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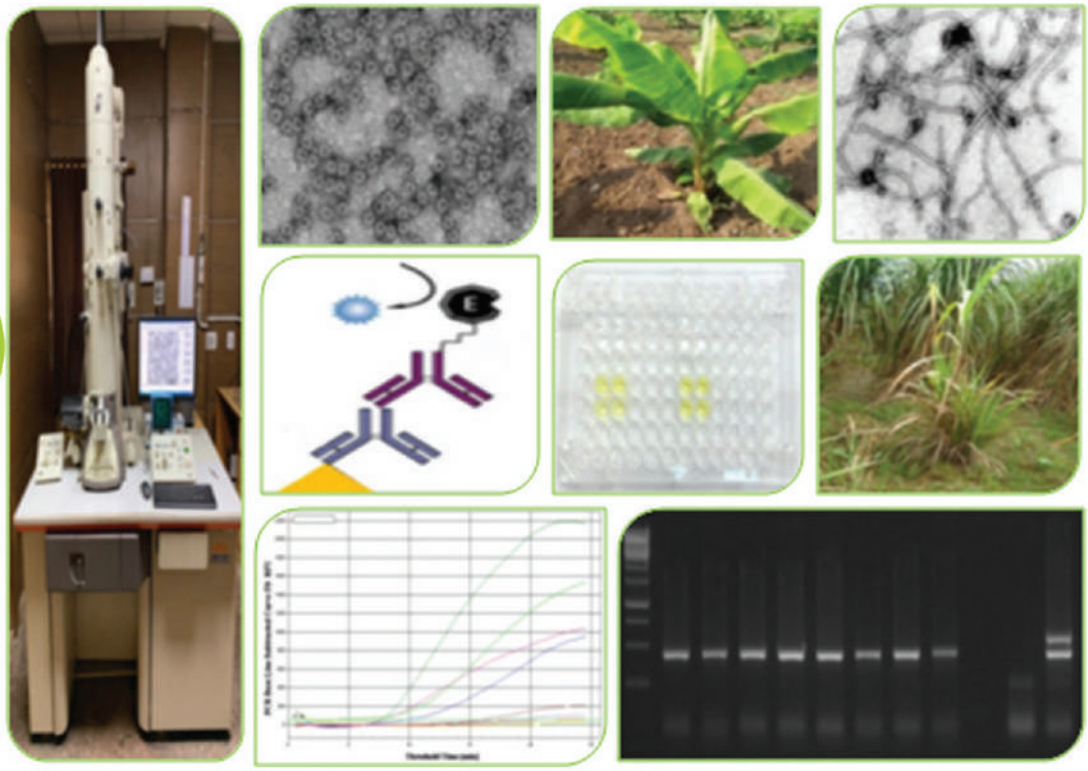
World Bank – ICAR funded
National Agricultural Higher Education Project
Centre for Advanced Agricultural Science and Technology (CAAST)
on "Genomics Assisted Crop Improvement and Management"

"Shaping human resources to harness the global gene pool for sustainable agriculture"

Practical Manual

"Genome Assisted Diagnosis of Plant Viruses, Viroids and Phytoplasmas"

October 15 - 24, 2019



Advanced Centre for Plant Virology
Division of Plant Pathology
ICAR-Indian Agricultural Research Institute, Pusa, New Delhi – 110012
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NAHEP sponsored

Short Term Training Programme

on

Genome assisted diagnosis of plant viruses, viroids and phytoplasmas

October 15-24, 2019

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About NAHEP-CAAST at IARI, New Delhi

Centre for Advanced Agricultural Science and Technology (CAAST) is a new initiative and student centric subcomponent of World Bank sponsored **National Agricultural Higher Education Project (NAHEP)** granted to The Indian Council of Agricultural Research, New Delhi to provide a platform for strengthening educational and research activities of post graduate and doctoral students. The ICAR-Indian Agricultural Research Institute, New Delhi was selected by the NAHEP-CAAST programme. NAHEP sanctioned Rs 19.99 crores for the project on "**Genomic assisted crop improvement and management**" under CAAST programme. The project at IARI specifically aims at inculcating genomics education and skills among the students and enhancing the expertise of the faculty of IARI in the area of genomics.

