METHODS MANUAL
Pigeonpea Sterility Mosaic Virus:
Detection & Screening for Resistance

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For the training course on
Methods for the detection of Pigeonpea Sterility Mosaic Virus and
Screening for Sterility Mosaic Disease Resistance

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1. Preface

For several diseases of dicotyledonous plants caused by agents transmitted by eriophyid mites, such agents have failed to be isolated and characterised. Most of these diseases were reported from temperate countries of the northern hemisphere, but an exception is sterility mosaic disease (SMD) affecting pigeonpea. This disease, reported in 1931, from Bihar State, India, is now recognized in all the pigeonpea growing countries of South-East Asia. SMD is particularly important in India where >80% of the global pigeonpea is grown and where SMD causes devastating yield losses every year. SM is one of few crop diseases that have baffled pathologists for decades. In our recent efforts substantial progress has been achieved in characterising the SMD causal agent. It was shown to be a distinct virus, provisionally named, Pigeonpea sterility mosaic virus (PPSMV). Additionally, methodologies were established for PPSMV detection and for the precise identification of broad-based durable resistance sources. Through this training program we aim to disseminate these technologies with the hope that scientists in the developing countries can utilise the techniques to formulate eco-friendly strategies for SMD management.

The course manual deals with the application of enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) techniques for PPSMV detection to assist screening pigeonpea germplasm for durable resistance to this disease. The tests developed are simple, and suitable for application in developing countries. Though the technology may appear to be complicated to a novice, every effort has been made to simplify the procedures. It is our objective that the knowledge gained will be used towards sustainable pigeonpea production in the Indian subcontinent.

The information and methods presented in the course are developed from a project entitled ‘Characterization of the causal virus of pigeonpea sterility mosaic disease: a step towards attaining sustainability of pigeonpea production in the Indian subcontinent’ funded by the United Kingdom Department for International Development (DFID; Project No R7452). Some basic procedures have been adapted from our and other people’s work and we credited this where applicable.

We would like to acknowledge with gratitude financial support from the United Kingdom Department for International Development (DFID), under the Crop Protection Program (Project No. R7452). The views expressed in this manual do not necessarily those of DFID.
Abbreviations

BTB  Bromothymol blue  
CV  Cultivar  
DAS-ELISA  Double antibody sandwich-enzyme-linked immunosorbent assay  
DNA  Deoxyribonucleic acid  
dH2O  Distilled water  
dNTPs  Deoxynucleotide phosphates  
ELISA  Enzyme-linked immunosorbent assay  
IC-RT-PCR  Immuno capture-reverse transcription-polymerase chain reaction  
Ig  Immunoglobulin  
IgG  Immuno-γ-globulin  
kb  Kilo base  
kbp  Kilo base pair  
kDa  Kilo Dalton  
PAGE  Polyacrylamide gel electrophoresis  
PBS  Phosphate buffer saline  
PCR  Polymerase chain reaction  
pi  Post-inoculation  
PNC  Penicillinase  
PPSMV  Pigeonpea sterility mosaic virus  
RNA  Ribonucleic acid  
RT-PCR  Reverse transcription-polymerase chain reaction  
SMD  Sterility mosaic disease  
SM  Sterility mosaic  
VLP  Virus-like particles  
WIB  Western immuno blotting

Symbols/Units

A  Absorbance  
cm  Centimeter  
°C  Degree centigrade  
g  grams  
h  hours  
l  Liter  
lb/sq.in  Pounds per square inch  
M  Moles  
mM  Millimoles  
min  minutes  
mil  Milliliter  
mg  Milligram  
μl  Microliter  
μg  Microgram  
ng  Nanogram  
nm  Nanometer  
OD  Optical density  
pH  Hydrogen ion concentration  
%  Per cent  
rpm  Revolutions per minute  
sec  Seconds  
v  Volume  
w  Weight
2. Pigeonpea Sterility Mosaic Disease – An Introduction

The sterility mosaic disease (SMD) is the most important disease of pigeonpea in the Indian subcontinent. SMD is characterized by a bushy and pale green appearance of plants, excessive vegetative growth, stunting, reduction in leaf size, leaf distortion and mosaic of leaves (Fig 1). Complete, or partial, cessation of flowering (sterile) occurs. Symptoms are often masked in older plants. However, when ratooned the newly produced leaves show clear symptoms. SMD symptoms depend on the pigeonpea genotype and they are categorized into three types: (i) severe mosaic and sterility; (ii) mild mosaic with partial sterility; and (iii) chlorotic ring spots without any noticeable sterility (Fig 2). Susceptibility of the plant decreases as age of the plant increases. The yield loss varies with the cultivar and age at which infection occurs. Highest disease incidence and losses are in ratoon and perennial pigeonpea. A susceptible genotype infected at an early stage of crop growth usually show complete sterility with an yield loss of up to 90%. Estimated annual yield losses due to SMD are over US$282 million in 1993.

Since its first description in 1931 from the Bihar state, India, little progress has been made in understanding the nature of the causal pathogen. However, convincing evidence was obtained to show that the causal agent is a virus. The SMD agent is transmitted by the eriophyid mite vector, Aceria cajani (Fig 3). This disease is confined to pigeonpea growing countries of south Asia. Lack of sensitive techniques for the rapid and unambiguous detection of the SMD causal agent, and scarce information on SMD epidemiology hampered the development of eco-friendly disease management strategies.
2.1. SMD etiology

Recently, using a new purification method, the SMD causal agent was isolated and confirmed as a virus, provisionally named as Pigeonpea sterility mosaic virus (PPSMV). The purified PPSMV preparations consist of slender highly flexuous filamentous virus-like particles (VLPs) of c. 3-10 nm in diameter (Fig 4), a major virus-specific protein of c. 32 kDa and 5-7 major RNA species of c. 0.8 – 6.8 kb. Polyclonal antiserum to PPSMV virus-VLPs preparations was produced in a rabbit. The partial nucleotide sequence of some cDNA clones made to PPSMV RNA revealed no significant sequence matches to any of the known viral sequences in the database. Oligonucleotide primers were developed for the detection of PPSMV by reverse transcription-polymerase chain reaction (RT-PCR).

PPSMV polyclonal antibodies in all SMD-affected plants detected the virus using ELISA, and the virus-specific 32 kDa protein in Western immunoblotting (WIB). In such assays, the virus was detected consistently in all SMD-affected pigeonpea plant samples from several different locations in India, but not in samples from symptom-free pigeonpea plants from the same locations. In experimental studies, all pigeonpea plants inoculated with viruliferous A. cajani and those plants grafted-inoculated with SMD-affected tissue, were infected with the virus as assessed by ELISA and WIB, but not any uninfected pigeonpea plants.

2.1.1. Mechanical transmission

Purified PPSMV preparations were not infective to plants. However, PPSMV was transmitted to Nicotiana benthamiana by mechanical sap inoculation using freshly extracted SMD-affected pigeonpea leaf sap (Fig. 5), but not to pigeonpea or several other herbaceous hosts tested. The transmission efficiency by mechanical inoculation to N. benthamiana was low (10-30% infection), and visible symptoms and detection of virus in these plants occurred only after more than 40 days post-inoculation (pi). Inoculum prepared from fresh sap extracts of PPSMV infected N. benthamiana was transmitted to N. benthamiana and N. clevelandii, but not pigeonpea or other herbaceous hosts.

2.1.2. Cytopathology

Ultrastructural studies of leaves from SMD-affected pigeonpea cultivars, ICP8863 showing sever mosaic symptoms and ICP2376 showing chlorotic ringspots, and PPSMV-infected N. benthamiana, revealed quasi-spherical, membrane bound bodies (MBBs) (Fig. 6) of c. 100-150 nm and
amorphous electron-dense material (EDM). These structures were distributed singly or in groups, in the cytoplasm of all cells, but not in conductive tissues. Fibrous inclusions (FIs), composed of randomly dispersed fibrils with electron lucent areas, were present in the cytoplasm of palisade cells and rarely in mesophyll cells of the two-pigeonpea cultivars. Immuno-gold labelling using antiserum to PPSMV, specifically labelled the MBBs and associated EDM, but not the FIs, indicating MBBs and EDMs contain the 32 kDa nucleoprotein and that FIs could probably be the non-structural protein component of the virus. The MBBs and associated inclusions are similar in appearance to those reported for plants infected with the eriophyd mite-transmitted High Plains virus and the agents of unidentified aetiology associated with rose rosette, fig mosaic, thistle mosaic, wheat spot chlorosis and yellow ringspot of budwood.

2.1.3. Transmission characteristics
The transmission characteristics of PPSMV to pigeonpea by its vector A. cajani were studied. Non-viruliferous A. cajani colonies were generated by ‘float-leaf technique’. The transmission efficiency of single A. cajani was up to 53% but was 100% when >5 mites per plant were used. A. cajani acquired PPSMV after a minimum inoculation access period (IAP) of 90 min. No latent period was observed. Starvation of A. cajani prior to, or following, PPSMV acquisition reduced the minimum AAP and IAP periods to 10 min and 60 min, respectively, and mites retained virus for up to 13 h. None of the mites that developed from eggs taken from PPSMV-infected leaves transmitted the virus indicating that it is not transmitted transovarily. Taken together, these data suggest a semi-persistent mode of transmission of PPSMV by A. cajani.

2.1.4. Taxonomy
PPSMV has some properties similar to virus species in the genera Tospovirus and Tenuivirus and with the eriophyd mite-transmitted High plains virus (HPV) but is distinct from these and from all other characterized viruses. The combination of novel properties shown by PPSMV and HPV suggest that they may constitute species in a new genus of plant viruses.

2.1.5. Host range
Twenty-nine commonly occurring weed species in the families Amaranthaceae, Asteraceae, Solanaceae, Boraginaceae, Convolulaceae, Tiliaceae, Euphorbiaceae, Laminaceae, Sapindaceae and Leguminaceae occurring naturally in pigeonpea fields of ICRISAT, Patancheru were analyzed for PPSMV and A. cajani. Of the plants observed only 2 of 12 plants of Chrozophora rotteri (Euphorbiaceae) tested positive for PPSMV in DAS-ELISA. No overt symptoms or mites were noticed on these two PPSMV-infected plants. Observations revealed that C. rotteri was susceptible to PPSMV, but it did not support A. cajani multiplication and therefore may not act as an inoculum source for PPSMV and mites in...
the fields. A. cajani were found on all the 11 Hibiscus penduliformis (Malvaceae) observed (5 to 9 mites/leaf). When mites from H. penduliformis were transferred onto indicator plants (pigeonpea cv. ICP8863), they developed typical SMD symptoms and were positive for PPSMV in DAS-ELISA. Hibiscus penduliformis plants observed in the fields were present close to pigeonpea plants. It is likely that mites carried by wind currents from pigeonpea might have entangled in the highly pubescent H. penduliformis leaves.

In experimental studies by inoculating various crop and weed species with vector mites, Phaseolus vulgaris cvs. Bountiful, Kintoki and Topcrop were infected with PPSMV. Affected plants showed stunting, reduction in leaf size, mosaic and crinkling and malformation of flowers and pods 20 days pi (Fig 7). However, none of these plants supported mite multiplication. Experiments indicated that A. cajani could acquire and transmit PPSMV from P. vulgaris. Although, P. vulgaris is infectible with PPSMV by mite inoculation, it did not support mite multiplication. Further studies are required to determine the significance of P. vulgaris as an inoculum source for PPSMV in the field.

Several accessions of wild Cajanus species tested positive for PPSMV and these plants supported mite multiplication, confirming earlier reports that they can harbour virus and vectors and act as potential sources of inoculum in the field.

Recent and past studies on SMD host range indicate that hosts of PPSMV include several accessions of cultivated and wild pigeonpea, N. benthamiana, N. clevelandii, P. vulgaris and C. rottleri. In the field, pigeonpea, its wild relatives, and C. rottleri were naturally infected with PPSMV, but only some wild Cajanus species supported A. cajani. However, H. penduliformis was infested with A. cajani, but was free from PPSMV. Under experimental conditions, P. vulgaris, but not Nicotiana species, were infected with PPSMV by vector mites, even though these later species were infected by mechanical sap inoculation. These studies show that PPSMV infects plants outside the genus Cajanus but, because mites are highly host specific, only accessions of Cajanus genus were found to support their multiplication. Therefore, only the cultivated and wild accessions of pigeonpea serve as potential sources of PPSMV under field conditions. Some weed species, such as H. penduliformis may act as a refuge for mite survival and may therefore aid the spread of SMD.

**Fig 7.** Phaseolus vulgaris cv. Topcrop infected with PPSMV. (A) Apical portion of the infected plant; (B) Leaf symptoms of PPSMV infected P. vulgaris (right) and healthy control (left).
2.1.6. Variability in host plant resistance

Screening trials for sources of SMD resistance initiated under a collaborative project between ICRISAT and the Indian Council of Agricultural Research (ICAR) have identified pigeonpea varieties with field resistance to SMD infection. By screening nearly 15,000 germplasm accessions about 400 lines resistant (no overt symptoms) or tolerant (no sterility or ring spots symptoms) to the SMD were identified. Most of these genotypes were shown to possess location-specific resistance. The resistance mechanism is not known, but is presumed to be resistant to either the pathogen, the vector, or to both agents. Three factors were attributed to the location-specific variation observed in SMD resistance: (i) variability in the pathogen, (ii) variability in the mite vector, (iii) the plant genotype and environment interaction.

The role of mite vector and its influence on host-plant resistance was studied using DNA-based markers to (i) determine whether different species of Aceria mites are involved as vectors; (ii) assess the diversity amongst A. cajani populations and (iii) understand the variation in SMD resistance shown by different pigeonpea genotypes with respect to the mite vector. This study suggested that A. cajani on pigeonpea across the Indian subcontinent constitutes one population and that no other Aceria species or A. cajani biotypes that differ in virus transmission ability are involved in PPSMV transmission. This indicates that host plant resistance across the Indian subcontinent is influenced by biotypes (strains) of PPSMV.

Previous studies using a set of 7 differential pigeonpea genotypes, indicated existence of at least 5 variants of SMD. Our recent studies using the differential cultivars indicated that PPSMV at Patancheru is a mild strain compared to that of the strain endemic in Bangalore and Coimbatore regions. Further wider studies to determine the PPSMV strains are underway.

2.1.7. SMD diagnosis

Until now, SMD recognition and selection of resistant lines is based solely on symptom expression. Disease confirmation based on symptoms alone is complicated by the fact that symptoms are governed by many biotic and abiotic factors. Pigeonpea is a cross-pollinated crop and in addition to environmental factors, genotypic variability induced as a result of cross-pollination, is also likely to play an important role in symptomatology.

Polyclonal antiserum was produced to PPSMV in a rabbit. These have been shown to be very effective in detecting PPSMV in plant tissues, utilizing double antibody sandwich (DAS)-ELISA [Detailed in section 5.1]. This assay is simple, sensitive, and cost effective, and can easily be adaptable to conditions in developing countries. For sensitive detection of PPSMV, RT-PCR-based method has been developed [Detailed in section 5.2]. These tests are now being used routinely for PPSMV detection in plants and in mites.

2.1.8. Screening for SMD-resistance

A system for screening of pigeonpea genotypes under laboratory conditions has been standardized [Detailed in 5.4]. Plants raised in growth chambers are inoculated at the 2-leaf stage with vector-mites by the leaf-stapling technique. Plants are monitored for disease symptoms and also tested for PPSMV in DAS-ELISA. Resistant genotypes (asymptomatic and ELISA negative) are tested again by graft inoculation. Since PPSMV is not
mechanically transmissible to pigeonpea, graft transmission tests are performed to confirm its resistance to virus. This method of screening pigeonpea genotypes confirmed for the first time that, there are genotypes that are (i) resistant to PPSMV and mites, (ii) resistant to mites, but not to PPSMV (iii) resistant to PPSMV, but not to mites and (iv) susceptible to PPSMV and mites. Using a combination of ELISA, mite transmission by leaf stapling and transmission by grafting, it is now possible to determine in 4 to 5 weeks, the precise nature of mechanism of resistance to SMD.

2.1.9. Selection of broad-based durable SMD resistant pigeonpea genotypes

A global pigeonpea germplasm collection is held in trust by ICRISAT following on international agreement with Food and Agriculture Organization (FAO). Many cultivated genotypes in this collection have been found to show location-specific resistance to SMD and a large number of genotypes are yet to be evaluated for SMD resistance. New technologies developed for SMD monitoring are being used for screening genotypes. These include cultivated and wild Cajanus species and also breeding lines from crosses between wild and cultivated short duration pigeonpea genotypes. High yielding and SMD-resistant genotypes will be selected and evaluated further for resistance to more than one PPSMV-biotype using new screening techniques. Genotypes that show resistance to more than one virus biotype will be selected. Promising genotypes will be identified and utilized in breeding programs. These efforts will lead to an understanding of the inheritance of SMD resistance and the development of pigeonpea cultivars with broad-based resistance.

