pigeonpea one month after

Figure: 14. RT-PCR Detection of PPSMV in staple inoculated pigeonpea and *Phseolus vulgaris* var topcrop plants



Lane-**M**. DNA molecular weight marker, lane-**1**. healthy *P. vulgaris*. var topcrop, **2**. healthy pigeonpea, **3**. SMD infected *P. vulgaris* var topcrop and **4**. SMD infected pigeonpea **5**. Control

planting of barrier plants resulted in attaining canopy height difference of about 1 ft between the barrier and the susceptible pigeonpea plants. No infection was noticed on barrier plants where as, SMD symptoms occurred 3 weeks after planting of susceptible pigeonpea. Observations after one month of planting, 5 percent of the plants were infected and the incidence gradually increased to 90% in two and a half months. However, 100% SMD infection was noticed in the check plot, where no barrier of ICP8136 was used. This suggests that barrier plants failed to check the spread of *A. cajani* from hedge to the test rows of pigeonpea.

V. DISCUSSION

The main purpose of this investigation was to study the *Aceria cajani* - PPSMV relationships, to understand the epidemilogy of SMD and to identify the SMD resistant sources. For understanding epidemiology of any virus disease, an important requirement is a knowledge of virus-vector relationships. Virus-vector relationships of PPSMV was reported by Reddy *et al* (1989). However, this research was done when the causal virus was not identified and consequently tools did not exist for precise virus diagnosis. PSMD has recently been shown to be caused by a virus (Kumar *et al*., 2000) and, the sensitive diagnostic tools for the precise virus identification have been developed (Kumar *et al*., 20001a), therefore time was ripe to undertake reinvestigation of PPSMV relationships with its mite vector *A. cajani*

In order to study any virus-vector relationships, it is essential to produce and maintain virus free cultures of the vector. Therefore techniques were developed to produce and maintain a PPSMV-free or non-viruliferous culture of *A. cajani*. The use of floating leaflets of a PPSMV-immune cultivar proved to be a simple and efficient means to generate non-viruliferous mites within 3 days. Previous methods that used plants of a PPSMV-resistant pigeonpea cultivar took more than 3 months to generate non-viruliferous *A. cajani* and the populations obtained after that period remained low (Ghanekar *et al*., 1992).

Our study has shown that single *A. cajani* can transmit PPSMV, but that transmission efficiency was not 100% unless 10 mites per plant were used (Table 5) Increased AAPs increased transmission of PPSMV by single *A. cajani*, but the

maximum transmission achieved with single mites was about 50% (Table 6). However, this compares with the much lower efficiency reported for the transmission of some other mite-transmitted viruses. For example, only 17% of individual *Eriophyes insidiosus* transmitted *Peach mosaic virus* (PMV) (Gispert *et al.*, 1998) and 1% of *A. tosichella* transmitted *Wheat streak mosaic virus* (WSMV) (Orlob, 1966). Compared to other vector mite species therefore, *A. cajani* shoul be considered as an efficient vector of PPSMV. One possible reason for the low efficiency of transmission by single mites may be differing efficiencies in the acquisition/transmission of mites at different stages in their life cycle. Another is that different populations of individual mite species may differ in vector efficiency, as has been shown recently for the transmission of High Plains virus (HPV) by *A. tosichella* (Seifers *et al.*, 2002).

For successful transmission of PPSMV, *A. cajani* requires a minimum of 15 min AAP and 90 min IAP but these times were decreased to 10 min and 60 min respectively, when mites were starved prior to feeding (Tables 9 a & b). These differences in time following starvation were probably due to the feeding behaviour by starved mites soon after they were transferred onto fresh leaves. Under normal transfer conditions, mites spent some time searching for a suitable site on the leaf surface before feeding. Viruliferous mites lost the ability to transmit PPSMV after feeding for 2-10 h on healthy plants. (Table 8) and there was no apparent latent period associated with transmission. It is possible that mites inoculate PPSMV immediately on feeding but that a minimum of 90 min feeding is required to inject sufficient amount of virus for infection to occur.