Objectives:

1. **To develop online teaching facility and online courses for enhancing the teaching and learning efficiency, and scientific communications skills**
2. **To develop and/or strengthen state-of-the art next-generation genomics and phenomics facilities for producing quality PG and Ph.D. students**
3. **To develop collaborative research programmes with institutes of international repute and industries in the area of genomics and phenomics**
4. **To enhance the skills of faculty and PG students of IARI and NARES**
5. **To generate and analyze big data in genomics and phenomics of crops, microbes and pests for genomics augmentation of crop improvement and management**

IARI's CAAST project is unique as it aimed at providing funding and training support to the M.Sc. and Ph.D. students from different disciplines who are working in the area of genomics. It will organize lectures and training programmes, and send IARI students and covering students from several disciplines. It will provide opportunities to the students and faculty to gain international exposure. Further, the project envisages developing a modern lab named as **Discovery Centre** that will serve as a common facility for students' research at IARI.

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PREFACE

Plant viruses, viroids and phytoplasma are causing huge economic losses in agriculture. It has been estimated that per year plant viruses can cause as much as 56 billion dollar loss worldwide. This situation may be worsened by recent climate change events and the associated changes in disease epidemiology. Reliable and early detection methods are still one of the main and most effective actions to develop control strategies for plant viral diseases. During the last two decades, considerable progress has been made to develop tools with high specificity and low detection limits for use in the detection of these plant pathogens.

Nowadays the genomic sequences of most of the plant viruses, viroids and phytoplasma are available which have provided foundation for designing molecular tools for diagnosis and control of these pathogens. For most of plant viruses coat protein gene sequences have been used for *in-vitro* expression and expressed proteins have been used for production of antibodies and standardization of ELISA. Novel approaches have been developed for genome amplification for linear and circular viruses for their characterization and detection. 16S rRNA and other housekeeping genes have been used for detection and characterization of phytoplasma.

The main objective of this training programme is to educate young students and research scholars on advances made in the genome assisted detection of viruses, viroids and phytoplasma using advanced molecular tools. The training programme will focus on hands on training in various diagnostic methods in the laboratory as well as lectures by eminent experts on current topics so as to enable the participants to apply the same in their research areas particularly plant disease diagnostics.

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R. P. Pant, Ph.D.
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Date: 30.09.2019

भा.कृ.अनु.प.
ICAR

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अधिष्ठाता एवं संयुक्त निदेशक (शिक्षा) – कार्यकारी
Dr. (Mrs.) Rashmi Aggarwal
Dean and Jt. Director (Education) - Acting

Foreword

The ICAR-Indian Agricultural Research Institute, New Delhi has made significant contributions in developing crop protection and production technologies for all major crops in India. The institute has core strength in the area of genomics and modern research facilities for conducting advanced genomics programmes. ICAR - IARI has major focus on research and academic programme on diagnosis of plant viruses, viroids and phytoplasma based on knowledge of genomics of the pathogens.

Plant diseases are important production constraints in global agriculture and India is no exception. In recent years, a number of new diseases have emerged which are major threats in several parts of the country. This has drawn attention of all the stake holders especially the developmental agencies, growers, policy makers and scientists. The modern molecular biological tools have accelerated the research on genome characterization of pathogens or pathogenomics and application of genomic sequences in disease management strategies. The genomics tools have added in the development of specific diagnostics of pathogens particularly viruses, viroids and phytoplasma in a big way. With this background, the Centre for Advanced Agricultural Science and Technology (CAAST) under NAHEP is organizing a Training programme on **"Genome assisted diagnosis of plant viruses, viroids and phytoplasmas"** in the Division of Plant Pathology, ICAR – IARI for the benefits of M.Sc. and Ph. D. students of SAUs and other central universities. I am sure that this training on modern diagnostics of viruses, viroids and phytoplasma will be highly useful to the Post Graduate and Ph.D. students of Plant Pathology.

(Rashmi Aggarwal)

Dated: 10th October, 2019

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The Head
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Sub: Allotment of Institute Continuous Number (ICN)

Sir,

With reference to your letter No. **nil** dated 03/10/2019 on the subject mentioned above, we are pleased to allot the following Institute Continuous Number (ICN) to the publication entitled **"Genome Assisted Diagnosis of Plant Viruses, Viroids and Phytoplasmas"** being brought out by you:

TB-ICN: 226/2019

This number may kindly be printed in bold on the right hand top corner of the cover page as well as on the back of the inner title page (i.e., copyright page) of the publication. The approved uniform size for the technical bulletin is: Demy Octavo (5.6"×8.75").