2.2. Concluding remarks

Sterility mosaic, Fusarium wilt and pod borer are serious threats to pigeonpea production in the Indian subcontinent. Although in annual incidence SMD is next to that of wilt, but SMD has been shown to cause significantly more crop losses than wilt. An integrated approach to tackle these three problems is vital to increase the pigeonpea production in south Asia. Significant progress has been made to enable in devising strategies for the management of wilt and pod borer. However, the previous lack of information on the causal agent and the absence of diagnostic tools have hindered progress to develop management strategies for SMD.

After seven decades of SMD description, ICRISAT, in collaboration with the Scottish Crop Research Institute, Scotland, has made a breakthrough in the identification of the causal agent of SMD. Information generated has lead to the development of efficient monitoring and screening technologies leading to identification of broad-based durable SMD-resistant pigeonpea genotypes and to understand the epidemiology of the SMD. Identification of SMD resistance in wild Cajanus species, some of which are also resistant to wilt and pod borer, is a major step towards an integrated approach to reduce losses substantially to biotic constraints. These efforts will contribute to sustainable pigeonpea production in the Indian subcontinent.
Diagnosis is as much an ‘art’ as it is ‘science’. The ‘scientific’ part is the technology used to detect pathogens. The art lies in the synthesis of information obtained from the case history, symptoms and results of laboratory tests to determine the virus(es) involved in inducing disease. Detection of a virus in a plant does not necessarily prove that the virus causes the disease. To establish that the virus detected causes the disease, Koch’s postulates should be proved. Nevertheless constant association of a virus with a set of symptoms is often used as the ‘proof’ that the virus detected causes the disease. Disease diagnosis based on symptoms is unreliable for the reason that different viruses may cause similar symptoms and that different symptoms may be induced by one virus. Many abiotic stresses and other pathogens such as phytoplasma may cause symptoms characteristic of virus infection. Even after one become familiar with the symptoms typically caused by a virus in a particular plant, it is essential to confirm the diagnosis with reliable methods.

Several factors influence the method to be used for virus detection. These include:
- Facilities and expertise available
- Type of virus suspected to be present
- Host plant
- Time available

Any detection method should be rapid and highly specific for the target virus, and should detect virus present in low amounts in the plant tissue and detection at an early stage of disease development.

Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are the most widely used virus detection methods because of their rapidness and sensitivity. However, PCR-based methods require expensive laboratory equipment, whereas ELISA requires little or no special equipment and is particularly suitable for use in developing countries.

3.1. ELISA: A serology-based method

3.1.1. Principles of antibody production

An antigen is a molecule that can elicit production of antibodies when introduced into warm-blooded animals. Proteins, peptides, carbohydrates, nucleic acids, lipids, and many other naturally occurring or synthetic compounds can act as antigens, especially those having a molecular weight of 10,000 daltons or higher with a definite molecular structure and which are not normal constituents of the animal being immunized. Antibodies are glycoproteins, which are produced as a result of immune response following introduction of antigens. Blood serum containing antibodies is referred to as antiserum.

When antigens are introduced, into an animal, a series of interactions between macrophages, T lymphocytes, and B-lymphocytes lead to antibody production. The first exposure of animals to antigens leads to a relatively weak reaction, referred to as the primary response. A series of specialized events occur during the primary response. These events prepare the animal to respond with quick and intense production of antibodies (secondary response) when the antigen is reintroduced. Both the primary and secondary responses occur in plasma cells. When antigens are first introduced, antigen presenting cells (APCs), (Langerhans cells in the skin, dendritic cells in the spleen and lymph nodes and monocytes in the blood), T cells and B cells act in concert to stimulate the production of antibodies. Many techniques for the preparation and
introduction of antigens, such as selection of appropriate injection site (intramuscular, subcutaneous, intravenous, intraperitoneal etc.), mixing of antigen with adjuvants etc. influence the uptake of antigen by the APCs. Adjuvants act by protecting the antigen from being rapidly degraded in the blood stream, and they also contain substances that stimulate the secretion of host factors that facilitate the macrophage movement to the site of antigen deposition and increase the local rate of phagocytosis.

After an antigen is engulfed by APCs, it is partially degraded, appears on the cell surface of APC and binds to it with a cell-surface class II glycoprotein. In the next step, antigen-glycoprotein complex on the APC binds to T-cell receptors. This leads to T-cell proliferation and differentiation. While T-cells are proliferating, antigens are also processed by virgin B-cell lymphocytes in a similar manner as by APC’s. However, the uptake of antigen by B-cells is specific, unlike that by APC’s. As in the case of APC’s, the antigen forms a complex with a surface antibody (Class II protein) on the B-cell surface. This complex also stimulates the same helper T-cells, which now bind to B-cells. This leads to division of B-cells and the production of the antibodies. Therefore the contact between B cells and helper T-cells is a major event in the regulation of production of antibodies.

In order for a compound to be good antigen, it should possess one or more epitopes (an antigenic determinant of defined structure), which can bind to the surface antibody on virgin B cells. After the antigen is dissociated, each epitope should be able to bind simultaneously to both the Class II protein and T-cell receptor. Any epitope that is exposed is expected to stimulate strong response to antibody production.

3.1.2 Structure of immuno-gammaglobulins and function

Antibodies are glycoproteins present in the serum and tissue fluids of mammals. They are referred to as immunoglobulins (Igs) because of their role in adaptive immunity. Although all antibodies are immunoglobulins, it is important to realize that not all the immunoglobulins produced by a mammal have antibody activity. There are five classes of antibodies, IgG, IgM, IgA, IgE, and IgO, separated on the basis of the number of Y-like units and the type of heavy-chain polypeptide they contain. There are also significant differences within each class of gammaglobulins.

The basic polypeptide structure of the immunoglobulin molecule is shown in the Fig 1. It contains a unit of two identical light polypeptide chains and two identical heavy polypeptide chains linked together by disulfide linkage. The class and subclass of an immunoglobulin molecule are determined by the type of heavy chain. The most common immunoglobulin is IgG and therefore the description given is for IgG.

IgG molecule contains one structural “Y” unit (Fig 1). The two arms of Y are made of two identical light chains of molecular weight 23,000 daltons and two identical heavy chains of molecular weight 53,000 daltons. Each light chain is linked to the heavy chain by non-covalent bonds and by one covalent disulfide bridge. Each light-heavy chain pair is linked to another IgG by disulfide bridges between the heavy chains. Carboxytermini of the two heavy chains fold together and form the “FC” domain. The region between the Fab and Fc fragments is called the “hinge”. Digestion of IgG with pepsin yields two Fab fragments attached to each other by disulfide bonds and a Fc fragment.
In both heavy and light chains, at the N-terminal portion, the amino acid sequences vary greatly from IgG to IgG. In contrast, in the Fc portion (C-terminal portion of both heavy and light chains) the sequences are identical. Hence the Fab domain contains "Complementary Determining Regions (CDRs)" or hypervariable regions. The six CDR's (three on either side of Fab) comprise the antigen combining site or "paratope" region of IgG. The antigen binds to IgG at this paratope region. The paratope is about 110 amino acid residues in length (both for light and heavy chain). The constant region of the light chain is also about 110 amino acids but the constant region of the heavy chain is about 330 amino acid residues in length.

The antigen-combining site (paratope region in IgG) is a crevice between the variable regions of the light and heavy-chain pair. The size and shape of crevice can vary because of differences in the variable light and variable heavy regions, as well as differences in the amino acid sequence variation. Therefore specificity between antigen and antibody results from the molecular complementarity between determinant groups on the antigen (called "Epitope") and the paratope region of the IgG. A single antibody molecule has the ability to combine with a range of different antigens. Stable antigen-antibody complexes can result when there are a sufficient number of short-range interactions between both, regardless of the total fit. This interaction can be as a result of non-covalent bonds (hydrogen bonds, salt bridges, electrostatic charges), hydrophobic bonds, van der Waals' forces and so on. Therefore it is important to realize that the interaction between antigen and antibody is not covalent and therefore is reversible. Various factors such as pH, temperature, detergents, and solvent conditions can influence these interactions.

3.1.3. Polyclonal antibodies
These are obtained from serum of an animal following injection with an antigen, which contains many antigenic sites. Therefore the antibodies produced react with more than one epitope.

3.1.4. Monoclonal antibodies
They are produced by a single antibody-producing B lymphocyte, immortalized either by mutation or fusion with a myeloma cell line. They react with a single epitope.

3.1.5. Production of polyclonal antibodies to viruses
If it is possible to use both polyclonal and monoclonal antibodies (MAbs) for virus detection. Polyclonal antibodies are cheaper to produce than MAbs and also can be highly specific when made to highly purified antigen. Since polyclonal antibodies consist of heterologous populations of antibodies with variable sensitivities, they tend to be broadly specific and widely applicable to different serological tests. Therefore for routine virus
detection polyclonal antibodies are highly suitable.

3.1.5.1. Preparation of virus antigens for antibody production
The viral genome can code for a number of proteins. Of all the proteins, the structural protein(s) [coat protein or capsid protein or nucleoprotein] or non-structural proteins, such as inclusion body proteins accumulate to a high concentration in the plants compared to other proteins encoded by the virus genome. The majority of antisera produced for plant viruses are to the coat protein(s). Inclusion body proteins can also be used for antibody production (eg. potyviruses).

The best source from which to obtain large quantity of coat protein is the purified virus, largely devoid of host plant components. Purification of viruses is accomplished by various physcio-chemical techniques. There are several important points to consider prior to purifying viruses from plants. They include selection of suitable host plant for virus maintenance, procedures for purification and methods for monitoring purity. The quality of the antiserum produced will depend largely on the purity of the virus preparation used for immunization.

3.1.5.2. Recombinant antigens
Recombinant DNA technology allows cloning of plant viral nucleic acids and express their genes in prokaryotic and eukaryotic systems. This facilitates large-scale expression of proteins in vitro. For this it is essential to know the sequence of protein encoding gene (for example, coat protein sequence, if the antibodies are to be produced to the coat protein). The gene of interest is inserted at a suitable site in an expression vector (eg., pET) to express in *Escherichia coli*. This leads to production of virtually unlimited quantities of gene product of interest. Expressed protein can be purified and utilized in the production of antiserum.

3.1.5.3. Choice of animals
Any warm blooded animal can be used for antibody production e.g., Rabbits, chickens, guinea pigs, rats, sheep, goats and horses. When small animals such as rats and mice are used, only small quantity of serum can be obtained. Although large animals such as goats and horses can provide large volumes of serum, large amounts of antigen are required for immunizing these animals. The rabbit is the most commonly used animal for antibody production.

3.1.5.4. Immunization
Injection of an antigen into an animal is accomplished either by intramuscular or subcutaneous injections or intravenous.

For injection the antigen preparation should be emulsified with an adjuvant (1:1 proportion). The most commonly used adjuvant is Freund’s adjuvant, which consists of paraffin oil and an emulsifier, mannide monooleate (incomplete). Complete adjuvants, in addition to these two components, contain heat-killed *Mycobacterium tuberculosis* or *M. butyricum* or a similar acid-fast bacterium. Emulsification with adjuvants results in very slow release of antigen, thereby stimulating excellent immune response. Antigen concentration required may vary from 100 µg/ml to 500 µg/ml. A normal immunization schedule followed for rabbits is given below.
• Four subcutaneous injections (multiple sites) at weekly intervals (for first injection use Freund's complete adjuvant and for the 2nd, 3rd and 4th use incomplete adjuvant). Five injections are usually adequate to obtain good immune response.
• If the titer of the antibody is low, either an intravenous (for intravenous injection adjuvants should not be used) or an intramuscular injection should be given as a booster.

3.1.5.5. Blood collection and serum preparation
Blood is collected from rabbits by making an incision in the marginal vein of the ear. It is preferable to collect the blood in sterile containers. The blood is allowed to clot at room temperature for 2 - 3 h (this can also be done by exposure at 37 °C for 30 min). After overnight refrigeration, the serum is collected with a Pasteur pipette and then centrifuged at 5,000 rpm for 10 min.
**Note:** It is important to starve rabbits for at least 24 h before blood collection to minimize concentration of lipids

3.1.5.6. Storage of antisera
• For long-term storage of antisera at 4 °C it is essential to add either glycerol (1:1) or sodium azide to a concentration of 0.02%.
• In lyophilized form antisera can be stored at -20 °C indefinitely for many years without losing potency.
• Antisera can be stored at -70 °C.
• It is advisable to store serum in small aliquots of 1.0 ml or less.
• Antisera should not be frozen and thawed repeatedly. This leads to aggregation of antibodies thereby affecting antibody activity by steric interference of the antigen-combining site or by generating insoluble material, which may sediment during centrifugation.

3.1.6 Enzyme-linked immunosorbent assay
Enzyme-linked immunosorbent assays are solid-phase assays in which each successive reactant is immobilized on a plastic surface and the reaction is detected by means of enzyme-labelled antibodies. The principle of amplification of the reaction between viral antigens and their antibodies by utilizing an enzyme and its substrate, was described by Avrameas (1969). The microplate method currently being used widely for virus detection and the term ELISA was introduced by Voller et al. (1976).

ELISA is one of the most widely used serological tests for the detection of plant viruses because of its simplicity, adaptability and sensitivity. In this immunospecificity is recognized through the action of the associated enzyme label on a suitable substrate. ELISA detects only viral antigens and it does not give a measure of infective virus concentration.

The basic principle of the ELISA lies in immobilizing the antigen onto a solid surface, or capturing antigen by specific antibodies, and probing with specific immunoglobulins carrying an enzyme label. The enzyme retained in the case of positive reaction is detected by adding the suitable substrate. The enzyme converts substrate to product, which can be easily recognized by its colour. There are two types of ELISA procedures; ‘direct’ and ‘indirect’ ELISA. In the ‘direct’ procedure, IgG's extracted from virus-specific antisera or in some cases polyclonal antisera, are used for coating the solid surface to trap the antigen, and the same IgG’s labelled with an enzyme are employed for detection. In this case the antigen gets sandwiched between IgG’s and thus is
referred to as the double-antibody sandwich (DAS) form of ELISA. The DAS-ELISA has limitations in that test is not suitable for (a) virus detection in disease surveys unless it is targeted to a specific virus, (b) when adequate antisera are not available for IgG extraction and conjugation and (c) for probing a single antigen with several different antisera.

In the simplest ‘indirect’ ELISA procedure, antigen is bound to the solid surface of ELISA plate. In the second step unconjugated antigen-specific detecting antibodies (primary antibody) is added. Primary antibody is detected by the enzyme-labelled second antibody (anti Fc or anti IgG). The second antibody is produced in a different animal than that used for producing primary antibody. The main advantage of the indirect ELISA procedure is that one enzyme conjugate (of antiglobulin antibody or protein A) can be utilized with all the systems. This assay is particularly suitable for (a) virus detection in disease surveys, (b) testing the presence of virus in seed and (c) for determining serological relationships, particularly when specific conjugates cannot be prepared. It is also more economical to perform than the DAS form.

3.1.6.1. Choice of antibodies
Antibodies produced in any experimental animal are suitable for ELISA. In some test procedures crude antisera can be used. For DAS-ELISA only purified IgGs can be used for conjugation with an enzyme. IgG’s produced in a heterologous animal or second antibody (e.g., anti-rabbit IgGs produced in goat) used in the ‘indirect ELISA’ procedure are commercially obtained.

3.1.6.2. Choice of antigens
One of the major advantages of ELISA is that it can be used on crude plant/insect extracts, and on partially purified and purified virus preparations.

3.1.6.3. Choice of enzyme labels
The two-enzyme labels that are widely used are alkaline phosphatase (ALP) and horseradish peroxidase (HRP). Urease and penicillinase (β-lactamase) have subsequently been introduced. Reaction kinetics of HRP is not linear and some of its substrates are hazardous to the operator. Urease and isozymes of peroxidase are known to be present in seeds and plant extracts, thus limiting their application in plant virus detection. ALP and its substrate, p-nitrophenyl phosphate, are very expensive and are not readily available in developing countries. ALP has certain limitations for use in the detection of viruses in insects.

Penicillinase has several advantages over the ALP system:
- It is less expensive than ALP and HRP
- Enzyme and substrate are available in some developing countries
- Penicilloic acid produced as a result of penicillinase activity on penicillin substrate is less toxic
- The substrate has longer shelf-life than the other enzyme substrates
- Visual reading of results is easier than for the ALP system
- Penicillinase is not known to occur in higher plants.