A. cajani retained PPSMV for up to 6 h when feeding and for more than 13 h without access to a susceptible host (Table 8). This explains the ability of *A. cajani* to

transmit PPSMV after being carried in wind currents to new plants. Although *A. cajani* remained alive without feeding for up to 30 h in a moist chamber, they did not survive when transferred to plants. It is unlikely therefore that in nature the mites survive for very many hours without feeding. Viruliferous *A. cajani* did not retain PPSMV for life as shown by serial transmission studies using single mites (Table 8), confirming an earlier study (Nene & Reddy, 1976). It is not surprising therefore that we found no evidence for transovarial transmission. Indeed, none of the eriophyid mite-borne viruses are reported to multiply in their vector nor are they transmitted through the egg of their vector.

Transmission details for most other eriophyid mite-borne viruses are not well defined due to the inherent difficulties in manipulating such tiny creatures. To date, the best-studied relationship is that of WSMV and its vector *A. tosichella* (previously known as *A. tulipae*). WSMV is transmitted by all stages of its vector, and is retained through the moult, but not through the egg. However, adults could transmit only if they acquired the virus during their immature stages; they could not acquire the virus as adults and then transmit it. *A. tosichella* acquired WSMV in a minimum AAP of 15 minutes (Slykhuis, 1955), although 50% transmission of WSMV was only achieved with AAP of 16 h AAP (Orlob, 1966). Once acquired, WSMV was transmitted by *A. tosichella* for at least 4 days after transferring them from WSMV-immune plants back to WSMV-susceptible plants (Del Rosario and Sill, 1965). Furthermore, ultrastructural studies showed WSMV particles in the midgut of viruliferous mites, where they persisted for at least 5 days (Slykhuis, 1955; Paliwal and Slykhuis, 1967); occasionally particles were also found in the haemocoel and salivary glands (Paliwal, 1980). Based on these findings, it was suggested that the mode of transmission was circulative but transmission by regurgitation, although unlikely, was not ruled out (Paliwal, 1980). In

less detailed studies, the uncharacterized agent of Fig mosaic disease was reported to be transmitted in a persistent manner by *A. ficus* with a 6-7 h latent period in the vector, to be retained through the moult, and to be transmitted by viruliferous mites to for up to 10 days (Proeseler, 1969; 1972). No latent period is reported for any other eriophyid mite-borne plant pathogen.

Based on our data, the transmission of PPSMV by *A. cajani* is best considered to be in a semi-persistent manner. Studies on two other mite-transmitted viruses also indicate a semi-persistent mode of transmission. Thus, transmission of PMV by *E. insidiosus* required an AAP of 3 days and an IAP of 6 h with no latent period (Gispert, *et al.*, 1998), and of *Ryegrass mosaic virus* (RgMV) by *Abacarus hystrix* requiring an AAP of at least 2 h with mites losing infectivity after 24 h (Mulligan, 1960; Slykhuis & Paliwal, 1972).

PPSMV has several novel properties and shows close similarities with HPV and to disease agents transmitted by eriophyid mites that cause Rose rosette and Fig mosaic (Oldfield & Proeseler, 1996; Ahn *et al.*, 1996, 1998; Kumar *et al.*, 2001; 2002). In the light of our studies reported here, a re-assessment of the suggested persistent transmission of the agent of Fig mosaic disease by *A. ficus* may be worthwhile.

Finally, the finding that PPSMV infection of pigeonpea greatly increased the reproduction of *A. cajani* compared to healthy plants, confirmed field observations (Reddy *et al.*, 1980). Similarly, greatly increased numbers of *Cecidophyopsis ribis*, the mite vector of *Blackcurrant reversion virus*, were reported on blackcurrant plants

infected with this virus compared to healthy plants (Thresh, 1964). Therefore, there is a beneficial relationship between the vector mite and the virus.

Attempts made on sap transmission of PPSMV resulted in virus transmission to *Phaseolus* plants but it was inconsistent with very low efficiency. However, mechanical transmission of PPSMV was achieved from pigeonpea to *Nicotiana benthamiana* and *N. clevelandii* plants with difficulty (Kumar *et al.*, 2002). Inconsistency in sap transmission may be due to is instability of PPSMV in the plant sap and moreover pigeonpea is a woody host, containing high polyphenolic complexes that might interfere with the virus infectivity (Kumar *et al.*, 2002). However, PPSMV was transmitted by grafting with higher efficiency (over 80%) using petiole grafting method (Table 4).