As per the policy guidelines approved by the Director, the manuscript of the publication should be got screened by a Technical Committee or a competent referee before publication, and 5 (five) copies of the publication should be sent to us as well as to the Head, Library Services, IARI for information and record.

Yours faithfully,

(G.P. Rao) 11.10.19
Incharge, Publication Unit

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CONTENTS

LECTURES

Introduction

Genome organization and use of genomic sequences for their nucleic acid based detection

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Viruses are small obligate intracellular parasites, comprising of a nucleic acid core surrounded by a protective protein coat. They are unique pathogens with the ability to infect all types of life forms from animals and plants to microorganisms like bacteria. Over 3700 viruses and viroids have been recognized and approximately a third of these are plant viruses (ICTV 2015). Plant viruses are major constraint in Indian agriculture as they damage crops through numerous diseases, resulting in reduced plant vigor, huge yield losses, unmarketable crops or even plant death. The majority of plant viruses are composed of single-stranded RNA (ssRNA, about 75% of plant viruses) with the virus genome in the sense orientation (i.e. member families *Bromoviridae*, *Closteroviridae*, *Luteoviridae* and *Potyviridae*). Other plant viruses possess single-stranded RNA genomes in an ambisense or antisense orientation (i.e. members of families *Bunyaviridae* and *Rhabdoviridae*), double-stranded (ds) RNA genomes (i.e. members of families *Reoviridae*, *Endoviridae*), ssDNA genomes (family *Geminiviridae*), or dsDNA genomes (family *Caulimoviridae*). Plant virus genomes are small (~4-20 kb). They make very efficient use of the limited amount of genomic nucleic acid they possess and code for only a few genes in a very compact manner. Viral genome may be circular (as in all known plant DNA viruses) or linear. The entire genome may occupy either one nucleic acid molecule (monopartite genome, in the genera *Potyvirus* and *Tobamovirus*) or several nucleic acid segments (multipartite genome, 11 in some members of the genus *Nanovirus*).

Compared to the other plant pathogens like fungus and bacteria, which are studied since long, the study on viruses are relatively new and much more difficult to manage. Traditionally, plant virus studies in India were based on transmission, host reactions, particles morphology and serology. Studies on plant viral genome sequence redefined plant virus identification to a finest level. A large number of viral sequences are available in databases (National Centre for Biotechnology Information (NCBI); European Molecular Biology Lab (EMBL), DNA Databank of Japan (DDBJ) etc.) which laid foundation for designing modern molecular diagnostic tools for control of plant viruses. Some of the nucleic acid based methods used for diagnosis and identification of disease causing plant viruses have been summarized here.

Polymerase Chain reaction (PCR) and its variants for amplification of viral genomes

PCR is the most commonly used technique to make billions of copies of specific DNA segment or complete genome of virus under study using a primer pair and a temperature-

mediated DNA polymerase in a thermal cycler. Primer pair specific to a particular DNA segment can be designed using sequences of same or related virus species available in GenBank nucleotide database of National Centre for Biotechnology Information (NCBI). PCR assay can be used to amplify or make copies of the whole genome either as a single long fragment or as copies of multiple small overlapping fragments using primers specific to overlapping segments which are joined together to get the full genome sequence. PCR assay has been developed into several techniques using a number of modifications to the basic procedure e.g. reverse transcriptase PCR (RT-PCR), nested PCR, multiplex PCR, real time or quantitave PCR (qPCR).

Reverse transcriptase PCR (RT-PCR) assay

Viruses containing RNA genome cannot be amplified using standard PCR assay. Reverse transcriptase is an enzyme which reverse transcribes the RNA template into complementary DNA (cDNA). In RT-PCR assay, the first step is to reverse transcribe the RNA template into cDNA using reverse transcriptase enzyme followed by a standard PCR assay for final amplification of cDNA template. For RNA viruses, reverse transcription of viral RNA into complementary DNA (cDNA) is a mandatory step prior to PCR. RT-PCR assay can also be utilized to measures RNA expression level of a particular gene by combining with the real-time PCR (qPCR). Primers designed to conserved regions or genes found in the viral genome hybridize with multiple members of a particular viral family. Oligo $(dT)_{18}$ primers can be used to produce cDNA from entire viral genome for those viruses that have a poly A tail.