Penicillinase breaks down penicillin into penicilloic acid, and this is detected either by the rapid decolorization of a starch-iodine reagent or by utilizing acid-sensitive pH indicators.
3.2 PCR: A nucleic acid-based virus detection method

3.2.1 Nucleic acid-based methods

Serological methods have major disadvantage that they are based on the antigenic properties of the virus structural proteins. Thus immunological approaches ignore the rest of the virus genome. It is possible that viruses that are distantly related or not related, as determined by serological methods, may have highly conserved sequences in the genes other than the coat protein gene or that serologically related viruses may have very little sequence homology. In addition, there are instances where immunological procedures have limited application such as the detection of viroids, satellite RNAs, viruses that lack particles (e.g. Groundnut rosette virus), viruses which occur as extremely diverse serotypes (e.g. Indian peanut clump virus) and viruses that are poor immunogens or are difficult to purify. For these agents, detection is often possible only by using nucleic acid-based methods such as nucleic acid hybridization assays and PCR.

In instances where nucleic acid-based methods and serological methods provide similar information, detection sensitivity, and specificity, and are equally convenient, serological methods like ELISA be the preferred method. This is particularly so in developing countries because serological methods are easier to perform, cost effective and the required reagents are readily available.

3.2.2 The composition of nucleic acids

Nucleic acids are polynucleotides, that is they consists of nucleotides joined together in a long chain. Each nucleotide is made up of a base, a sugar and a phosphate group. The differences between DNA and RNA (i) the sugar is ribose in RNA but deoxyribose in DNA, (ii) the bases in DNA are adenine (A), cytosine (C), guanine (G) and thymine (T) but in RNA the bases are A, C, G and Uracil (U) in place of T. In polynucleotide the bases are side branches on a ‘backbone’ chain made of alternating sugar and phosphate groups. The carbon atoms in the sugar molecule are numbered by convention. Thus the backbone is constructed by joining the 3’ and 5’ carbon atoms through a phosphate. As a result every linear nucleic acid molecule that has 5’-end usually terminating in a phosphate group and a 3’ end, which usually terminates in a hydroxyl (OH) group.

Because of their structure, bases are able to join in particular pairs by hydrogen bonding. This is called base pairing. Adenine (A) will bond to T (in DNA) or U (in RNA) by making two bonds, G will bond to C by

Fig 2. DNA molecule
making three bonds. The bonds form between polynucleotide chains running in opposite direction (see Fig 2). The bonding can be within a molecule, which will make a loop, or between separate molecules. When two sequences of nucleotides are able to base pair they are said to be complementary, the structure formed is double-stranded molecule. The process of two polynucleotides joining to form a double-stranded structure is called ‘annealing’ (renaturation), the reverse process, when chains separates to from a single stranded molecules, is called ‘melting’ (denaturation).

3.2.3. Polymerase chain reaction
The PCR provides a simple ingenious method to exponentially amplify specific DNA sequence by in vitro DNA synthesis. The three essential steps to PCR include (a) melting of target DNA, (b) annealing of two oligonucleotide primers to the denatured DNA strands and (c) primer extension by a thermostable DNA polymerase. Newly synthesized DNA strands serve as targets for subsequent DNA synthesis as the three steps are repeated up to 35 times (see Fig 2 in section 5.2). The specificity of the method derives from the synthetic oligonucleotide primers, which base pair to and defines each end of the target sequence to be amplified. PCR has the power to amplify a specific nucleic acid present at an extremely low level, from a complex mixture of heterologous sequences. PCR has become an attractive technique to exploit for the diagnosis of viruses through the detection of the viral genome.

3.2.3.1. Basic PCR
PCR process amplifies a short segment of a longer DNA molecule. A typical PCR reaction includes thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dATP, dGTP, dCTP and dTTP collectively termed dNTPs), reaction buffer, magnesium and optional additives and the template. The components of the reaction are mixed and the reaction is placed in a thermal cycler, which is automated instrument that takes the reaction through a series of different temperatures for varying periods of time. This series of temperatures and time adjustments is referred to as one cycle of amplification. Each PCR cycle doubles the amount of template sequence (amplicon) in the reaction.

Each cycle of PCR consists of initial denaturation of the target DNA by heating to >90 °C for 15 seconds to 2 min. In this step, the two intertwined strands of DNA separate from one another. In the second step, the temperature is reduced to approximately 45-60 °C. At this step oligonucleotide primers can form stable associations (anneal) with the separated target strands and serve as primers for DNA synthesis. This step lasts approximately 30-60 seconds. Finally, the synthesis of new (primer extension) DNA begins when the reaction temperature is raised to the optimum for the thermostable DNA polymerase, which is around 70-74 °C. This step lasts for 30-120 seconds depending on the amplicon size. This step completes one cycle. After 20-35 cycles, the amplified nucleic acid can be analyzed for size, quantity, sequence or can be used for further experimental procedures such as cloning.

3.2.3.2. PCR optimization
The following factors influence the amplification of products during PCR:
- Magnesium ion concentration
- Reaction buffer
- Enzyme choice and concentration
- Primer design
- Template
- Cycle parameters
- Nucleic acid cross-contamination

**Magnesium ion concentration:** It is the critical factor affecting the performance of Taq DNA polymerase. Reaction components, including template, chelating agents present in the sample (e.g., EDTA), dNTPs and proteins, can affect the amount of free magnesium. In the absence of adequate free magnesium, Taq DNA polymerase is inactive. Excess free magnesium reduces enzyme fidelity and may increase the non-specific amplification. For this reason it is important to determine empirically, the optimal concentration of MgCl₂ for each reaction. This can be done by preparing a reaction series in 0.5 mM increments by adding 2, 3, 4, 5 or 6 µl of a 25 mM MgCl₂ stock to a 50 µl reaction.

**Reaction buffer:** The basic ingredients of a PCR reaction buffer are; NaCl, KCl, EDTA, DTT, Triton X-100, Nonidet-P 40, Tween-20, glycerol and tris-HCl, pH 8. The composition of these components varies depending on the type of thermostable polymerase in consideration. The manufacturer supplies reaction buffer in 10x concentration along with the thermostable DNA polymerase. For most of the PCRs, use of this buffer at recommended concentration yields good amplification.

**Enzyme:** The choice of the enzyme to use depends on the several factors. Taq DNA polymerase is the most popular thermostable DNA polymerase. This enzyme possesses relatively high processivity and is the least expensive enzyme. However, this enzyme lacks 3'-5' exonuclease (proof reading) activity and it has high error incorporation rate compared to other enzymes. For accurate amplification of the PCR product thermostable enzymes with proof reading activity are recommended (e.g: Pfu, Tli).

Generally, 1 U of Taq DNA polymerase in a 50 µl reaction is sufficient for good yield of product. Inclusion of more enzyme does not significantly increase product yield. Further, this lead to likelihood of generating artifacts associated with 5'-3' exonuclease activity associated with Taq DNA polymerase resulting in smearing in agarose gels. Pipetting errors are the most frequent cause of excessive enzyme levels. Accurate dispensing of submicroliter volumes of enzyme solutions is difficult. We strongly recommend the use of reaction master mixes, sufficient for the number of reactions being performed to overcome this problem. The master mixes will increase the initial pipetting volumes of reactants and reduce pipetting errors.

**Primer design:** PCR primers (oligomers or oligonucleotides) generally range in length from 15-30 bases and are designed to flank the region of interest. Primers should contain 40-60% G+C and care should be taken to avoid sequences that would produce internal secondary structure. The 3’-end of the primers should not be complementary to avoid the production of primer-dimers in the PCR reaction. Ideally both primers should anneal at the same temperature. The annealing temperature is dependent upon the primer with the lowest melting temperature. Regardless of primer choice, the final concentration of the primer in the reaction must be optimized. We recommend adding 50 pmol of primer (1 µM final concentration in a 50 µl reaction) as a starting point for the optimization.

**Template:** successful PCR amplification depends on the amount and quality of the template. Reagents commonly used to purify nucleic acids (salts, guanidine, proteases, organic solvents and SDS) are potent inhibitors of DNA polymerases. The amount of template required for successful amplification is
dependent upon the complexity of the DNA sample and depends on percent target DNA of interest. Too much of target DNA or too little, results in poor or no amplification.

**Cycle parameters:** The sequence of the primers is major consideration in determining the temperature of the PCR amplification cycles. For primers with a high melting temperature it may be advantageous to use high annealing temperatures. The higher temperature minimizes nonspecific primer annealing, increasing the amount of specific product and reduce primer-dimer formation. Allow a minimum extension time of 1 min for a cycle and increase it by a min for every 1 kb of amplicon (2 min extension for 2 kb target).

Certain unwanted reactions can occur in PCR, and these usually begin at room temperature once all components are mixed. These unwanted reactions can be avoided by incorporating ‘hot start’ method. In this thermostable enzyme is added into the reaction mixtures after heating the reaction minus enzyme to 90°C. However, this method is tedious and can increase the chances of contamination.

**Nucleic acid cross-contamination:** It is important to take great care to minimize the potential for cross-contamination between samples and to prevent carryover of RNA and DNA from one experiment to another. Use positive displacement pipettes or aerosol resistant tips to reduce contamination during pipetting. Wear gloves and change them often. Wherever possible prepare master mixes by mixing all reagents and at the end, add template into the reaction tube.

### 3.2.4. RT-PCR

Most of the viral and sub-viral pathogens have RNA genome. In this case RNA is first reverse transcribed in order to produce a complementary (c)DNA copy using the enzyme reverse transcriptase and a primer. In the first cycle of PCR thermostable DNA polymerase synthesis complementary strand to the first strand cDNA. The resultant double stranded cDNA is amplified exponentially by PCR process (see Fig 2 in section 5.2).

RT-PCR uses Moloney murine leukemia virus (MoMLV) or Avian myeloblastosis virus (AMV) reverse transcriptase (RT). Taq DNA polymerase performs second strand cDNA and subsequent amplification during PCR. The viral RT enzymes are inactivated at elevated temperatures. Therefore first strand reaction must be performed at 37-48°C. The maximum recommended temperature for optimum RT enzyme activity is 42°C. Efficient first cDNA can be completed in 20-60 min. RNA exhibiting significant secondary structure must be denatured for efficient reverse transcription. Generally, incubation at 42°C for 45 min yields good yield of first strand cDNA. For RNA templates with high secondary structures, a denaturation step can be incorporated by incubating primers and RNA in a separate tube at 70°C for 10 min, then quench on ice and proceed to RT step.

The purity and integrity of the total RNA extracted from the leaf tissue of interest is critical for successful and consistent results in RT-PCR. The extraction procedure for RNA isolation consists of (a) effective disruption of tissue, (b) inactivation of ribonuclease (RNase) activity and (c) separation of RNA from protein, carbohydrates, polysaccharides etc. It is very difficult to inactivate RNase and hence several precautions have to be followed to prevent RNA degradation due to RNase activity, during or after extraction. Use autoclaved solutions and baked glassware (bake in an overnight 200°C overnight). Always use disposable gloves as a precaution against RNase in the fingertips. Include potent
RNase inhibitors (SDS, guanidine thiocyanate, β-mercaptoethanol) in the extraction buffer to inactivate the enzyme and carry all steps at 4 ᵒC to minimize RNase activity.

3.2.5. Immuno capture-RT-PCR
A technique that combines trapping or capture of the virus using virus specific antibodies and RT-PCR is called immuno-
capture (IC)-RT-PCR (see Fig 3, section 5.2). In this method virus from crude sap trapped using antivirus antibodies. Trapped virus particles are disrupted to release the viral RNA and is reverse transcribed for amplification by RT-PCR. This method circumvents the need for RNA isolation.
Crop losses caused by plant virus diseases can be prevented in various ways. Over the years three main categories of control measures have been adopted for minimizing virus-induced crop losses. They are (i) removing virus sources, for example by producing virus-free planting stocks of vegetatively propagated plants, or by removing volunteer plants or plant remains left from the previous crops; (ii) preventing virus spread usually by killing vectors or interfering with their activity; and (iii) growing the virus-resistant/tolerant varieties of crops. The third option is the most economical for farmers and easily adaptable. Because of this, host resistance has become one of the primary control methods for reducing losses from virus diseases. This form of control is relatively inexpensive for plant producers to implement and is “environmentally friendly”.

The attempts to breed improved crop plants relies on selection, more often intentional, to eliminate the most readily infectible and sensitive types and to select genotypes with superior performance in the field. When the range of genetic variation found in a crop species does not meet the required degree of virus resistance, then related crop species can be screened for the identification of resistance. If the useful source of resistance is identified in cultivated species or closely related and sexually compatible species, it can be used for crossing with a cultivar having desirable agronomic traits. The strategy for breeding depends on the crop species, nature of the reproductive-biology (self-pollinated or self-incompatible), type of cultivar (F1 hybrid, homozygous line or vegetative clone) and inheritance of the resistance (monogenic, oligogenic or polygenic; dominant or recessive). In case of resistant sources available only in related wild species that are difficult or impossible to use in crossing, techniques of interspecific crosses such as in vitro culture of immature embryo can be used to introduce resistance.

The basic requirement for successful breeding programs for virus resistance involves, selection and crossing appropriate parents, and then making selections from among their progeny, backed, where possible, by knowledge about the genetic control of resistance. This also possible without detailed knowledge of the genetic mechanism for resistance. The final objective is to combine the resistance with good agronomic traits.

4. Screening genotypes for virus resistance

For any strategy of breeding for virus resistance, good knowledge of the virus and its different strains, and diagnostic tools for their unambiguous detection are essential. The plants to be tested should generally be young and uniform in stage of development. It is essential to use susceptible control plants to ensure that the inoculum used on test plant produces typical symptoms.

Virus transmission onto test plants can be achieved by various means. Mechanically transmissible virus can be inoculated by sap inoculation. The inoculation can be done manually or using inoculation gun. If the virus is not readily sap transmissible (e.g: luteoviruses; PPSMV) virus vectors (fungi, nematodes, insects, mites) can be used for inoculation purpose. In this case viruliferous vectors need to be reared on infected plants. In case of vegetatively propagated crops such as raspberry, graft inoculation can be used. After inoculation the plants should be
protected from other viruses to avoid confusions.

Appearance of symptoms often forms the basis of screening. It is advisable to monitor presence of virus in symptomless plants with sensitive serological or nucleic acid-based detection tools. In case where inoculation response is highly variable in the plant population, from complete resistance to partial resistance with different grades of symptom intensities in between, scoring system often denoted by a scale, can be used.

Large-scale evaluation of genotypes is often carried out under field conditions. This is possible only if the disease recurs at the same area on particular crop every year owing to the presence of vectors and of virus reservoir hosts nearby and there is no risk of mixed infections. Alternatively, growing host plants of the vectors and the virus, inter-spreading the test plants to increase the vector population, allows more consistent disease spread onto test plants evaluated in the field. In any case test plants should be evaluated for presence or absence of virus by diagnostic tools. The screening done under field conditions for 2-3 years takes into account the field resistance. This does not ensure test plants performance against different strains of the virus. The multilocal screening for resistance helps in exposing the genotype to diverse geographic isolates of the virus.

In case of seed transmitted viruses, initial screening of seed material for virus by ELISA is essential. Seed tested positive should be eliminated from the screening trial.

4.2. Selection for SMD resistance
Several different methods have been used to identify pigeonpea accessions with useful levels of resistance to sterility mosaic disease (SMD). The PPSMV is not transmissible to pigeonpea by mechanical sap inoculation. Therefore viruliferous mite vectors have been used for PPSMV transmission. 'Leaf-stapling' or 'infector row' methods wherein an infected leaflet or infected plant, carrying mites aid in virus transmission onto the healthy plants, are the popular screening methods used for resistance screening. Selection of resistant genotypes is based on visual symptoms. In one method pigeonpea genotypes were categorized based on disease incidence (scoring by symptoms) as (1) resistant (0-10% incidence); (2) moderately resistant (11-30% SMD incidence) and (3) susceptible (>30% SMD incidence). However, this categorization is disadvantaged with the fact that various pigeonpea genotypes show different symptoms and different effects on yield. Screening pigeonpea germplasm at ICRISAT resulted in identification of four types of host response to PPSMV infection, genotypes that show (i) severe mosaic and sterility (eg. ICP8863), (ii) mild mosaic with partial sterility (eg. ICP8862), (iii) chlorotic ringspots without any noticeable sterility (eg. ICP2376 reaction to PPSMV Patancheru isolate) and (iv) no visible symptoms (eg. ICP7035). Genotypes showing mild mosaic and chlorotic ring spot symptoms can be classified as tolerant to SMD, genotypes with severe mosaic symptoms as susceptible type and the genotypes showing no visible symptoms as resistant. Additionally, if the resistance selection is based on symptoms, it is essential to record observations at least twice, once during early stage of the plant growth (20-30 days following germination) and again before maturity. In this case new growth, especially at the base of the stem should be observed for symptoms. Early observation is important to monitor chlorotic ringspots or mild mosaic symptoms, which get masked with the plant growth. In case of late infection symptoms
may not appear even if the genotype is susceptible to PPSMV. However, when plants are ratooned symptoms appear prominently on the new growth.