None of the reports published so for looked into the detection of PPSMV in *Acaeria cajani* because the necessary tools were not available. The virus detection in *A. cajani* was attained using DAS-ELISA and Dot-immunobinding assay (DIBA). The virus could be detected in DAS-ELISA only when more than 50 mites were used. However, the virus could be detected by DIBA utilizing as few as five mites. It is likely that, the dilutions used in making mite extracts may not have permitted the detection of virus in less than 50 mites by DAS-ELISA. The only report of detection of virus in the vector is in case of WSMV transmitted by *A. tosichella* using immuno-fluorescent microscopy and DIBA (Mahmood *et al.*, 1997). Attempts to detect PPSMV in the mite vector by RT-PCR utilizing more than 100 mites did not yield successful results. The dilutions used for making the mite extracts may not have permitted the amplification of RNA required for detection by RT-PCR. Additionally, it is possible that RNA extracted from filamentous virus like PPSMV may not be amenable for amplification (Dr. A. T

Jones, Personal communication). However, the attempts made were preliminary and require further investigation.

The response of host differentials confirms that the virus strains present at ICRISAT, Patancheru and at Bangalore are different and Bangalore strain appears to be more virulent than that of Patancheru strain. Based on the differential response to PPSMV at different locations, occurrence of five different strains of PPSMV has been reported (Reddy *et al.*, 1993a). However, reaction of host differentials alone is not enough to differentiate the virus strains. Further confirmation using molecular methods such as, genome sequencing of these strains is needed to unravel the variation in PPSMV strains.

A number of genotypes have been shown to be resistant to PSMD in multilocational trials conducted over a period of 15 years (Reddy *et al.*, 1989). Few genotypes showed resistance at more than one locations. Therefore, there was a need to identify broad-based resistance to PSMD. With this aim many wild relatives of pigepnepa were screened for PSMD resistance at ICRISAT and Bangalore under glasshouse conditions. ICP15614, ICP15700, ICP15701, ICP15712, ICP15728 and ICP15734 were found to be resistant at both the locations. These accessions were also evaluated by graft inoculation to determine if the resistance is to the virus. ICP15614 was found to be resistant to the vector as well as to the virus. Rest of the genotypes showed overt symptoms. Therefore the resistance observed under lab conditions is likely to be due the vector resistance.

A preliminary attempt was made on the inheritance of virus resistance in a cross between wild pigeonpea, *Cajanus scaraboeides* (resistant to SMD) and Pant A_2 (a cultivated pigeonpea variety, with a good agronomic traits, but susceptible to SMD). The generations were advanced until F_3. Susceptibility was found to be dominant over resistance. SMD resistance is governed by a recessive gene and supports the observations made by previous workers (Sharma *et al.*, 1984 and Srinivas *et al.*, 1997 b & c).

Various weeds present in SMD affected pigeonpea fields were tested for PPSMV presence by DAS-ELISA. Only *Chrozophora rottleri* (Family-Euphorbiaceae) was found to be infected by PPSMV (Table 16). Interestingly this is the first report of natural infection of PPSMV to a host other than that of *Cajanus* species. This weed was not found to be colonized by mites. Therefore, it is unlikely that it will act as a source of virus inoculum for SMD spread.

Hibiscus penduliformis (Family – Malvaceae) was found to harbour *A. cajani* under field conditions. In laboratory tests also the mites survived on this leaf floated on water surface, for two days. Additionally mites have retained the virus for the entire period, therefore the mites may not have fed on this leaf. Pubescent nature of *H. penduliformis* leaf may have prevented the vector from escaping from the leaf surface after landing.

Under glasshouse conditions, various cultivars of *Phaseolus vulgaris*, Bountiful, Kintoki and Topcrop, could be infected by the leaf stapling technique. None of the plants supported mite multiplication. However, non-viruliferous mites could acquire the virus from leaves of all the three cultivars of *Phaseolus vulgaris* hence they may act as

a source of inoculum for mites. This is the first report of transmission of PPSMV by *A. cajani* to other hosts out side the *Cajanus* species

Intercropping was earlier experimented for reducing SMD incidence (Siddappaji *et al.*, 1979; Bhatnagar *et al.*, 1984 and Zote *et al*, 1988). This did not yield successful results. In this study, effect of using a barrier pigeopea crop which is resistant to virus but supports mite multiplication was attempted. The aim of this experiment was to trap the mite vector prior to its colonization on the main crop. However, in a single experiment performed, no difference in the SMD incidence was noticed between the treated and check plots. Since the vector dissemination is by wind (Reddy *et al.*, 1989), three rows of barrier crop did not prevent vector colonization on to the main crop. It is worth while to repeat this experiment utilizing at least ten rows of barrier crop.