Multiplex PCR assay

Multiplex PCR is an advanced version of standard PCR assay in which two or more DNA or RNA segments of varying length are targeted and amplified in a single reaction using a set of multiple primers and a temperature-mediated DNA polymerase in a thermal cycler. The primer pairs specific for different segments of DNA are designed in such a manner that all different primer pairs have nearly same annealing temperature but different amplicon size, so that they could be easily visualized and differentiated through gel electrophoresis. The target DNA segment may be present in genome of a single virus/organism or may be present on two or more genomes of different viruses. Thus, multiplex PCR is an important tool for confirming presence of mixed infection of viruses in a sample. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Multiplex PCR is very useful for simultaneous detection of different genes in a single reaction as individual detection of different segments of DNA by standard PCR assay is not only expensive but also time consuming.

Real-time or quantitative PCR (qPCR) assay

Real-time PCR, also known as quantitative PCR (qPCR) is an improved version of conventional PCR assay in which a real-time thermal cycler monitors the amplification of a targeted DNA fragment during the PCR assay (i.e. in real time), not at the end of the assay as in conventional assay.

Nested PCR assay

It is a modification of conventional PCR technique intended to amplify specific DNA segment by utilizing two pair of primers along with DNA polymerase enzyme thus avoiding amplification of unexpected non-specific PCR amplicons. Non-specific amplification is a very common problem faced with standard PCR assays due to non-specific binding of primers on the template DNA. Nested PCR assay is also very useful for the samples in which template DNA is available in less amount which cannot be amplified by conventional PCR assay in sufficient amount that can be visualized through agarose gel electrophoresis. Nested PCR involves the use of two primer sets and two successive PCR reactions. In this technique, a primer pair for a specific region of DNA is designed as it is designed for conventional PCR assay. This round of PCR is known as the first round PCR assay. For nested round PCR assay. Amplicons resulting from the first PCR reaction are used as template for a second set of primers and a second amplification step thus increasing sensitivity and specificity of amplicons produced of specific DNA segment. Amplicons from nested PCR assays are detected in the same manner as in PCR assay.

Isothermal amplification

Isothermal amplification techniques are a group of techniques which are carried out at a single specific temperature (mostly at room temperature) and do not require cycling of reaction cocktail between different temperatures as it is done in conventional PCR assays. Isothermal amplification techniques are sequence-specific and do not require costly thermal cycler machines. However, some common advantages are that isothermal techniques are extremely fast, specific and cost-effective in terms of working-hours involved to carry out the reaction. Various isothermal amplification assays have been developed e.g. rolling circle amplification (RCA), recombinase polymerase amplification (RPA), loop-mediated thermal amplification (LAMP), etc.

Loop-mediated thermal amplification (LAMP)

The LAMP reaction is a variant of gene amplification reactions which is carried out at constant temperature (60-65°C) using either two or three sets of primers in the presence of a polymerase enzyme with single strand displacement activity in addition to replication activity. The reaction is usually completed within one hour with sensitivity similar to that of

standard PCR. It is a very sensitive, easy and time efficient method of gene amplification. Four different primers (two specially designed inner and two outer primers) identify six different regions on the target gene, which makes LAMP reaction highly specific. An additional pair of primer "loop primers" can further accelerate the reaction. Initially, all four primers are used in the LAMP reaction, but later in the reaction only the inner primers are utilized for strand displacement DNA synthesis (Notomi *et al.*, 2000; 2014). Final products of a LAMP reaction can be analyzed using simple real-time detection and visual inspection methods such as turbidity (Mori *et al.*, 2001, 2004) or fluorescence detection methods (Tomita *et al.*, 2008) because of the large output and high specificity of the amplification products. The turbidity detection method uses the turbidity of magnesium pyrophosphate, a byproduct of DNA synthesis, as an indicator whereas the fluorescence detection method utilizes fluorescent chelation reagents. Additionally, detection using normal DNA probes is also possible. When the final LAMP reaction product is run through agarose gel, multiple bands of variable lengths are observed under UV light indicating successful amplification of specific DNA segment.