Screening genotypes using vectors (leaf-stapling method) and assessment based on symptoms does not provide information on type of resistance offered by the host plant. Furthermore, screening of wild Cajanus accessions, which have been suggested to contain useful resistance genes for diseases and pests, was difficult because susceptible wild accessions seldom showed visible symptoms. Moreover, several factors influence the expression of symptoms: (i) genotype x pathogen interaction (ii) environment (iii) PPSMV strains, (iv) mite numbers and (v) age of the plant at which infection occurs. Thus symptoms based methods are unreliable.

Previously progress in developing broad-based SMD resistant material has been hindered by the lack of information on the SMD causal agent and the absence of diagnostic tools. Now the SMD causal agent has been identified and diagnostic tools are available for its detection. This led to the development of a new scheme for rapid screening of genotypes for SMD resistance (detailed in section 5.4). Either for selection of resistance or for critical studies such as inheritance of resistance, it is necessary to inoculate test plants at seedling stage with leaflets containing 5-10 viruliferous mites per leaf and observations should be made at regular intervals (at least once in 2 weeks). Transmission of PPSMV by vector mites (leaf-stapling method) occurs if the test accession is susceptible to mites as well as to the virus. Failure of virus transmission suggests that the test accession could possess resistance to vector or to virus, or to both. To confirm this precisely, it is essential to test the accessions by graft inoculation, which facilitates reliable testing for virus resistance. Previously the ‘tissue implant grafting’ method was used for establishing SMD, but this method resulted in a very low level (about 12%) of virus transmission. Now a new method “petiole-grafting” has been established which results in >85% of virus transmission.

This scheme has confirmed for the first time existence of various genotypes that are (i) resistant to PPSMV and mites, (ii) resistant to mites, but not to PPSMV, (iii) resistant to PPSMV, but not to mites and (iv) susceptible to PPSMV and mites.
5. Laboratory Exercises
5.1. Enzyme-linked Immunosorbent Assay (ELISA)

The basic principle of ELISA technique involves immobilizing the antigen onto a solid surface or captured by specific antibodies bound to the solid surface, and probing with specific immunoglobulins carrying an enzyme label. The enzyme retained in the case of positive reaction is detected by adding a suitable substrate. The enzyme converts the substrate to a product, which can be recognized by its colour.

Polyclonal antibodies to Pigeonpea sterility mosaic virus (PPSMV) were produced in a rabbit. These were used to detect the virus in plant tissues by double antibody sandwich (DAS)-ELISA (Fig 1). In DAS-ELISA polyclonal antibodies are coated onto the ELISA plate followed by the addition of antigen. The trapped antigen is detected by the addition of penicillinase (PNC)-labeled PPSMV-specific immunoglobulins (IgGs). The positive reaction is detected by adding sodium-penicillin-G (substrate). Penicillinase converts sodium penicillin-G into penicilloic acid, and this is detected by utilizing an acid-sensitive pH indicator, bromothymol blue (BTB). In the case of positive reaction the bluish-green colour of the PNC substrate turn apple green to orange-yellow (see Fig 1). No colour change will occur in the case of negative reaction.

5.1.1 MATERIALS

- **ELISA plates**: Several brands are available. For high binding ‘Nunc-Maxisorp’ plates are recommended.
- **Micropipettes**: 1-40 µl, 40-200 µl and 200-1000 µl single channel pipettes. 40-200 µl multichannel pipette. Several brands are available (eg: Eppendorf, Finpipette, Gilson). Those with adjustable volumes are preferable.
- **ELISA plate reader**: Manual or automatic provided with a 620 nm filter.
- **PPSMV polyclonal antibodies**.
- **Mortars and pestles**
- **Muslin cloth**
- **pH meter**
- **Light box**
- **Incubator**
- **Refrigerator**

5.1.2. Solutions

5.1.2.1. Carbonate buffer or coating buffer, pH 9.6

\[
\begin{align*}
\text{Na}_2\text{CO}_3 & \quad 1.59 \text{ g} \\
\text{NaHCO}_3 & \quad 2.93 \text{ g}
\end{align*}
\]

Distilled water to 1 l [No need to adjust pH]
5.1.2.2. 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 to 2 l
No need to adjust the pH

5.1.2.3. Phosphate buffered saline Tween (PBS-T)

PBS 1 l
Tween-20 0.5 ml

5.1.2.4. Antibody buffer (PBS-TPO)

PBS-T 100 ml
Polyvinyl Pyrrolidone (PVP) 40,000 MW 2.0 gm
Bovine serum albumin (Sigma Cat. No. A6793) 0.2 gm

5.1.2.5. Distilled water - Tween

Distilled water 2 l
Tween 20 (0.05% v/v) 1 ml

5.1.2.6. Substrate buffer (BTB buffer)

1. Dissolve 15 mg bromothymol blue (BTB) in 50 ml of 0.01 M NaOH. Neutralise the alkali by adding 0.1 N HCl, until the pH of the solution is 7.2 (or to the appearance of bluish-green colour). Make up the volume to 100 ml with distilled water. [Note: the final concentration of NaOH in 100 ml solution is 0.005 M and that of BTB is 0.015% w/v].

2. Add sodium penicillin-G (potassium penicillin-G or procaine penicillin also suitable) at 0.5 mg/ml (w/v) concentration and adjust the pH to 7.2 using either HCl or NaOH. Store the mixture at room temperature.

Note: It is absolutely essential to adjust the substrate buffer pH to 7.2 before use. BTB solution alone is stable for several months at room temperature, but with substrate (penicillin) it is stable only for few days.
5.1.3. Extraction of PPSMV IgG by sodium sulphate method

5.1.3.1. Materials

- **36% Na₂SO₄**: Dissolve 36 g Na₂SO₄ in 80 ml distilled water. Make up the volume to 100 ml.
- **18% Na₂SO₄**: Mix 36% Na₂SO₄ with equal volumes of distilled water.
- **PBS**: See section 5.1.2.2.
- **Sodium azide**
- **UV-Spectrophotometer**
- **Magnetic stirrer**
- **Dialysis tube**
- **Low speed centrifuge (table top or floor models)**

5.1.3.2. Procedure

1. Dilute 1 ml of crude antiserum with 1 ml of distilled water.
2. Add 2 ml of 36% Na₂SO₄, drop by drop.
3. Immediately collect the precipitate by centrifugation at 6,000 rpm/10 min at room temperature.
4. Suspend the precipitate in 18% Na₂SO₄ and centrifuge at 6,000 rpm/10 min at room temperature. Repeat this again.
5. Dissolve the precipitate in 2 ml half strength PBS containing 0.02% sodium azide (1x PBS diluted 1:1 with distilled water).
6. Dialyze against 500 ml half-strength PBS containing 0.02% sodium azide. Change the buffer at least three times.
7. Remove IgGs from the dialysis bag, measure the concentration by reading the absorbance (230-300 nm) in a spectrophotometer. Normally, the preparation should be diluted 1:8 for measurement. At 280 nm, 1.4 optical density (OD) is considered to be equal to 1 mg/ml, when measure in 1 cm cuvettes.
8. Store IgGs at 4 °C in aliquots. **Note**: Do not freeze IgG preparations.

5.1.4. Conjugation of PPSMV IgGs with Penicillinase by glutaraldehyde method

5.1.4.1. Materials

- **PPSMV IgG fraction** (see 5.1.3)
- **PBS** (see 5.1.2.2)
- **Glutaraldehyde** (generally supplied as 25% v/v solution) (Sigma, Cat. No. G5882)
- **Sodium azide**
• Dialysis bag (0.6 cm dia)
• Bovine serum albumin (BSA) (Sigma, Cat. No. A7638)
• Penicillinase (PNC) (Sigma, Cat. No. P0389). Generally supplied as lyophilized powder. Store this at 4 °C. **Working solution:** Prepare PNC solutions of 1 mg/ml concentration in sterile distilled water and store in a refrigerator (4 °C).

### 5.1.4.2. Procedure

1. Place 500 µg/ml PPSMV IgGs diluted in PBS, in a dialysis bag and add 250 µg of penicillinase. Dialyse against PBS in a beaker for 1 h at room temperature.
2. Transfer the dialysis bag into a beaker containing 0.06% glutaraldehyde in PBS (Add 1 ml of 25% glutaraldehyde in 400 ml of PBS to get 0.06% v/v final concentration) and dialyse for 3-4 h at room temperature.
3. Replace glutaraldehyde with 500 ml of PBS containing 0.02% sodium azide and dialyse for 18 h at 4 °C with at least three changes of buffer. For each change replace with 500 ml PBS containing sodium azide.
4. Transfer the conjugate into a new glass or plastic vial and add BSA at 5 mg/ml concentration. Store in small aliquots at 4 °C. If stored properly shelf life of enzyme IgG conjugate should exceed 12 months. **Note:** Do not freeze the conjugate.
5. Estimate the optimum working concentration of the enzyme conjugate by performing ELISA using various dilutions.

### 5.1.6. Cross-absorption of PPSMV-antiserum

To minimize the non-specific reaction as a result of presence of antibodies to host plant antigen, cross-absorption of PNC-conjugated PPSMV IgGs with healthy leaf extracts is recommended. For this grind healthy leaves in antibody buffer to give 1:10 w/v dilution (10 mg/ml), then filter through a double layer of muslin cloth. Use this extract for preparing the required dilution of PPSMV-IgG-PNC conjugate. This step reduces the non-specific reaction due to neutralization of antibodies to host antigen. **Note:** Cross-absorbed enzyme conjugates can be stored for a maximum period of 3 weeks at 4 °C.

• **Preparation for 15 ml PPSMV-IgG-PNC conjugate (sufficient for one ELISA plate using 100 µl reaction volume)**
  1. Grind 150 mg of healthy pigeonpea leaf (ICP 7035 or ICP8863) in 4 ml of PBS-TP0.
2. Filter the extract through double layer muslin cloth and make up the volume to 15 ml using PBS-TPO.
3. Dilute PNC-conjugated PPSMV IgGs in healthy pigeonpea leaf extract, to 1:1,500 dilution and incubate at 37 °C or room temperature for 45-60 min with gentle shaking.

**Precautions**

- Perform all incubation steps in a humid chamber to provide uniform temperature [a small plastic box suitable to fit ELISA plate, with moist paper towels covering the bottom of the box].
- Rinse the glassware intended for storing penicillin-BTB solution with water thoroughly. Presence of even traces of detergent or soaps will buffer the reaction.
- Use new ELISA plates as supplied by the manufacturer. Do not wash or rinse them prior to use.
- In each plate always include positive (PPSMV infected sample), negative (healthy) and buffer controls.

**5.1.7. Double antibody sandwich (DAS)-ELISA**

1. **Coating ELISA plates with antibodies:** Dilute PPSMV polyclonal antiserum to 1:10,000 in carbonate coating buffer and dispense 100 µl into each well of the ELISA plate. Cover the plate, place it in a humid chamber and incubate at 37 °C for 1 h or in a refrigerator (4 °C) overnight.

2. Wash the plate with three changes of PBS-T, allowing 3 min at each wash.
   **Note:** Antibody coated plates can be stored for up to 4-6 weeks in a refrigerator. In this case coat the well of the ELISA plate with antibodies, incubate and wash the plate as above. After final wash, fill the wells of the ELISA plate with PBS-T, cover with a lid and store in a refrigerator (4 °C).

3. **Preparation of leaf extract:** Grind test samples in PBS-TPO at a rate of 100 mg/ml buffer and dispense 100 µl into each well of the antibody coated ELISA plates, incubate in a humid chamber for 1 h at 37 °C or in a refrigerator (4 °C) overnight.

4. Wash the plate with three changes of PBS-T, allowing 3 min for each wash.

5. Cross-absorb the PNC conjugated PPSMV IgGs as described in section 5.1.6. dispense 100 µl of this into each well of the ELISA plate. Keep the plates in a humid chamber and incubate at 37 °C for 1 h.

6. Wash the plates with three changes of distilled water-Tween, allowing 3 min for each wash. **Note:** Traces of PBS-T left in wells is adequate to buffer the reaction between penicillin and penicillinase and therefore preventing the color change.
7. Add 100 µl of PNC substrate and incubate for 1 h at room temperature or for the intervals depending on the development of orange-yellow colour in the case of positive reaction. Observe plate on X-ray film light box for recording color changes. Results recorded after long intervals (>2 hrs) may not be accurate. Measure absorbance at 620 nm in an ELISA plate reader. **Note:** Absorbance value of the positive reaction will be lesser than negative.

8. In the case of positive reaction the bluish-green colour of BTB will turns to apple-green, and then to orange-yellow color. Apple-green color indicates week positive and orange yellow indicates a strong positive. Normally 0.2 mg/ml BTB gives an optical density of >2 units and positive reaction gives less than 0.1 optical density (O.D) units.
5.2. Reverse Transcription-Polymerase Chain Reaction

RT-PCR is used for the amplification of viruses containing RNA as their genome. During RT-PCR, the target RNA is first reverse-transcribed to a complementary DNA (cDNA) copy using the enzyme, reverse transcriptase (RT). During the first cycle of PCR, a second strand of the DNA is synthesized from the first-strand cDNA. The resultant dsDNA copy is then amplified in vitro by PCR by the simultaneous primer extension of complementary strands of DNA. This involves repeated cycles of heat denaturation of the cDNA (to separate the two DNA strands), annealing of primers to the complementary sequences and extension of the annealed primers with a thermostable DNA polymerase (Taq polymerase) in the presence of four deoxyribonucleotides (dNTPs). Since the extension products are complementary to and capable of binding primers, subsequent cycles of amplification double the amount of target DNA synthesized in the previous cycle (Fig 2). The result is exponential accumulation of the specific target DNA of interest.

Oligonucleotide primers, SM-1 and SM-2 are designed for the detection of PPSMV by RT-PCR. This primer pair amplifies a 321 bp product corresponding to the RNA-5 segment of PPSMV genome.

5.2.1. Isolation of total RNA from leaf tissue

Obtaining high quality intact RNA is the first and the critical step in performing RT-PCR. Many procedures are currently available for the isolation of total RNA from prokaryotes and eukaryotes. The essential feature of any protocol is to obtain large amount of intact RNA by effectively lysing the cells, avoiding the action of contaminating nucleases, in particular RNase. RNA isolation is difficult when processing certain tissues like pigeonpea, which is rich in polyphenols, tannins, polysaccharides and nucleases making it difficult to get clean RNA preparations. The protocols described here for RNA isolation from pigeonpea are being used successfully at ICRISAT for RT-PCR experiments.

Precautions
- Use autoclaved solutions, glass- and plasticware.
- Always wear disposable gloves as a precaution to avoid RNase contamination.
- Where possible use DEPC-treated water (see section 5.2.2.2.1)

5.2.1.1. Isolation of total RNA using Qiagen plant RNeasy RNA isolation kit

This kit is designed to isolate high quality total RNA from small amounts of starting material. The procedure is simple and fast (<30 min). In this procedure, leaf material is first lysed and homogenised in the presence of a denaturing buffer, which rapidly inactivates the RNase to ensure isolation of intact RNA. Ethanol is added to the lysate to provide appropriate binding
conditions and the sample is then applied to an RNeasy minicolumn built with a silica-gel-based membrane. Total RNA binds to the membrane and contaminants are efficiently removed. High-quality RNA is then eluted in distilled water.

5.2.1.1.1. Materials

- QIAGEN Plant RNeasy mini kit (Genetix, New Delhi, India)
- Variable speed microcentrifuge (table top model)
- Sterile 1.5 ml and 2 ml eppendorf tubes
- Sterile mortars and pestles
- Liquid nitrogen
- Absolute ethanol (molecular biology grade)

5.2.1.1.2. Procedure

1. Grind 100 mg of leaf material under liquid nitrogen to a fine powder using a mortar and pestle.
2. Transfer the tissue powder to a 2 ml eppendorf tube.
3. Add 450 µl of RLT buffer (supplied with the kit) and 5 µl of α-monothioglycerol (or β-mercaptopoethanol) and mix vigorously (in a vortex shaker).
4. Transfer the lysate into the QIAshreder spin column (supplied with the kit) and centrifuge for 2 min at maximum speed (14,000 rpm) in a microcentrifuge.
5. Transfer flow-throw fraction (lysate) from QIAshreder to a new 2 ml tube without disturbing the cell-debris pellet.
6. Add 0.5 volumes (usually 250 µl) of absolute ethanol to the lysate and mix well by pipetting.
7. Apply the sample into an RNeasy mini spin column (supplied with the kit) and centrifuge for 15 sec at 10,000 rpm.
8. Discard the flow-throw.
9. Add 700 µl of RW1 buffer (supplied with the kit) into mini column and centrifuge for 15 sec at 10,000 rpm.
10. Discard the flow-throw.
11. Add 500 µl of RPE buffer (supplied with the kit) into mini column and centrifuge for 15 sec at 10,000 rpm.
12. Discard the flow-throw.
13. Repeat the steps 11 and 12.
14. Transfer the RNeasy column into a new 1.5 ml collection tube and centrifuge for 1 min at 10,000 rpm to dry the RNeasy membrane.
15. Transfer RNeasy column into a new 1.5 ml tube and add 30-50 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge at 10,000 rpm for 1 min to elute RNA.
16. Store RNA at –20 °C.
5.2.1.2. Isolation of total RNA by phenol-chloroform method

This is a relatively inexpensive procedure to separate RNA from proteins and other contaminants. In this RNA from leaf extract is selectively partitioned into the aqueous phase after extracting in the presence of phenol-chloroform. RNA from aqueous phase is precipitated in the presence of salt by adding 2.5 volumes of ethanol.