VI. SUMMARY

Investigations on Pigeonpea sterility mosaic disease (PSMD) caused by a virus, Pigeonpea sterility mosaic virus (PPSMV), transmitted by an eriophyid mite vector, *Aceria cajani,* was carried out with special reference to virus-vector relationships, identification of SMD resistant sources and identification of alternate sources of PPSMV infection at ICRISAT, Patancheru and UAS, Bangalore during 1999-2002. The findings of these investigations are summarized below.

Sap transmission of PPSMV occurred on *Phaseolus vulgaris* var Topcrop with 0.1 M Phosphate buffer (pH 7.0). Of the three grafting methods tried to establish an efficient grafting method for PPSMV transmission, petiole grafting resulted in maximum virus infection (87%). However, PPSMV was not transmitted by dodder.

A new method called 'float leaf technique' was developed to generate non-viruliferous *A. cajani* colony. It is simple and convenient method where large number of non-viruliferous mites can be generated in just three days.

Efficiency of single mite to transmit PPSMV was found to be about 40 percent. However, more than five mites per plant resulted in 100 percent infection. Minimum 15 min of AAP and 1.5 h of IAP are required for *A. cajani* to acquire and transmit PPSMV respectively. No latent period is involved in virus transmission. Increase in AAP and IAP resulted in increased transmission efficiency. However, at least 15 h of AAP, and more than 5 h of IAP is required to attain 100 percent PPSMV transmission. Serial transmission experiments using single mite revealed that, *A. cajani* lose PPSMV between 2 to 10 upon feeding on a susceptible pigeonpea plants. Mites did not retain

the virus after first or second transfers. *A. cajani* retained PPSMV even after 13 h of starvation on dried pigeonpea leaf. Starvation of mites influenced acquisition and inoculation access periods. However, no transovarial transmission of PPSMV was observed. Based on this data, the transmission of PPSMV by *A. cajani* is best considered to be in a semi-persistent manner.

A. cajani preferred SMD infected pigeonpea plants for its multiplication and survival. However, younger leaves of SMD infected plants supported higher mite number than the old or matured leaves and the mite number was found to be positively correlated with the PPSMV concentration in leaves.

PPSMV was successfully detected in SMD affected pigeonpea leaves by DAS-ELISA, and by RT-PCR. The virus was also detected in, *A. cajani* by DAS-ELISA and by Dot-immunobinding assay (DIBA) but not by RT-PCR. However, more than 50 mites are required for PPSMV detection by ELISA and at least 5 mites by DIBA.

Reaction of host differentials observed at ICRISAT, Patancheru was different from that of Bangalore indicating that the PPSMV strain present at ICRISAT is different from the Bangalore strain.

Screening of advanced pigeonpea breeding lines at ICRSAT and at Bangalore, none of the lines were found to be resistant except ICP7035. However, ICPL93001, ICPL96048, ICPL96053, ICPL96061, ICPL99044, ICPL99046, ICPL99051 and ICPL99087 at ICRISAT, and ICPL96061, ICPL97087 and ICPL99092 at Bangalore showed tolerance to PPSMV. Mite population was found to be low on all the lines at Bangalore when compared to ICRISAT.

Of 62 wild accessions of pigeonpea screened at ICRISAT and at Bangalore for PSMD resistance in glass house genotypes, ICP15614, ICP15700, ICP15701, ICP15712, ICP15728 and ICP15734 were found to be resistant at both the locations with no mite infestation.

Studies on inheritance of PSMD resistance by crossing *Cajanus scaraboeides* (resistant to SMD) and Pant A_2 (a cultivated pigeonpea variety, with a good agronomic traits, but susceptible to SMD) revealed that susceptibility is dominant over resistance and resistance to SMD is governed by a single recessive gene.