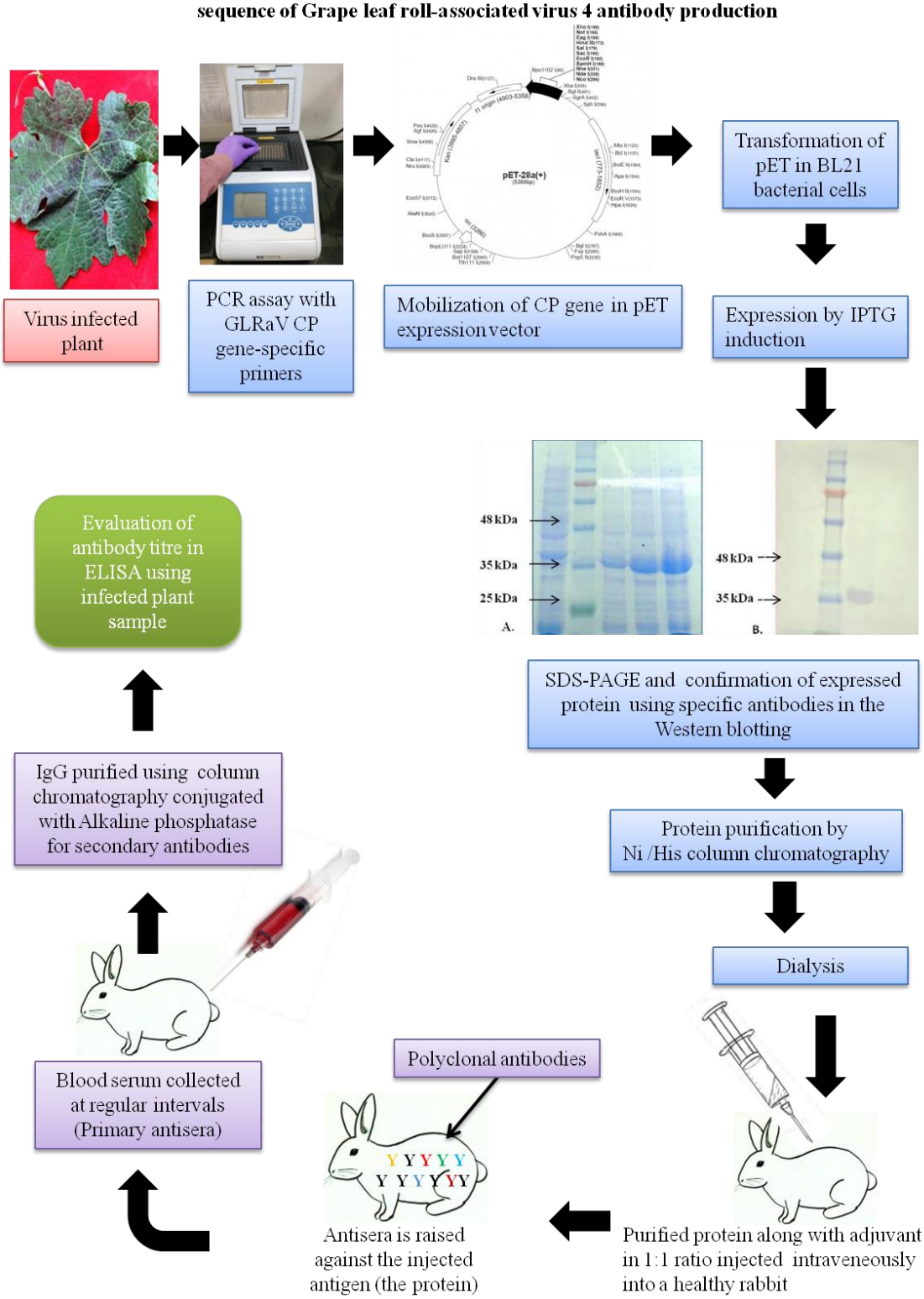
Rolling circle amplification (RCA) assay