5.2.1.2.1. Materials

- Sterile mortars and pestles
- Sterile eppendorf tubes 0.5 ml, 1.5 ml and 2 ml
- **1 M Tris-HCl, pH 8.0**: Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust the pH to 8.0 with conc. HCl. Adjust volume to 1 l with distilled water. Sterilize by autoclaving.
- **0.1 M Trish-HCl, pH 7.6**: Dissolve 12.11 g of Tris base in 800 ml of distilled water. Adjust the pH to 7.6 with conc. HCl. Adjust volume to 1 l with distilled water. Sterilize by autoclaving.
- **10% SDS**: Dissolve 10 g of sodium dodecyl sulfate (SDS) in 1 l of autoclaved distilled water. Warm to assist dissolution of SDS. No need to sterilize by autoclaving.
- **0.5 M EDTA**: Add 186.1 g of EDTA to 800 ml water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8 with 1 M NaOH (EDTA dissolves in solutions above pH 8). Make up to 1 l with distilled water. Sterilize by autoclaving.
- **3 M sodium acetate**: Dissolve 24.612 g of sodium acetate in 80 ml distilled water. Adjust the pH to 5.2 with glacial acetic acid. Adjust volume to 100 ml. Sterilize by autoclaving.
- **Phenol:chloroform**: Mix equal amounts of redistilled phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1 M Tris-HCl, pH 7.6. Store the mixture under 0.01 M Tris-HCl pH 7.6 at 4 °C in a dark bottle.
  
  **Caution**: Phenol is highly corrosive, can cause severe burns and is carcinogenic. Wear gloves and protective clothing when handling phenol. Any areas of skin that comes in contact with phenol should be rinsed with a large volume of water. DO NOT USE ETHANOL. Carry all steps involving phenol-chloroform in a fume hood. Care must be taken in disposing phenol-chloroform solutions.
- DEPC-treated water (see section 5.2.2.2.1)
- Chloroform: Isomyl alcohol (IAA) (24:1 v/v) mixture: To 96 ml of chloroform add 4 ml of IAA. Store the bottle at 4 °C.

5.2.1.2.2. Procedure

1. Grind 150 mg leaf material in liquid nitrogen to a fine powder.
2. Add 1 ml of extraction buffer (0.1 M Tris-HCl, pH 8.0 containing 2% SDS and 2 mM EDTA) and 1 ml of phenol-chloroform mixture (1:1 v/v).
3. Transfer the contents into a 2 ml eppendorf tube, vortex vigorously and then heat the samples at 70 °C for 5 min.
4. Centrifuge at 12,000 rpm for 10 min in a microcentrifuge.
5. Collect the upper aqueous phase carefully and add equal volumes of phenol-chloroform mixture and vortex vigorously.
6. Centrifuge at 12,000 rpm for 5 min.
7. Take the upper aqueous phase carefully and add equal volumes of chloroform and vortex vigorously.
8. Centrifuge at 12,000 rpm for 5 min.
9. Carefully collect the upper aqueous phase and to this add 1/10 (v/v) 3 m sodium acetate, pH 5.2 and 2.5 volumes of cold absolute ethanol. Store at –20 °C overnight.
10. Centrifuge at 12,000 rpm for 10 min. Carefully discard the supernatant. Rinse the pellet with 70% ethanol. Carefully discard the supernatant.
11. Dry the pellet at room temperature and resuspend the pellet in 100 µl of RNase-free water and store at –20 °C.

5.2.2. RT-PCR

Precautions
RT-PCR is a highly sensitive technique. Care must be taken to avoid cross-contamination and carryover of template, to prevent false amplifications. The following tips may help in getting good results with RT-PCR.

- Autoclave all solutions used in PCR. This degrades any extraneous DNA/RNA and nucleases.
- Divide reagents into aliquots to minimize the number of repeated samplings necessary.
- Avoid splashes by using tubes, which do not require much effort to open and collect the contents to the bottom by brief spinning before opening the tubes.
- Ensure that all the reaction components are added as per the required concentration. Failure would result in blank PCR gel.
- Use positive displacement pipettes with disposable tips.
- **Wherever possible, prepare master reaction mixture by premixing all reagents except template. Distribute into individual reactions then add the template directly into each tube.**
• Always use a positive control (known positive) and a negative control (no ‘template’ control) to ensure the specificity of the RT-PCR reaction. A successful RT-PCR should give amplification in positive control and there should not be any bands in negative control.

5.2.2.1. Materials

• Thermal cycler
• Sterile 0.2 ml, 0.5 ml and 1.5 ml Eppendorf tubes
• Oligonucleotide primers
  SM-1: 5’ACA TAG TTC AAT CCT TGA GTG CG3’
  SM-2: 5’ATA TTT TAA TAC ACT GAT AGG A3’
• Template RNA
• Moloney murine leukemia virus-RT (MoMLV-RT. Cat.# M1701, Promega)
• RNase inhibitor (Rnasin Cat.# N251A, Promega)
• Dithiothreitol (DTT) (Sigma grade)
• Taq Polymerase (Cat.# M668, Promega)
• Four deoxynucleotide triphosphates, 100 mM stock (Promega, Cat.# U1330)
  (dATP, dGTP, dCTP, dGTP)
• RNase free water
• Mineral oil (optional)
• Crushed ice
• Micropipettes (1-10 µl, 1-40 µl, 40-200 µl and 200-1000 µl single channel pipettes).
• Microfuge

5.2.2.2. Solutions

5.2.2.2.1. RNase free water
Treat distilled water with 0.1% diethylpyrocarbonate (DEPC; Sigma) for 12 h at 37 ºC. Then autoclave for 15 min at 15 lb/sq.in to destroy DEPC.

Caution: DEPC is a suspected carcinogen and should be handled with care.

Note: DEPC reacts rapidly with amines and cannot be used to treat solutions containing buffers such as Tris. Autoclaving degrades DEPC and therefore is safe to use DEPC-treated autoclaved water for preparation of Tris buffers.

5.2.2.2.2. 10 mM dNTP mixture
Mix 10 µl of each dATP, dCTP, dGTP and dTTP from a 100 mM stock and makeup to 100 µl with RNase free water. The final concentration of each dNTP in this mixture is 10 mM.
5.2.2.2.3. 25 mM MgCl₂
Usually supplied with Taq enzyme by the manufacturer.
If necessary, prepare by dissolving 0.508 g of MgCl₂·6H₂O in 100 ml RNase-free water. Sterilise by autoclaving, aliquot and store at -20 °C.
Note: Magnesium chloride solution can form a gradient of different concentrations when frozen. Therefore vortex well prior to using it.

5.2.2.2.4. 0.1 M DTT
Dissolve 154 mg of DTT in 10 ml of RNase-free water, aliquot and store at -20 °C

5.2.2.3. RT-PCR reaction
5.2.2.3.1. First strand cDNA synthesis (RT reaction)
1. Add the following reagents in a sterile 0.2 ml (or 0.5 ml depending on the thermal cycler) Eppendorf tubes. Keep the tubes in crushed ice during setting up of the reaction:
   (composition given is for one reaction).
   - 5x MMLV RT buffer (supplied with the enzyme) 4 µl
   - 25 mM MgCl₂ 2 µl
   - 0.1 M DTT 2 µl
   - 10 mM dNTP mixture 0.5 µl
   - SM 1 primer 0.5 µl (5 ng)
   - SM 2 primer 0.5 µl (5 ng)
   - RNasin 10 U
   - MMLV RT 100 U
   - Total RNA 1-4 µl
   - Sterile dH₂O to 20 µl
   - Total volume 20 µl
2. Incubate the reaction at 42 °C for 45 min. (During incubation period set up the PCR reaction see 5.2.2.3.2)
3. Terminate RT reaction by heating tubes at 94 °C for 5 min.

5.2.2.3.2. PCR reaction
1. Add the following in a sterile 0.2 ml (or 0.5 ml depending on thermal cycler) tubes.
   (composition given is for one reaction)
Note: Mineral oil overlay on the reaction mixture is not necessary if the thermal cycler is provided with a heated coverlid. For machines without heated coverlid, overlay PCR reaction with 10 µl of mineral oil to prevent evaporation.

2. Place the PCR tubes in the thermal cycler and use the following PCR programme for the amplification.

3. RT-PCR programme:

Perform PCR amplification in a thermal cycler using the following parameters: one cycle of denaturation for 5 min at 94 °C, followed by 35 cycles of amplification by denaturation at 92 °C for 45 sec, primer annealing at 55 °C for 45 sec and primer extension at 72 °C for 90 sec and finally incubate at 72 °C for 5 min for extension.

5.2.2.4. Analysis of RT-PCR products

Analyse 30 µl of PCR products in a 1% agarose gel as described in section 5.4.1

5.2.3. Immuno capture (IC)-RT-PCR

A technique that combines capturing or trapping of the virus using virus-specific antibodies and RT-PCR is called immuno capture-RT-PCR. In this the virus is first trapped from crude plant extract using antivirus antibodies, trapped virus particles are disrupted, the released viral RNA is reverse transcribed with specific primers into cDNA and finally amplified by PCR (Fig 3). The IC-RT-PCR circumvents the need for isolating RNA from test material and is highly specific and sensitive.
5.2.3.1. Materials
- Materials and chemicals listed in section 5.2.2.1
- PPSMV polyclonal antiserum
- Mortars and pestles (sterilise by autoclaving)
- Healthy and SMD-affected leaf material

5.2.3.2. Solutions
Solutions listed in section 5.2.2.2

5.2.3.2.1. Sterile carbonate coating buffer
See section 5.1.2.1. Sterilize by autoclaving.

5.2.3.2.2. Sterile 1x PBS
See section 5.1.2.2. Sterilize by autoclaving

5.2.3.2.3. Wash buffer
Sterile PBS containing Tween-20 to the final concentration of 0.05% (v/v)

5.2.3.2.4. Antigen extraction buffer
PBS-T containing 1% PVP (w/v), 0.2% ovalbumin

5.2.3.3. IC-RT-PCR Procedure
5.2.3.3.1. Coating of tubes with antibodies
1. Coat sterile polypropylene 0.5 ml microcentrifuge tubes with 30 µl of 1:500 PPSMV polyclonal antibodies diluted in carbonate coating buffer.
2. Incubate tubes at 37 °C for 2 h or 4 °C overnight.
3. Take out the contents with a micropipette and wash the tubes three times with 100 µl of wash buffer. Give 3 min interval between each wash.
   **Note:** To use antibody-coated tubes at a later period do not empty the PBS-T after final wash. Store tubes at 4 °C.

5.2.3.3.2. Antigen extraction and coating
4. Grind 100 mg of test leaf material in 1 ml of antigen extraction buffer (1:10 w/v) in a sterile mortar.
5. Load 30 μl into the antibody-coated tubes and incubate at 37 °C for 1-2 h.
6. Wash tubes with PBS-T as given in the step-3.
7. After final wash, aspirate remaining drops of wash buffer and allow tubes to dry by leaving lids open for 10 min at room temperature.
8. Then place tubes on ice
9. Add the following into reaction tube and incubate tubes at 50 °C for 5 min.
   - RNase free water 10 μl
   - SM-1 primer 0.5 μl
   - SM-2 primer 0.5 μl
   - 10 mM dNTPs 0.5 μl
10. Place tubes on ice for 1 min. Then add the following in to each tube and incubate tubes at 42 °C for 45 min.
    - 5X MMLV RT PCR buffer 4 μl
    - 25 mM MgCl₂ 2 μl
    - 0.1 M DTT 2 μl
    - RNasin 10 U
    - MMLV RT 100 U
    - Sterile distilled water to 20 μl
    - Total volume 20 μl
11. Terminate RT reaction by heating tubes at 94 °C for 5 min.
12. Subsequent steps are similar to those given in 5.2.2.3.2.

5.2.3.4. IC-RT-PCR cycle programme
Similar to the one given in section 5.2.2.3.2.

5.2.3.5. Analysis of IC-RT-PCR products
Analyse 30 μl of IC-RT-PCR products in a 1% agarose gel as described in section 5.4.1
5.3. Gel Electrophoresis of RT-PCR Products
Electrophoresis through agarose or polyacrylamide gels is the standard method used to analyse PCR amplified products. The phosphate groups in the DNA backbone carry uniform net negative charge at neutral or alkaline pH. During electrophoresis regardless of base composition, the DNA molecules move towards anode under a constant driving force provided by the net negative charge. Consequently, the rate of migration of a DNA molecules depends on its size than on the molecular weight, the smallest moving fastest. However, the migration rate is affected by such factors as, DNA conformation, buffer composition and presence of intercalating dyes. These techniques are simple, rapid to perform and DNA in the gel can be identified by staining with low concentrations of intercalating fluorescent dyes, such as ethidium bromide. As little as 1 ng of DNA can be detected in the gels by direct observation under ultraviolet light. The choice of gels to be used depends on the size of the fragments being separated. Polyacrylamide gels have high resolving power and are most effective for separating DNA fragments differed by 1-500 bp. These are run in a vertical configuration in a constant electric field. Agarose gels have low resolving capacity than polyacrylamide gels but are easy to prepare and has greater separation range. These are run in a horizontal configuration. For routine separation of RT-PCR products agarose gels are preferred.

5.3.1. Agarose Gel Electrophoresis
Agarose gels are prepared by melting agarose in the desired buffer until a clear transparent solution is obtained. The molten agarose solution is poured into a mould (boat) and allowed to harden. Upon hardening the agarose forms a matrix, the density of which depends on the concentration of the agarose.

5.3.1.1. Materials
- Horizontal electrophoresis unit
- Power supply
- Agarose (electrophoresis grade; BioRad, Cat.# 162-0125)
- UV Transilluminator (302 nm wave length)

5.3.1.2. Solutions
5.3.1.2.1. 10x Electrophoresis buffer (TBE buffer, pH 8.3)
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>(0.45 M)</td>
<td>54 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>(0.45 M)</td>
<td>27.5 g</td>
</tr>
</tbody>
</table>
5.3.1.2.2. Working solution (0.5x)
To 5 ml of 10x TBE buffer add 95 ml of sterile distilled water. The final concentration of Tris-base, boric acid and EDTA in working solution is 0.045 M, 0.045 M and 0.001 M, respectively.

5.3.1.2.3. 5x Sample buffer (Gel loading buffer)
Bromophenol blue (0.25%) 5 mg
Xylene cyanol FF (0.25%) 5 mg
Glycerol (30%) 3 ml
Sterile distilled water to 10 ml

5.3.1.2.4. 1% Ethidium bromide solution
Ethidium bromide 100 mg
Distilled water 10 ml
Store in a dark coloured bottle at 4 °C.
Working solution (0.5 µg/ml): To 100 ml water or molten agarose, add 5 µl of 1% ethidium bromide.
Caution: Ethidium bromide is a carcinogen. Gloves should be worn when handling and care must be taken to dispose materials containing this substance.

5.3.1.3. Procedure
1. Prepare agarose at the desired concentration (w/v) in 1x TBE buffer (for 1% gel, dissolve 1 g agarose in 100 ml buffer) and boil in a microwave oven or on a hot water bath, with intermittent shaking until all the agarose is completely dissolved. Replace evaporation loss with distilled water.
2. Seal the edges of the gel tray with a tape and place the comb at one end of the tray surface.
3. Cool the agarose solution to about 50 °C and pour into the gel tray to a thickness of 4-5 mm and allow the gel to set. Note: It will take about 20 min for agarose to harden.
4. Remove the tape and place the gel tray in the electrophoresis unit and fill the unit with 0.5x TBE buffer so that there is 2-3 mm of buffer over the gel surface. Then remove the comb.

0.5 M EDTA, pH 8 (0.01 M) 20 ml
Distilled water to 1 liter
It is not necessary to adjust pH. Sterilize by autoclaving and store at room temperature.
carefully. **Note:** Wells should be towards cathode end (black colour leads). The migration of DNA will be towards anode (red colour leads).

5. Mix 6 µl of loading buffer to 30 µl of PCR product and load slowly into the wells. Avoid overloading of the wells.


7. Connect electrophoresis unit to the power pack and turn on power supply until the bromophenol blue dye reaches the bottom of the gel. (Approximately 60 min at 100 V, for DNA to migrate 7 cm from the wells in a 1% gel)

8. Remove the gel from the tray and stain in ethidium bromide solution (0.5 µg/ml) in water for 15 min with gentle agitation. Then destain by soaking the gel in water for 5 min.