Weeds present in SMD affected pigeonpea field were tested for natural infection of PPSMV. *Chrozophora rottleri* (Family-Euphorbiaceae) was found to be infected with PPSMV as detected by ELISA tests and they were not infested with mites. However, none of the weeds tested for virus presence supported *A. cajani* with the exception of *Hibiscus penduliformis* (Family – Malvaceae). Mites from this weed transmitted PPSMV when inoculated back on to pigeonpea

Cultivated crop species and the weeds were tested in glasshouse by leaf stapling technique. PPSMV infection occurred only on *Phaseolus vulgaris* and its varieties such as Bountiful, Kintoki and Topcrop. Symptoms on these host species appeared as stunting of plant, reduction in the size of leaves, flower and pods. Young leaves showed mosaic and crinkling type of symptoms. Presence of PPSMV in these plants was confirmed by DAS-ELISA and by RT-PCR. Non-viruliferous mites exposed infected *Phaseolus* leaves for 3-4 h, when transferred on to pigeonpea plants, resulted in PPSMV transmission.

Observations on behavior of *A. cajani* on non-host like sorghum revealed that, mites do attempt to escape from the non-host plant using its caudal setae.

Field experiment conducted using PPSMV resistant pigeonpea cv ICP8136 as a barrier, failed to check the spread of *A. cajani* from hedge to the test rows of pigeonpea.

Future line of work

- Develop a variety with broad based multiple resistance by back cross breeding using wild and cultivated pigeonpeas

- Thorough investigation is needed on alternate sources of PPSMV by looking for natural infection by PPSMV in SMD endemic areas

- Light must be thrown on how actually *Aceria cajani* survives during off season (summer)

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*Originals not seen.

APPENDIX

APPENDIX

Buffers required for DAS-ELISA (Penicillinase system)

Carbonate(Coating) Buffer

Na2Co3	:	1.59 g
NaHCo3	:	2.93 g
Distilled Water:		1.0 L.

pH of the buffer should be 9.6. No need to adjust the pH.

Phosphate buffer saline (PBS), pH 7.4

Na2HPO4	:	2.38 g
KH2PO4	:	0.4 g
KCl	:	0.4 g
NaCl	:	16.0 g
Distilled water:		2 L.

PBS-Tween (Washing) buffer

PBS	:	1 L.
Tween-20	:	0.5 ml

Antigen Extraction buffer

PBS-Tween	:	100 ml
Polyvinyl Pyrollidone (PVP)	:	2.0 g

Antibody buffer (PBS-TPO)

PBS-Tween	:	100 ml
Polyvinyl Pyrollidone (PVP)	:	2.0 g
Ovalbumin	:	0.2 g

Distilled water-Tween

Distilled water	:	1 L.
Tween-20	:	0.5 ml

Substrate buffer

Dissolve 15 mg bromothymol blue (BTB) in 50 ml of 0.01 M NaOH. Neutralize the alkali by adding conc. HCl drop wise. Make up the volume to 100 ml. Incorporate Sodium Pencillinase-G at 0.5 ml^{-1} and adjust the pH to 7.2 using either HCl or NaOH.

Buffers required for Dot Immuno-binding Assay (DIBA)

Coating buffer pH 9.6

Na2HCo3	:	1.59 g
NaHCo3	:	2.93 g

Dissolve in about 900 ml distilled water, adjust the pH to 9.6; make up the volume to 1 L.

Tris-buffered saline (TBS) pH 7.5

Tris (0.02 M)	:	4.84 g
NaCl (0.15 M)	:	58.48 g

Dissolve in about 1.9 L distilled water, adjust the pH to 7.5 and make up the volume to 2L.

TBS-Tween

TBS	:	1 L.
Tween-20	:	0.5 ml

Blocking solution

TBS	:	100 ml
Non fat dried milk powder:		5 g.

Antibody buffer

TBS	:	100 ml
Non fat dried milk powder:		5 g.

Substrate solution

One tablet of Fast red TR/Naphthol in 10 ml of Tris buffer.

Buffers and reagents required for RT-PCR

RNase free water

Treat distilled waster with 0.1% diethylpyrocarbonate (DEPC; Sigma) for 12 h at 37 °C. Then autoclave for 15 min at15lb/sp. Inch to destroy DEPC.