Rolling circle amplification is particularly used for the amplification of viruses with circular DNA genomes using exo-resistant random hexamer primers or species specific primers and utilizing the strand displacement activity of Phi29 DNA polymerase. RCA is a sequence independent amplification, carried out overnight at isothermal temperature 30˚C. As random hexamer primers are employed in RCA, the prior sequence information of the targeted viral genomes is not required. Thus, it has the potential to amplify novel circular viral genomes of members of *geminiviridae*, *caulimoviridae* and *nanoviridae.* The RCA product after restriction digestion needs to be sequenced for confirmation of viral origin. Random primed RCA was employed to identify the shorter Banana streak OL virus variants causing the leaf streak disease of banana in India (Baranwal *et al.*, 2014). Although, RCA is less suitable for larger genomes as the amplification efficiency decreases with the length of the circular DNA template. The probability of strand breaks increases with the length of the DNA molecule, resulting in termination of the RCA.

Recombinase polymerase amplification (RPA) assay

Recombinase polymerase amplification (RPA) is an isothermal amplification assay which is rapid, highly specific and sensitive. It is an alternative technique to the PCR assays which requires a costly thermal cycler. In RPA, the isothermal amplification of specific DNA fragments is achieved by the combination of enzymes and proteins, *viz.*, the recombinase, single-stranded binding proteins (SSB) and strand displacing polymerase, used at room temperature (i.e. 37-40^{o}C).

Polyclonal antibodies raised against expressed coat protein gene sequence of Grape leaf roll-associated virus 4 antibody production



For detailed procedure please refer Rai *et al.*, 2018

This technique can produce sufficient quantity of amplicons in 10-15 minute which a normal polymerase chain reaction (PCR) produces in 1.5 to 2 hour reaction time and the final product could be easily visualized on an agarose gel. RPA employs two sets of primers which are combined with the recombinase that targets the dsDNA and facilitates strand displacement. The displaced single stranded DNA strand is stabilized by SSB proteins and the polymerase initiates synthesis. RPA products can be visualized on gel after purification, although alternate methods, such as fluorescence and/or hybridization. Crude sap or purified DNA samples may be utilized as template in RPA assay. RPA can be modified into reverse transcription recombinase polymerase amplification (RT-RPA) assay by adding reverse transcriptase enzyme in the reaction mixture to amplify a RNA template in a single set of reaction.

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Genome assisted virus classification and diagnostics

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Viruses are simple and unique pathogens that are difficult to manage. History of plant viruses is more than hundred years old. Presently, there is greater emphasis on genomics which includes sequencing of viral genome, gene function, transgenic resistance, genome editing etc (molecular era from 1990 onward).

More than 1500 plant virus species affecting field and horticultural crops have been documented globally (refer ICTV 2018 Master Species List MSL 34), while the Indian plant virus database developed in 2015 has listed 168 plant virus species. Until 1995, plant virus classification was either host or disease-oriented. Subsequently, a unified virus classification system based on the intrinsic properties of the virus was followed by all virus workers. Based on the nature of the genome, plant viruses have been classified in to viruses with (i) ssDNA genome (586); (ii) retroid viruses (67); (iii) dsRNA genome (93); (iv) negative sense RNA genome (64) and (v) positive sense RNA genome (752). Further ICTV (2019) has approved the creation of a higher Taxonrealm *Riboviria*, which includes viruses with positive sense strand, negative sense strand and double strand genome RNA that use RdRp for replication. Also number of Ranks (approved by ICTV 2019) in virus classification has increased to 11 i.e., Realm (1), Phylum (1), Subphylum (2), Class (6), Order (14), Suborder (7), Family (143), Subfamily (64), Genus (846), Subgenus (59) and Species (4958).

Virus diagnostics has gained importance during the last 3-4 decades due to increased travel, traffic and trade. This has led to the development of immuno- and nucleo-based diagnostics ranging from ELISA to PCR, real time PCR, multiplex PCR, LAMP PCR, macro/micro-arrays and deep or next generation sequencing (NSG). More recently NSG coupled with high performance computing (HPC) has resulted in the identification of newer viruses with the help of a public web interface such as VirFind (http://virfind.org/j) and VirusDetect (http://virusdetect.feilab.net).