9. Observe the gel under UV Transilluminator using UV protective goggles or a full safety mask that efficiently blocks UV light. Photograph the gel using an orange filter fitted camera.

   **Caution:** UV radiation is very dangerous to the skin and particularly to the eyes. **It is absolutely essential to use UV-protective goggles.**

### 5.3.2. Polyacrylamide Gel Electrophoresis (PAGE)

Two PAGE systems are commonly used. 1. Continuous system (Weber and Osborn system) and 2. Discontinuous system (Laemmli system), which is most widely used system. In discontinuous system the gel consists of two parts, resolving gel located at the bottom, which has pore size that permits the sieving of the macromolecule to be analyzed. On top of this is a stacking gel, which has large pore size to exert no sieving effect. During electrophoresis, the DNA molecules are concentrated as a sharp band by isotachophoresis (steady state sieving) in the stack gel. The DNA is then separated according to their size in a resolving gel.

#### 5.3.2.1. Materials

- Vertical slab gel electrophoresis unit [Refer to the equipment user manual]
- Power supply
- Acrylamide
- Bisacrylamide
- Ammonium per sulphate
- TEMED

#### 5.3.2.2. Solutions

5.3.2.2.1. Stack gel buffer (1 M Tris-HCl, pH 6.8)
Tris base 12.1 g
Dissolve in 70 ml distilled water, adjust pH to 6.8 with 1 N HCl and make up to 100 ml with distilled water.

5.3.2.2.2. Resolving gel buffer (1 M Tris-HCl, pH 8.8)
Tris base 12.1 g
Dissolve in 70 ml distilled water, adjust pH to 8.8 with 1 N HCl and make up to 100 ml with distilled water.

5.3.2.2.3. Acrylamide/Bis (30:0.8 w/w) mixture
Acrylamide 30 g
Bis acrylamide 0.8 g
Distilled water to 100 ml
Store this solution at 4 °C in amber coloured bottle or wrap the bottle with aluminium foil to avoid exposure to light.
Caution: Acrylamide is a neurotoxin. Direct contact with skin or inhalation of acrylamide should be avoided. Prepare this solution in fume hood and always wear gloves.

5.3.2.2.5. 10% ammonium persulphate (APS)
APS 100 mg
Distilled water 1 ml
Note: Always prepare fresh solution before use.

5.3.2.2.6. TEMED (Sigma, Cat.# T9281)
Store at 4 °C.

5.3.2.2.7. Electrode (running or tank) buffer, pH 8.3
Tris base (25mM) 3 g
Glycine (250 mM) 14.4 g
Distilled water to 1 litre. No need to adjust pH. Store at room temperature.

5.3.2.2.8. Plug gel composition
Note: Plug gel is used to seal the bottom of the gel mould. Use of this depends on the type of electrophoresis unit.
Acrylamide: Bis mixture 1.75 ml
5.3.2.2.9. Resolving gel composition

**Note:** The concentration of the gel depends on the size of the products being separated. Usually 10% is the preferred concentration for separation of PCR products of size between 10-1000 bp.

<table>
<thead>
<tr>
<th>Gel concentration</th>
<th>10%</th>
<th>12%</th>
<th>14%</th>
<th>16%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide: Bis mixture</td>
<td>10 ml</td>
<td>12 ml</td>
<td>14 ml</td>
<td>16 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>11.25 ml</td>
<td>11.25 ml</td>
<td>11.25 ml</td>
<td>11.25 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>9 ml</td>
<td>7 ml</td>
<td>5 ml</td>
<td>3 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

5.3.2.2.10. Stacking gel composition (4%)

- Acrylamide: Bis mixture 1.5 ml
- Stacking gel buffer 1.25 ml
- Distilled water 7 ml
- TEMED 15 µl
- 10% APS 200 µl

**Note:** Mix acrylamide:bis solution, gel buffer, distilled water and TEMED mix well, then add APS, swirl the flask and immediately pour into the gel mould.

**Caution:** Unpolymerized acrylamide is a neurotoxin. Gloves should be worn when preparing this solution.

5.3.2.3. Procedure

1. Assemble the vertical slab gel apparatus in casting mode as per the manufacturer instructions.
2. Pipette 1 ml of the plug gel solution into the gel mould from a corner and allow it to set. **Note:** Some units may not require sealing the bottom with a plug gel.
3. Pour resolving gel solution into the gel mould leaving about 3 cm space for stacking gel. Gently overlay with water. A sharp water-gel interface will be visible with the polymerisation of the gel.

4. Decant the water overlay by gently tilting the gel mould.

5. Pour stacking gel solution into the gel mould and insert a comb and allow the gel to set. Care must be taken not to trap air bubbles below the comb’s teeth.

6. Carefully lift the comb straight-up, without disturbing the wells. Wash the wells with water to remove unpolymerised acrylamide.

7. Fill the lower tank of the electrophoresis unit with electrode buffer.

8. Insert the gel mould into the electrophoresis unit making sure not to trap air bubbles under the gel.

9. Fill the upper tank with electrode buffer. Avoid direct pouring of buffer into the wells.

10. Mix the DNA sample with gel loading dye and load into the wells.

11. Connect electrophoresis unit to the power supply. Connect the cathode lead to upper chamber and anode lead to the lower chamber. Turn power on and set the appropriate voltage.

12. Turn off the power supply when tracking dye (bromophenol blue or xylene cyanol) reaches nearly to bottom of the gels.

   **Note:** Under constant applied voltage and buffer temperature, migration of the tracking dye depends on the percentage of the resolving gel (see Table 1).

13. Remove the gel and stain in ethidium bromide solution for 15 min. Destain the gel for 5 min in distilled water and observe the gel under UV transilluminator.

   **Note:** PAGE gels are very fragile; care must be taken while handling to avoid breaking the gel.

### Table 1

**Dye migration in polyacrylamide non-denaturing gels of different strengths**

<table>
<thead>
<tr>
<th>% Acrylamide</th>
<th>Xylene cyanol (Migration equivalent to base pairs of DNA)</th>
<th>Bromophenol blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>460</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>260</td>
<td>65</td>
</tr>
<tr>
<td>8</td>
<td>160</td>
<td>45</td>
</tr>
<tr>
<td>12</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>20</td>
<td>45</td>
<td>12</td>
</tr>
</tbody>
</table>
5.4. Screening for SMD Resistance

The following methods are established for rapid screening of pigeonpea genotypes and to evaluate ‘type’ of resistance offered by the test plants. In this, plants raised in growth chambers are inoculated at the two-leaf stage with mites by the leaf-stapling technique. Plants are monitored for disease symptoms and tested for PPSMV by DAS-ELISA. Resistant genotypes (asymptomatic and ELISA negative) are tested again by graft inoculation. Since PPSMV is not mechanically transmissible to pigeonpea, graft transmission tests are performed to confirm its resistance to virus. The complete scheme is depicted in Fig 5.

5.4.1. Leaf stapling method

In this leaflets from diseased plants infested with mites are stapled onto the primary leaves of the test seedlings. Mites from the diseased leaf migrate onto the test seedling and their feeding results in virus transmission onto the test plant. This is the most efficient (100% infection if the genotype is susceptible) method for PPSMV transmission to pigeonpea and permits also testing of young seedlings.

5.4.1.1 Materials

- Mx-10 stapler (Max Co., Ltd., Japan)
- Pigeonpea seedlings at primary leaf stage (usually 14-18 days old)
- Leaflets from SMD-affected pigeonpea plant
- Binocular light microscope (10-40X).

5.4.1.2. Procedure

1. Collect leaflets from SMD affected pigeonpea plants.
2. Observe leaflets under a binocular microscope for mite infestation. Note: about 30-40 mites per leaf are required for efficient transmission.
3. Fold diseased leaflet (if larger in size) in a way that the under surface of the disease leaflet comes in contact with both surfaces of the test plant. Staple these leaves together using a stapler. Alternatively, if the disease leaflet is smaller, staple these two leaves in a way that under surface of test leaf and mite-infested leaf are in contact.
4. Inoculate SMD-susceptible pigeonpea genotypes as control (e.g.: ICP8863; TTB7). These plants show symptoms 12-14 days following staple inoculation.
5. Observe inoculated plants for visible symptoms. Assay test plants by DAS-ELISA (section 5.1).
6. Positive result indicates that test plants are susceptible to virus. A negative result indicates that the test plants possess inherent resistance to virus or to mites or to both.

5.4.2. Grafting method

In graft transmission freshly cut surfaces of infected and healthy plant tissues are brought together to allow virus movement from infected plant to healthy plant. There are several forms of graft-inoculation methods. ‘Petiole grafting’ developed for PPSMV transmission from pigeonpea to pigeonpea, including to its wild relatives, is simple to perform and results in high virus transmission rate (80-90% if the genotype is susceptible). In this method SMD-affected leaflet (scion) is used to graft to stems of the test plant (stock plant) (see Fig 4).

5.4.2.1. Materials

- Surgical blade
- Scissors
- Cellophane tape
- Polythene bags
- SMD-affected plants

- Plant material
  - Test plant or stock plant: A healthy rooted plant (about 25 days old). Use at least 5 plants per accession for graft inoculation studies.
  - Scion (detached tissue): Leaflet from SMD-affected plant. **Note:** Render virus source plants free from mites by repeatedly spraying with acaricides. For routine use, establish SMD cultures by graft-inoculation and maintain in a mite-free area.

5.4.2.2. Procedure

1. Cut the primary branch at the terminal end of a test plant.
2. Make an incision of about 1 cm down the centre of the stem.
3. Take a leaflet from the donor plant and trim its petiole into a wedge shape and insert into the stem slit of the test plant. **Note:** Ensure that donor plants are free from mites. Also treat donor plant tissue in acaricide to eliminate any mites.
4. Bind the grafted portion tightly with a cellophane tape. **Note:** Ensure that contact surfaces between grafted parts fit neatly and closely, prior to binding.
5. Cover the grafted plants with plastic bags for maintaining high humidity for up to 7 days. **Note:** To avoid cross-contamination by mites maintain graft-inoculated plants in a mite-proof growth
cabinets or at least well away from the known sources of SMD-affected plants and spray with acaricides.

6. Observe plants for symptoms. A susceptible genotype takes about 20-25 days to show the symptoms. Assay all grafted plants for virus by DAS-ELISA (section 5.1). Negative result indicates that the test plants possess inherent resistance to virus infection.

5.4.3. Interpretation of results

- Leaf-stapling method:
  - ELISA positive and mite infestation occurred: Plants susceptible to virus and mites.
  - ELISA positive and no mites: Plants susceptible to virus, but not to mites.
  - ELISA negative and no mites: Plants resistant to virus or mites or to both. Test these plants by grafting.

- Graft-inoculation method:
  - ELISA positive: Plants susceptible to virus.
  - ELISA negative: Plants resistant to virus.
6. Appendices
## Appendix 6.1

**Certain Economically Important Virus Diseases of Crops in India**

(Compiled by Prof. P. Sreenivasulu, Dept. of Virology, SV University, Tirupati 517502)

<table>
<thead>
<tr>
<th>Crop</th>
<th>Disease</th>
<th>Causal virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td>Tungro</td>
<td><em>Rice tungro spherical virus</em> (RTSV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Rice tungro bacilliform virus</em> (RTBV)</td>
</tr>
<tr>
<td>Potato</td>
<td>Leaf roll</td>
<td><em>Potato leaf roll virus</em> (PLRV)</td>
</tr>
<tr>
<td></td>
<td>Rugose mosaic</td>
<td><em>Potato virus Y</em> (PVY)</td>
</tr>
<tr>
<td>Tomato</td>
<td>Leaf curl</td>
<td><em>Tomato leaf curl virus</em> (TLCV)</td>
</tr>
<tr>
<td>Chilli</td>
<td>Mosaic</td>
<td><em>Cucumber mosaic virus</em> (CMV)</td>
</tr>
<tr>
<td>Brinjal</td>
<td>Mosaic</td>
<td>CMV</td>
</tr>
<tr>
<td>Bhendi/Oakra</td>
<td>Yellow vein mosaic</td>
<td><em>Okra yellow vein mosaic virus</em> (OYMV)</td>
</tr>
<tr>
<td>Mungbean</td>
<td>Yellow mosaic</td>
<td><em>Mungbean yellow mosaic virus</em> (MYMV)</td>
</tr>
<tr>
<td>Urdbean</td>
<td>Yellow mosaic</td>
<td>MYMV</td>
</tr>
<tr>
<td>Soybean</td>
<td>Yellow mosaic</td>
<td>MYMV</td>
</tr>
<tr>
<td>French bean</td>
<td>Mosaic</td>
<td>Bean common mosaic virus (BCMV)</td>
</tr>
<tr>
<td>Chickpea</td>
<td>Stunt</td>
<td>Pea/Bean leaf roll virus (BLRV)</td>
</tr>
<tr>
<td>Pigeonpea</td>
<td>Sterility mosaic</td>
<td><em>Pigeonpea sterility mosaic virus</em> (PPSMV)</td>
</tr>
<tr>
<td></td>
<td>Yellow mosaic</td>
<td>MYMV</td>
</tr>
<tr>
<td>Groundnut</td>
<td>Bud necrosis</td>
<td>Peanut bud necrosis virus (PBNV)</td>
</tr>
<tr>
<td></td>
<td>Stem necrosis</td>
<td>Tobacco streak virus (TSV)</td>
</tr>
<tr>
<td>Sunflower</td>
<td>Necrosis</td>
<td>TSV</td>
</tr>
<tr>
<td>Cassava</td>
<td>Mosaic</td>
<td>Indian cassava mosaic virus (ICMV)</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>Streak mosaic</td>
<td>Sugarcane streak mosaic virus (SCSMV)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Leaf curl</td>
<td>Tobacco leaf curl virus (TLCV)</td>
</tr>
<tr>
<td>Small cardamom</td>
<td>Mosaic (Katte)</td>
<td>Cardamom mosaic virus (CdMV)</td>
</tr>
<tr>
<td>Black pepper</td>
<td>Stunt</td>
<td>CMV</td>
</tr>
<tr>
<td>Banana</td>
<td>Bunchy top</td>
<td><em>Banana bunchy top virus</em> (BBTV)</td>
</tr>
<tr>
<td></td>
<td>Infectious chlorosis</td>
<td>CMV</td>
</tr>
<tr>
<td></td>
<td>Streak</td>
<td><em>Banana streak virus</em> (BSV)</td>
</tr>
<tr>
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<td>Brack mosaic (Kokkan)</td>
<td><em>Banana bract mosaic virus</em> (BbrMV)</td>
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<td>Citrus</td>
<td>Tristeza</td>
<td><em>Citrus tristeza virus</em> (CTV)</td>
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<tr>
<td></td>
<td>Yellow mosaic</td>
<td><em>Citrus mosaic virus</em> (CiMV)</td>
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<td>Papaya</td>
<td>Ringspot</td>
<td><em>Papaya ringspot virus</em> (PRSV)</td>
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<tr>
<td>Watermelon</td>
<td>Bud necrosis</td>
<td>Watermelon bud necrosis virus (WBNV)</td>
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</table>
Appendix 6.2
List of Commonly Used Methods for the Detection of Plant Viruses
(Compiled by Prof. P Sreenivasulu, Dept. of Virology, SV University, Tirupati 517502)

• **Biological methods**
  • Visual detection based on symptoms
  • Transmission to indicator hosts

• **Microscopic methods**
  • Light microscopy of inclusions
  • Electron microscopy

• **Serological methods (protein-based)**
  • Polyclonal antibodies, monoclonal antibodies and recombinant antibodies
  • Agar gel single/double diffusion test
  • Immuno-fluorescent microscopy
  • Latex agglutination assay
  • Immuno filter paper assay
  • Enzyme immuno assays
    • Direct and indirect ELISAs
    • Dot immunobinding assay
    • Electroblot immunoassay
    • Tissue blotting/printing
    • Immunospecific electron microscopy

• **Nucleic acid-based methods**
  • dsRNA / DNA analysis
  • DNA/RNA probes- radio active, non radioactive molecular beacons
  • Nucleic acid hybridization assays on solid supports
  • In situ hybridization
  • PCR-based methods
    • Immuno Capture-PCR
    • Reverse Transcription-PCR
    • Multiplex-PCR
    • Print Capture-PCR
    • Spot Capture-PCR
    • PCR-ELISA
    • Isothermal Multiplex Aplidet RNA System
    • RT-PCR-ELOSA (Enzyme-linked oligosorbent assay)

• **Bioelectric recognition assay (BERA)**
Appendix 6.3
Common Conversions

Weight conversions

1 µg = 10^{-6} g
1 ng = 10^{-9} g
1 pg = 10^{-12} g
1 fg = 10^{-15} g

Spectrophotometric conversions

1 OD at A_{260nm} double-stranded DNA = 50 µg/ml concentration
1 OD at A_{260nm} single-stranded DNA = 33 µg/ml concentration
1 OD at A_{260nm} single-stranded RNA = 40 µg/ml concentration

SI Unit prefixed

<table>
<thead>
<tr>
<th>Prefix</th>
<th>Symbol</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exa</td>
<td>E</td>
<td>10^{18}</td>
</tr>
<tr>
<td>Penta</td>
<td>P</td>
<td>10^{15}</td>
</tr>
<tr>
<td>Tera</td>
<td>T</td>
<td>10^{12}</td>
</tr>
<tr>
<td>Giga</td>
<td>G</td>
<td>10^{9}</td>
</tr>
<tr>
<td>Mega</td>
<td>M</td>
<td>10^{6}</td>
</tr>
<tr>
<td>Kilo</td>
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<td>Milli</td>
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<tr>
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<td>Pico</td>
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<td>10^{-12}</td>
</tr>
<tr>
<td>Femto</td>
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<tr>
<td>Atto</td>
<td>a</td>
<td>10^{-18}</td>
</tr>
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Appendix 6.4
ICTV Classification of Plant Viruses
Appendix 6.5
Selected References

Pigeonpea
Annual Reports, All India Coordinated Research Project on Pigeonpea, Indian Institute of Pulses Research (IIPR), Kanpur 208 024, India.