10mm dNTP mixture

Mix 10µl of each dATP, dCTP, dGTP, dTTP from a 100mM stock and make up the to 100 µl with RNase free water. The final concentration of each dNTP in this mixture is 10mM.

25mM $MgCl_2$

Usually supplied with Taq enzyme by the manufacturer. If necessary, prepare by dissolving 0.508 g of $MgCl_2$. $6H_2O$ in 100 ml RNsae free water. Sterilize by autoclaving, aliquot and store at –20 °C.

0.1 M DTT

Dissolve 154 mg of DTT in 10 ml of RNase free water, aliquot and store at –20 °C.

Agarose Gel Electrophoresis

10x Electrophoresis buffer (TBE buffer, pH 8.3)

Tris base (0.45 M)	:	54 g
Boric acid (0.45M)	:	27.5 g
0.5 M EDTA, pH 8.0 (0.01 M):		20 ml
Distilled water	:	to 1 L.

It is not necessary to adjust the pH. Sterilize by autoclaving and store at room temperature.

5x Sample loading buffer

Bromophenol blue	(0.25%)	:	5 mg
Xylene cynol FF	(0.25%)	:	5 mg
Glycerol	(30%)	:	3 ml
Sterile distilled water		:	to 10 ml

1% Ethidium bromide solution

Ethidium bromide	:	100 mg
Distilled water	:	10 ml

Store in a dark coloured bottle at 4 °C

Buffers used for mechanical sap inoculation

TM buffer, 0.1 M (pH 7.0)

Dissolve 6.06 g Tris and 12.33g of $MgSO_4$, in 1 L distilled water, adjust the pH and add 750 µl of α-Monothioglycerol

0.1 M Phosphate buffer (pH 7.0)

Dissolve, 10.8 g of K_2HPO_4, 4.8 g of KH_2PO_4 in 1L. of distilled water, add 750 µl of α-Monothioglycerol

0.1 M, Sodium citrate buffer (pH 7.0)

Dissolve, 21 g of Citric acid, in 1L of 1N NaOH, adjust the pH to 7.0 by adding 0.1 N NaOH.

Extraction of immunogama-globulins (IgGs) from antisera

- To 1 ml of crude antiserum, add 1 ml of distilled water.
- Add 2 ml of 36% Na_2SO_4, drop by drop (36% Na_2SO_4, Dissolve 36 g Na_2SO_4 in 90 ml water and make up the volume to 100 ml)
- Immediately collect the precipitate, by centrifugation at 6000 rpm for 10 min and discard the supernatant.
- Add 18% Na_2SO_4,(mix 36% Na_2SO_4 with equal volume of distilled water) mix the precipitate and centrifuge at 6000 rpm for 10 min.
- Repeat washing in 18% Na_2SO_4
- Dissolve the precipitate in 2 ml half strength PBS with azide (PBS diluted to 1:1 with distilled water).
- Dialyze three time against half strength PB-azide: with at least 500 ml of buffer for each dialysis. The third dialysis should be left over night.
- Remove IgGs from the dialysis bag, measure the concentration by reading the absorbance (200-300 nm) in a spectrophotometer. Store IgGs in refrigerator in aliquots of 1 ml.

Conjugation of immunoglobulins with penicillinase

- Place IgG, 500^{-1}, in a dialysis bag and add 250 µg of penicillinase. Solutions oflgG and penicillinase can be made at hogher concentrations and mixed to get IgG mg ml^{-1} and 1 mg ml^{-1} penicillinase.

- Dialyse against PNS ina beaker for 1 h at room temperature.

- Transfer the dialysis bag (containing IgG and enzyme) into a beaker containing PBS with 0.06% glutaraldehyde (mix 1 ml of 25% glutaraldehyde in 400 ml PBS to get 0.06% glutaraldehyde) and dilyse for 3-4 h at room temperature.

- Replace the buffer containing glutaraldehyde with 500 ml PBS containing sodium azide (0.02%) and dialyze for 18 h at 4 °C with atleast three changes of buffer (for each change replace with 500 ml PBS containing azide).

- Transfer the conjugate into a new glass or plastic vial and bovine serum albumin at 5 mg ml^{-1} concentration. Store in small aliquots (100 ml) at 4 °C.

Scheme for SMD resistance screening