Sterility mosaic disease
Annual Reports, All India Coordinated Research Project on Pigeonpea, Indian Institute of Pulses Research (IIPR), Kanpur 208 024, India.


**Plant Virology**


All the virology on WWW. (http://www.tulare.edu/~dmsander/garry-fawweb.html)

Collection of digital images of crop diseases (http://www.shopapspress.org)

Descriptions of plant viruses CD-ROM (dpv.aab@hri.ac.uk)


**Erophyd mites**


**Molecular Biology & Serology**


Appendix 6.6
Glossary

(Compiled from www.inversonsoftware.com; www.virology.net; www.biotech-monitor.nl; arneson.cornell.edu/; www.unep-wcmc.org)

**Abiotic stress:** Outside (nonliving) factors which can cause harmful effects to plants, such as soil conditions, drought, extreme temperatures.

**Absorbance (optical density):** This is a measure of the amount of light absorbed by a suspension of bacterial cells or a solution of an organic molecule; it is measured by a colorimeter or spectrophotometer. Absorbance values are used to plot the growth of bacteria in suspension cultures and to gauge the purity and concentration of molecules (such as proteins) in solution. Absorbance is defined as a logarithmic function of the percent transmission of a wavelength of light through a liquid.

**Accession or entry:** A population or line in a breeding programme or germplasm collection; also an individual sample in a germplasm bank. A sample of a crop variety collected at a specific location and time; may be of any size.

**Adenine (A):** A nitrogenous base, one member of the base pair AT (adenine-thymine).

**Agarose gel electrophoresis:** A matrix composed of a highly purified form of agar that is used to separate larger DNA and RNA molecules ranging 20,000 nucleotides.

**Alternate host:** One of two kinds of plants on which a parasitic fungus (e.g., a rust) must develop to complete its life cycle.

**Alternative host:** A plant other than the main host that a virus can infect.

**Amino acid:** Any of 20 basic building blocks of proteins--composed of a free amino (NH2) end, a free carboxyl (COOH) end, and a side group (R).

**Amplification:** An increase in the number of copies of a specific DNA fragment; can be in vivo or in vitro.

**Amplify:** To increase the number of copies of a DNA sequence, in vivo by inserting into a cloning vector that replicates within a host cell, or in vitro by polymerase chain reaction (PCR).

**Anion:** A negatively charged molecule

**Anode:** A positive electrode in an electrolytic cell toward which anions migrate.

**Anneal:** The pairing of complementary DNA or RNA sequences, via hydrogen bonding, to form a double-stranded polynucleotide. Most often used to describe the binding of a short primer or probe.

**Antibody:** An immunoglobulin protein produced by B-lymphocytes of the immune system that binds to a specific antigen molecule.

**Antigen (Immunogen):** Any foreign substance, such as a virus, bacterium, or protein, that elicits an immune response by stimulating the production of antibodies.

**Antigenic determinant:** A surface feature of a microorganism or macromolecule, such as a glycoprotein, that elicits an immune response.

**Antiserum:** The serum from a vertebrate that has been exposed to an antigen and which contains antibodies that react specifically with the antigen.

**Antisense:** Nucleic acid that has a sequence exactly opposite to an mRNA molecule made by the body; binds to the mRNA molecule to prevent a protein from being made.

**Antisense RNA:** A complementary RNA sequence that binds to a naturally occurring (sense) mRNA molecule, thus blocking its translation.

**Asymptomatic:** Without signs or symptoms of disease.

**AT content:** The percentage of nitrogenous bases on a DNA molecule which are either adenine (A) or thymine (T) (from a possibility of four different ones, also including cytosine (C) and guanine (G)).

**AT/GC ratio:** The ratio of adenine-thymine base pairs to guanine-cytosine base pairs on a DNA molecule.

**Avirulent:** Not exhibiting virulence; nonpathogenic.

**Base:** one of the four chemical units (nucleotides) arranged along the DNA or RNA molecule.

**Base composition:** The relative proportions fo the four respective nucleotides in a given sequence of DNA or RNA.

**Base pair (bp):** A pair of complementary nitrogenous bases in a DNA molecule--
adenine-thymine and guanine-cytosine. Also, the unit of measurement for DNA sequences.

**Base sequence**: The order of nucleotide bases in a DNA molecule; determines structure of proteins encoded by that DNA.

**Bioassay**: The measurement of infective virus concentration in plant extracts.

**Biological control**: The deliberate use by humans of one species of organism to eliminate or control another.

**Biodiversity**: The variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems.

**Biotechnology**: The scientific manipulation of living organisms, especially at the molecular genetic level, to produce useful products. Gene splicing and use of recombinant DNA (rDNA) are major techniques used.

**Biotic stress**: Living organisms which can harm plants, such as viruses, fungi, and bacteria, and harmful insects.

**Biotype**: A subspecies of organism morphologically similar to but physiologically different from other members of the species.

**Blotting**: Following electrophoresis: the transfer of nucleic acids and/or proteins from a gel strip to a specialized, chemically reactive matrix on which the nucleic acids, etc. may become covalently bound in a pattern similar to that present in the original gel.

**Breeding line**: Genetic lines of particular significance to plant or animal breeders that provide the basis for modern varieties.

**Carrier**: Organism that carries a virus either in form of an infection or while it is in incubation.

**Cation**: A positively charged ion.

**Causal agent of disease**: That which is capable of causing disease.

**cDNA**: DNA synthesized from an RNA template using reverse transcriptase.

**cDNA library**: A library composed of complementary copies of cellular mRNAs.

**Chlorosis**: The loss of chlorophyll from the tissues of a plant, resulting from microbial infection, viral infection, the action of certain phytotoxins, the lack of light, to magnesium or iron deficiency, etc. Chlorotic tissues commonly appear yellowish.

**Central dogma**: Francis Crick's seminal concept that in nature genetic information generally flows from DNA to RNA to protein.

**Circulative transmission**: Virus transmission characterized by a long period of acquisition of the virus by a vector, a latent period of several hours before the vector is able to transmit the virus, and retention of the virus by the vector for a long period, usually several days. (Also termed persistent transmission)

**Cistron**: A DNA sequence that codes for a specific polypeptide; a gene.

**Clone**: An exact genetic replica of a specific gene or an entire organism.

**Cloning**: The mitotic division of a progenitor cell to give rise to a population of identical daughter cells or clones.

**Coalesce**: To merge or grow together into a similar but larger structure.

**Coat protein (capsid)**: The coating of a protein that enclosed the nucleic acid core of a virus.

**Codon**: A group of three nucleotides that specifies addition of one of the 20 amino acids during translation of an mRNA into a polypeptide. Strings of codons form genes and strings of genes form chromosomes.

**Complementary DNA or RNA**: The matching strand of a DNA or RNA molecule to which its bases pair.

**Complementary nucleotides**: Members of the pairs adenine-thymine, adenine-uracil, and guanine-cytosine that have the ability to hydrogen bond to one another.

**Control**: Economic reduction of crop losses caused by plant diseases.

**Cross-hybridization**: The hydrogen bonding of a single-stranded DNA sequence that is partially but not entirely complementary to a single-stranded substrate. Often, this involves hybridizing a DNA probe for a specific DNA sequence to the homologous sequences of different species.

**Cross-pollination**: Fertilization of a plant from a plant with a different genetic makeup.

**Crop rotation**: The practice of growing a sequence of different crops on the same land in successive years or seasons; done to replenish the soil, curb pests, etc.

**Cross-protection**: The protection conferred on a host by infection with one strain of a virus that prevents infection by a closely-related strain.

**Cultivar**: A cultivated variety (genetic strain) of a domesticated crop plant. A cultivated plant
variety or cultural selection. International term denoting certain cultivated plants that are clearly distinguishable from others by one or more characteristics and that when reproduced retain their distinguishing characteristics. In the United States, 'variety' is considered to be synonymous with cultivar (derived from 'cultivated variety').

**Dalton:** A unit of measurement equal to the mass of a hydrogen atom, 1.67 x 10E-24 gram/L (Avogadro's number).

**Degenerate primers:** Oligonucleotides designed to include a mixture of different sequences to allow for variation at particular nucleotide positions in a target sequence.

**Denature:** To induce structural alterations that disrupt the biological activity of a molecule. Often refers to breaking hydrogen bonds between base pairs in double-stranded nucleic acid molecules to produce in single-stranded polynucleotides or altering the secondary and tertiary structure of a protein, destroying its activity.

**Density gradient centrifugation:** High-speed centrifugation in which molecules "float" at a point where their density equals that in a gradient of cesium chloride or sucrose.

**Diagnostic:** A distinguishing characteristic important in the identification of a disease or other disorder.

**Diagnosis:** The evaluation of symptoms and laboratory tests which confirms or establishes the nature/origin of a disease.

**Differential host:** A plant host that on the basis of disease symptoms serves to distinguish between various strains or races of a given plant pathogen.

**Diploid:** A full set of genetic material, consisting of paired chromosomes one chromosome from each parental set.

**Disease:** An abnormal condition of a plant in which its physiology, morphology, and/or development is altered under the continuous influence of a pathogen.

**DNA (Deoxyribonucleic acid):** An organic acid and polymer composed of four nitrogenous bases-adenine, thymine, cytosine, and guanine linked via intervening units of phosphate and the pentose sugar deoxyribose. DNA is the genetic material of most organisms and usually exists as a double-stranded molecule in which two antiparallel strands are held together by hydrogen bonds between adeninethymine and cytosine-guanine.

**DNA diagnosis:** The use of DNA polymorphisms to detect the presence of a disease gene.

**DNA fingerprint:** The unique pattern of DNA fragments identified by Southern hybridization (using a probe that binds to a polymorphic region of DNA) or by polymerase chain reaction (using primers flanking the polymorphic region).

**DNA probe:** A fragment of DNA used to recognize a specific complementary DNA sequence, or gene(s). Probes can be employed, for example, to bind to the genetic material of microbes for purposes of detection, identification, or, in some cases, inactivation.

**DNA sequencing:** Procedures for determining the nucleotide sequence of a DNA fragment.

**Downstream:** The region extending in a 3' direction from a gene.

**Ecology:** The study of the interactions of organisms with their environment and with each other.

**Electron Microscopy:** An imaging method, which uses a focused beam of electrons to enlarge the image of an object on a screen or photographic plate.

**Electrophoresis:** The technique of separating charged molecules in a matrix to which is applied an electrical field.

**Encapsidation:** Process by which a virus' nucleic acid is enclosed in a capsid.

**Endemic:** Restricted to specified region

**Enzymes:** Proteins that control the various steps in all chemical (metabolic) reactions.

**Enzyme-linked immunosorbent assay (ELISA):** A sensitive, inexpensive assay technique involving the use of antibodies coupled with indicators (e.g., enzymes linked to dyes) to detect the presence of specific substances, such as enzymes, viruses, or bacteria.

**Epidemic:** A change in the amount of disease in a population in time and space.

**Epidemiology:** The science concerned with the determination of the specific causes of a disease or the interrelation between various factors determining a disease, as well as disease trends in a specific region.

**Epitope:** The region of antigen that triggers and interacts with antibody.

**Eradication:** Control of plant disease by eliminating the pathogen after it is established or by eliminating the plants that carry the pathogen.
**Escape:** Failure of inherently susceptible plants to become diseased, even though disease is prevalent.

**Etiology:** The study or theory of factors which cause disease.

**Exon:** A DNA sequence that is ultimately translated into protein.

**Express:** To translate a gene’s message into a molecular product.

**Flanking region:** The DNA sequences extending on either side of a specific locus or gene.

**GxE interaction:** Genotype by Environment interaction. Phenomenon that two (or more) varieties will react differently to a change of environment.

**Gene:** A locus on a chromosome that encodes a specific protein or several related proteins. It is considered the functional unit of heredity.

**Genetic code:** The three-letter code that translates nucleic acid sequence into protein sequence. The relationships between the nucleotide base-pair triplets of a messenger RNA molecule and the 20 amino acids that are the building blocks of proteins.

**Genetic disease:** A disease that has its origin in changes to the genetic material, DNA. Usually refers to diseases that are inherited in a Mendelian fashion, although noninherited forms of cancer also result from DNA mutation.

**Genetic engineering:** The manipulation of an organism’s genetic endowment by introducing or eliminating specific genes through modern molecular biology techniques. A broad definition of genetic engineering also includes selective breeding and other means of artificial selection.

**Genome:** The genetic complement contained in the chromosomes of a given organism, usually the haploid chromosome state.

**Genomic library:** A library composed of fragments of genomic DNA.

**Genotype:** The structure of DNA that determines the expression of a trait. Genetic constitution of the organism distinguished by physical appearance.

**Glycoprotein:** A protein molecule coated with carbohydrates.

**Hapten:** A small chemicals coupled to larger protein molecules (carriers). Small chemicals (hapten) serve as epitopes for binding to the antibodies on the B-cell surface.

**Haploid:** A single set of chromosomes (half the full set of genetic material), present in the egg and sperm cells of animals and in the egg and pollen cells of plants.

**Heredity:** The handing down of certain traits from parents to their offspring. The process of heredity occurs through the genes.

**Heterozygosity:** The presence of different alleles at one or more loci on homologous chromosomes.

**Heteroduplex:** A double-stranded DNA molecule or DNA-RNA hybrid, where each strand is of a different origin.

**Histopathology:** The study of pathology of cells and tissues; the microscopic changes characteristic of disease.

**Horizontal resistance:** In a given cultivar: the existence of similar levels of resistance to each of the races of a given pathogen.

**Host:** An organism that contains another organism.

**Hybrid:** An individual produced from genetically different parents. The term is often reserved by plant breeders for cases where the parents differ in several important respects. Hybrid are often more vigorous than either parent, but cannot breed true.

**Hybridization:** The hydrogen bonding of complementary DNA and/or RNA sequences to form a duplex molecule.

**Hybridoma:** A hybrid cell, composed of a B lymphocyte fused to a tumor cell, which grows indefinitely in tissue culture and is selected for the secretion of a specific antibody of interest.

**Hydrogen bond:** A relatively weak bond formed between a hydrogen atom (which is covalently bound to a nitrogen or oxygen atom) and a nitrogen or oxygen with an unshared electron pair.

**Hypersensitive:** The state of being abnormally sensitive. It often refers to an extreme reaction to a pathogen (e.g., the formation of local lesions by a virus or the necrotic response of a leaf to bacterial infection).

**Immune:** Cannot be infected by a given pathogen.

**Immunity:** A natural or acquired resistance to a specific disease.

**Inbred line:** Genetically (nearly) homozygous population, derived through several cycles of selfing (see below), also used for hybrid seed production.

**Incubation period:** The period of time between penetration of a host by a pathogen and the first appearance of symptoms on the host.

**Indexing:** A procedure to determine whether a given plant is infected by a virus. It involves
the transfer of a bud, scion, sap etc. from one plant to one or more kinds of indicator plants sensitive to the virus.

**Indicator host:** A plant species that gives characteristic symptoms to a specific virus. Used in virus diagnosis.

**Infection:** Condition in which virulent organisms are able to multiply within the cell and cause a response. Infection may or may not lead to visible symptoms.

**Infectious:** Capable of being transmitted by infection, with or without actual contact.

**Inoculate:** To introduce a microorganism into an environment suitable for its growth; to bring a parasite into contact with a host.

**Inoculation:** The act of inoculating; the placement of microorganisms or viruses at a site where infection is possible (the infection court).

**Inoculum:** The population of microorganisms introduced in an inoculation; the units of a parasite capable of initiating an infection.

**In situ:** Refers to performing assays or manipulations with intact tissues.

**In vitro:** (Literally “in glass.”) Cultivated in an artificial, non-living environment.

**In vivo:** Refers to biological processes that take place within a living organism or cell.

**Initiation codon:** The mRNA sequence AUG, coding for methionine, which initiates translation of mRNA.

**Intergenic regions:** DNA sequences located between genes that comprise a large percentage of the human genome with no known function.

**Intron:** A noncoding DNA sequence within a gene that is initially transcribed into messenger RNA but is later snipped out.

**Ion:** A charged particle.

**Isolate:** In plant pathology: a culture or subpopulation of a microorganism separated from its parent population and maintained in some sort of controlled circumstance; also, to effect such separation and control, for example to isolate a pathogen from diseased plant tissue.

**Isotope:** One of two or more forms of an element that have the same number of protons (atomic number) but differing numbers of neutrons (mass numbers). Radioactive isotopes are commonly used to make DNA probes and metabolic tracers.

**Land race:** Primitive or antique variety usually associated with traditional agriculture. Often highly adapted to local conditions.

**Legume:** A member of the pea family that possesses root nodules containing nitrogen-fixing bacteria.

**Local infection:** An infection affecting a limited part of a plant.

**Local lesion:** A localized spot produced on a leaf upon mechanical inoculation with a virus.

**Lyophilization:** Rapid freezing of a material at low temperature followed by rapid dehydration by sublimation in a high vacuum. A method used to preserve biological specimens or to concentrate macromolecules with little or no loss of activity. (Also freeze-drying)

**Masked symptoms:** Virus-induced plant symptoms that are absent under some environmental conditions but appear when the host is exposed to certain conditions of light and temperature.

**Mechanical inoculation:** Of plant viruses, a method of experimentally transmitting the pathogen from plant to plant; juice from diseased plants is rubbed on test-plant leaves that usually have been dusted with carborundum or some other abrasive material.

**Mass selection:** Selection of individual plants from a population. Mass selection may be positive and negative selection. Seeds from mass selection form the next generation.

**Messenger RNA (mRNA):** The class of RNA molecules that copies the genetic information from DNA, in the nucleus, and carries it to ribosomes, in the cytoplasm, where it is translated into protein.

**Molecular biology:** The study of the biochemical and molecular interactions within living cells.

**Molecular cloning:** The biological amplification of a specific DNA sequence through mitotic division of a host cell into which it has been transformed or transfected.

**Monoclonal antibodies:** Immunoglobulin molecules of single-epitope specificity that are secreted by a clone of B cells.

**Monoculture:** The agricultural practice of cultivating crops consisting of genetically similar organisms.

**Monogenic resistance:** Resistance determined by a single gene.

**Mosaic:** A common symptom induced in leaves by many plant virus infections in which there is a pattern of dark green, light green and sometimes chlorotic areas. This pattern is
often associated with the distribution of veins in the leaf. In monocotyledonous leaves it shows as stripes.

**Mottle:** A diffuse form of the mosaic symptom in plant leaves in which the dark and light green are less sharply defined. This term is frequently used interchangeably with mosaic.

**Multicomponent virus:** A virus in which the genome needed for full infection is divided between two or more particles (e.g., cowpea mosaic virus, brome mosaic virus, cucumber mosaic virus).

**Necrosis.** Localized death of cells or tissues (necrotic. Dead)

**Nitrocellulose:** A membrane used to immobilize DNA, RNA, or protein, which can then be probed with a labeled sequence or antibody.

**Nitrogen fixation:** The conversion of atmospheric nitrogen to biologically usable nitrates.

**Nitrogenous bases:** The purines (adenine and guanine) and pyrimidines (thymine, cytosine, and uracil) that comprise DNA and RNA molecules.

**Nodule:** The enlargement or swelling on roots of nitrogen-fixing plants. The nodules contain symbiotic nitrogen-fixing bacteria.

**Nomenclature:** A system of names, or naming, as applied to the subjects or study in any art or science.

**Noncirculative transmission:** Virus transmission characterized by a very short period of acquisition of the virus by a vector (e.g., an aphid), no latent period before the vector can transmit the virus, and a short period of retention by the vector after acquisition. (Also termed nonpersistent transmission.)

**Nontarget organism:** An organism which is affected by an interaction for which it was not the intended recipient.

**Northern hybridization:** (Northern blotting). A procedure in which RNA fragments are transferred from an agarose gel to a nitrocellulose filter, where the RNA is then hybridized to a radioactive probe.

**Nuclease:** A class of enzymes that degrades DNA and/or RNA molecules by cleaving the phosphodiester bonds that link adjacent nucleotides. In deoxyribonuclease (DNase), the substrate is DNA. In endonuclease, it cleaves at internal sites in the substrate molecule. Exonuclease progressively cleaves from the end of the substrate molecule. In ribonuclease (RNase), the substrate is RNA. In the S1 nuclease, the substrate is single-stranded DNA or RNA.

**Nucleic acids:** The two nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are made up of long chains of molecules called nucleotides.

**Nucleoprotein:** A compound of nucleic acid and protein.

**Nucleoside:** A building block of DNA and RNA, consisting of a nitrogenous base linked to a five carbon sugar.

**Nucleoside analog:** A synthetic molecule that resembles a naturally occurring nucleoside, but that lacks a bond site needed to link it to an adjacent nucleotide.

**Nucleotide:** A building block of DNA and RNA, consisting of a nitrogenous base, a five-carbon sugar, and a phosphate group. Together, the nucleotides form codons, which when strung together form genes, which in turn link to form chromosomes.

**Oligonucleotide:** A short DNA polymer composed of only a few nucleotides.

**Open pollination:** Pollination by wind, insects, or other natural mechanisms.

**Open reading frame:** A long DNA sequence that is uninterrupted by a stop codon and encodes part or all of a protein.

**Organelle:** A cell structure that carries out a specialized function in the life of a cell.

**Parasitism:** The close association of two or more dissimilar organisms where the association is harmful to at least one.

**Pathogen:** Organism which can cause disease in another organism.

**Pathotype:** An infrasubspecific classification of a pathogen distinguished from others of the species by its pathogenicity on a specific host(s).

**Pellet:** The material concentrated at the bottom of a centrifuge tube after centrifugation.

**Pesticide:** A substance that kills harmful organisms (for example, an insecticide or fungicide or acaricide).

**pH:** A measure of the acidity or basicity of a solution.

**Phenotype:** The observable characteristics of an organism, the expression of gene alleles (genotype) as an observable physical or biochemical trait.

**Phosphodiester bond:** A bond in which a phosphate group joins adjacent carbons through ester linkages. A condensation reaction between adjacent nucleotides results in a phosphodiester bond between 3' and 5' carbons in DNA and RNA.
**Plasmid (p):** A circular DNA molecule, capable of autonomous replication, which typically carries one or more genes encoding antibiotic resistance proteins. Plasmids can transfer genes between bacteria and are important tools of transformation for genetic engineers.

**Polycyclic:** Of a disease or pathogen: Producing many generations of inoculum and many cycles of infection during a single growing season.

**Polyetic:** Of plant disease epidemics: Continuing from one growing season to the next.

**Polygenic:** A character controlled by many genes.

**Polymorphism:** Difference in DNA sequence among individuals. Genetic variations occurring in more than 1% of a population would be considered useful polymorphisms for genetic linkage analysis. Compare mutation.

**Polyacrylamide gel electrophoresis:** Electrophoresis through a matrix composed of a synthetic polymer, used to separate proteins, small DNA, or RNA molecules of up to 1000 nucleotides. Used in DNA sequencing.

**Polyclonal antibodies:** A mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different epitope.

**Polymerase (DNA):** Synthesizes a double-stranded DNA molecule using a primer and DNA as a template.

**Polymerase chain reaction (PCR):** A procedure that enzymatically amplifies a DNA polymerase.

**Polypeptide (protein):** A polymer composed of multiple amino acid units linked by peptide bonds.

**Primer:** A short DNA or RNA fragment annealed to single-stranded DNA, to initiate synthesis of DNA by a DNA Polymerase or reverse transcriptase which extends a new DNA strand to produce a duplex molecule.

**Probe:** (1) A sequence of DNA or RNA, labeled or marked with a radioactive isotope, used to detect the presence of complementary nucleotide sequences. (2) A single-stranded DNA that has been radioactively labeled and is used to identify complementary sequences in genes or DNA fragments of interest.

**Propagative virus:** A circulative virus that replicates in its insect vector. Such a virus is said to be propagatively transmitted (e.g., potato yellow dwarf virus).

**Protein:** A polymer of amino acids linked via peptide bonds and which may be composed of two or more polypeptide chains.

**Purine:** A nitrogen-containing, single-ring, basic compound that occurs in nucleic acids. The purines in DNA and RNA are adenine and guanine.

**Pyrimidine:** A nitrogen-containing, double-ring, basic compound that occurs in nucleic acids. The pyrimidines in DNA are cytosine and thymine; in RNA, cytosine and uracil.

**Race:** A subspecies group of pathogens that infect a given set of plant varieties.

**Recessive:** Moving back and out of view. In genetics, a recessive gene is a gene that does not express its instructions when paired with a dominant gene.

**Recombinant:** A cell that results from recombination of genes.

**Recombinant DNA:** The process of cutting and recombining DNA fragments from different sources as a means to isolate genes or to alter their structure and function.

**Recombinant DNA technology:** A broad term referring to molecular cloning as well as techniques for making recombinant DNA or using it for specific purposes.

**Renature:** The reannealing (hydrogen bonding) of single-stranded DNA and/or RNA to form a duplex molecule.

**Resistance:** The ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor.

**Resistant:** Possessing resistance.

**Response:** The change produced in an organism by a stimulus.

**Reverse transcriptase (RNA-dependent DNA polymerase):** An enzyme isolated from retrovirus-infected cells that synthesizes a complementary (c)DNA strand from an RNA template.

**Ringspot:** A type of local lesion consisting of single or concentric rings of discoloration or necrosis, the regions between the concentric rings being green. The center of the lesion may be chlorotic or necrotic.

**RNA (ribonucleic acid):** An organic acid composed of repeating nucleotide units of adenine, guanine, cytosine, and uracil, whose ribose components are linked by phosphodiester bonds.

**RNA polymerase:** Transcribes RNA from a DNA template.
Rouging: The removal of diseased plants from a crop in order to prevent the spread of the disease.

Rosette: An abnormal condition in which the leaves form a radial cluster on the stem.

Rugose: Wrinkled.

Satellite RNA (viroids): A small, self-splicing RNA molecule that accompanies several plant viruses, including tobacco ringspot virus.

Satellite virus: A defective virus requiring a helper virus to provide functions necessary for replication. It may code for its own coat protein or various other products.

Secondary infection: Any infection caused by inoculum produced as a result of a primary or a subsequent infection; an infection caused by secondary inoculum.

Secondary inoculum: Inoculum produced by infections that took place during the same growing season.

Secondary organism: An organism that multiplies in already diseased tissue but is not the primary pathogen.

Secondary symptom: A symptom of virus infection appearing after the first (primary) symptoms.

Self-pollination: Pollen of one plant is transferred to the female part of the same plant or another plant with the same genetic makeup.

Selection: Natural selection is the differential contribution of offspring to the next generation by various genetic types belonging to the same populations. Artificial selection is the intentional manipulation by man of the fitness of individuals in a population to produce a desired evolutionary response.

Selective breeding: The selection of certain seeds or animals for reproduction in order to influence the traits inherited by the next generation.

Serology: Branch of science dealing with properties and reactions of sera, particularly the use of antibodies in the sera to examine the properties of antigens.

Serotype: A subdivision of virus strains distinguished by protein or a protein component that determines its antigenic specificity.

Southern hybridization (Southern blotting): A procedure in which DNA restriction fragments are transferred from an agarose gel to a nitrocellulose filter, where the denatured DNA is then hybridized to a radioactive probe (blotting).

Species: A classification of related organisms that can freely interbreed.

Spot: A symptom of disease characterized by a limited necrotic area, as on leaves, flowers, and stems.

Stem-pitting: A symptom of some viral diseases characterized by depressions on the stem of the plant.

Stringency: Reaction conditions—notably temperature, salt, and pH—that dictate the annealing of single-stranded DNA/DNA, DNA/RNA, and RNA/RNA hybrids. At high stringency, duplexes form only between strands with perfect one-to-one complementarity; lower stringency allows annealing between strands with some degree of mismatch between bases.

Substrate: A substance acted upon by an enzyme.

Supernatant: The soluble liquid action of a sample after centrifugation or precipitation of insoluble solids.

Suppression: A hypoplastic symptom characterized by the failure of plant organs or substances to develop

Susceptible: Vulnerable or predisposed to a disease (Lacking the inherent ability to resist disease or attack by a given pathogen; not immune).

Susceptibility: The inability of a plant to resist the effect of a pathogen or other damaging factor.

Symptoms: Any perceivable, subjective change in the organism or its functions that indicates disease or phases of disease.

Symptomatology: The study of symptoms of disease and signs of pathogens for the purpose of diagnosis.

Symptomless carrier: A plant that, although infected with a virus, produces no obvious symptoms.

Systemic: Spreading internally throughout the plant body.

Systemic infection: An infection resulting from the spread of virus from the site of infection to all or most cells of an organism.

Taq polymerase: A heat-stable DNA polymerase isolated from the bacterium Thermus aquaticus, used in PCR.

Taxonomy: Classification based on natural relationships.

Taxon: The named classification unit to which individuals, or sets of species, are assigned.
Higher taxa are those above the species level.

**Template:** An RNA or single-stranded DNA molecule upon which a complementary nucleotide strand is synthesized.

**Tolerance:** The ability of a plant to sustain the effects of a disease without dying or suffering serious injury or crop loss.

**Transcapsidation:** The partial or full coating of the nucleic acid of one virus with a coat protein of a differing virus.

**Transmission:** The transfer of a pathogen from one plant to another, or from one plant organ to another.

**Transcription:** The process of creating a complementary RNA copy of DNA.

**Transgenic:** An organism in which a foreign DNA gene (a transgene) is incorporated into its genome early in development. The transgene is present in both somatic and germ cells, is expressed in one or more tissues, and is inherited by offspring in a Mendelian fashion.

**Transgenic organism:** An organism formed by the insertion of foreign genetic material into a germ cell.

**Transgenic plant:** Genetically engineered plant or offspring of genetically engineered plants. The transgenic plant usually contains material from at least one unrelated organisms, such as from a virus, animal, or other plant.

**Translation:** The process of converting the genetic information of an mRNA on ribosomes into a polypeptide. Transfer RNA molecules carry the appropriate amino acids to the ribosome, where they are joined by peptide bonds.

**Upstream:** The region extending in a 5’ direction from a gene.

**Variation:** Differences in the frequency of genes and traits among individual organisms within a population.

**Variety:** An infrasubspecific rank which has no official standing in nomenclature.

**Vector:** 1. A living agent that transmits a pathogen from an infected plant to an uninfected one. 2. An autonomously replicating DNA molecule into which foreign DNA fragments are inserted and then propagated in a host cell. 3. Also living carriers of genetic material (such as pollen) from plant to plant, such as insects.

**Vein banding:** A symptom of virus-infected leaves in which tissues along the veins are darker green than other laminar tissue.

**Vein clearing:** A symptom of virus-infected leaves in which veinal tissue is lighter green than that of healthy plants.

**Viroid:** A plant pathogen that consists of a naked RNA molecule of approximately 250-350 nucleotides, whose extensive base pairing results in a nearly correct double helix.

**Virion:** The infectious unit of a virus.

**Virology:** The study of viruses and viral disease.

**Virulence:** The degree of ability of an organism to cause disease.

**Viruliferous:** Used to describe a vector containing a virus and capable of transmitting it.

**Virus:** An infectious particle composed of a protein capsule and a nucleic acid core, which is dependent on a host organism for replication. A double-stranded DNA copy of an RNA virus genome that is integrated into the host chromosome during lysogenic infection.

**Weed:** An undesirable plant.

**Wild relative:** Plant species that are taxonomically related to crop species and serve as potential sources for genes in breeding of new varieties of those crops.

**Wild species:** Organisms captive or living in the wild that have not been subject to breeding to alter them from their native state.

**Wilt:** A disease (or symptom) characterized by a loss of turgidity in a plant (e.g., vascular wilt).

**Witches' broom:** An abnormal form of plant growth, most common in woody plants, in which there is a profuse outgrowth of lateral buds to give a "witches' broom" appearance. The shoots may be thickened and may bear abnormal leaves.

**Wild type:** An organism as found in nature; the organism before it is genetically engineered.

**Yellowing:** A symptom characterized by the turning yellow of plant tissues that were once green.

**Yellows:** Any of a wide variety of plant diseases in which a major symptom is a uniform or non-uniform yellowing of leaves and/or other plant components. Yellows may be caused by fungi (e.g., celery yellows), viruses (e.g., sugar beet yellows virus), bacteria, protozoa (e.g., hartrot), spiroplasmas or phytoplasmas (e.g., coconut lethal yellowing).