Importance of genome organization and nucleic acid sequencing for detection of plant viruses

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Introduction

Detection of the pathogens is centered to systemic and epidemiological study of plant pathogens and development of disease management strategies. Diagnosis of a viral infection in the plant means to determine the infection caused by known or unknown virus and virus like pathogens (Bar-Joseph and Dawson, 2008; Fauquet *et al.*, 2005; Biswas *et al.*, 2004; Biswas and Varma, 2000; van Regenmortel *et al.*, 2000). Thus the detection techniques must be simple and rapid so that several plant samples can be tested at a time (Agrios, 2005). The diagnosis for plant virus is needed for the following basic purposes; the identification of viruses in known and in new hosts, the characterization of newly detected viruses, the identification of virus resistance in new breeding lines (Biswas, 2008, Bhattacharyya *et al.*, 2917). Further it is needed for selection and certification of virus and virus-like disease free planting material, reduction of spread of the diseases, and determination of the epidemiology of disease (Biswas *et al.*, 2009; Ahlawat and Pant, 2003).

Crop production losses attributed to plant viruses can be enormous especially when high value crops are at stake. Earlier, the diagnostic assays were based on symptoms expression, light and electron microscopy and isolation of viruses (Ahlawat and Pant, 2003). But serological tests using polyclonal antibodies have proved to be invaluable and retain their place as essential tools in pathogen identification Biswas, 2008). Recently, developments in biotechnology and molecular biology have widened the choice for techniques for disease diagnosis. This biotecgnological approach allows a higher level of sensitivity and discrimination in comparison with the classical techniques used earlier (Fauquet *et al.*, 2005; Biswas *et al.*, 2012). The nucleic acid based methods do not depend on the metabolic state of the pathogen or the state of gene expression as both coding and non coding regions of the genome can be targeted (Hull, 2014). Several numbers of biological, physical and molecular techniques have been developed. These techniques individually or in combination with them provided easy and authentic base for the detection and diagnosis of plan viruses. Importantly, the positive diagnosis should always involve the application of two or more of the available techniques simultaneously. The recent developments of virus taxonomy, an arrangement of the viruses into families and genera, and refinement of sensitive and rapid diagnostic techniques have simplified the viral diagnoses (Bar-Joseph and Dawson, 2008; Biswas *et al.*, 2012). The techniques involved in detection and diagnosis of plan viruses can be grouped

into different types based on (i) Biological assays, (b) Protein assays and (c) Nucleic acid assays.

Genome structure and organization

Plant viruses are classified into families/genera on the basis of virion morphology and genomic structure and genome organization (Fauquet *et al.*, 2005; Biswas *et al.*, 2012a, b) Viral genomes contain the information for the replication and expression of genes necessary for the functioning of the virus at the right time in the right place. All most plant viruses and some bacterial and animal viruses, genome is rather small with a few thousands. The genome of viruses consists of coding and non coding regions. The coding regions express the proteins required for the viral infection cycle and non coding regions control the expression and replication of the genome (Biswas *et al.*, 2019). A viral genome is composed of various cassettes. The "basic" cassette comprises the gene or genes and the nucleic acid sequences that replicate and express the genome. This is made up of the replicase enzymes, the sequences that initiate and/ or terminate the formation of a new viral genome and sequences and/or proteins that control expression. As a virus is totally dependent on its host for its multiplication, it can be considered to be advantageous for it to control its replication and to maintain its natural host in a living state for as long as possible (Agrios, 2005).

General properties of plant viral genomes

General properties of plant viral genomes includes kind of nucleic acid, number of genome pieces, open reading frame (ORFs), secondary structure, 5' and 3' end terminal and non coding structure,, nucleotide sequence, amino acid sequence and regulatory signals. Viral genomes are ssRNA, dsRNA, ssDNA, dsDNA, linear or circular (Hull, 2014). Genomes of almost all the plant viruses with RNA genomes and some bacterial and animal viruses are rather small (a few thousand nucleotides). Viruses with DNA genomes (e.g. lambda= 48,502 bp) often a circular genome. All ssRNA viruses produce dsRNA molecules and many linear DNA molecules become circular. Molecular weight per base pair is of about 660 and contour length (duplex length) per nucleotide is of 3.4 Å (van Regenmortel *et al.*, 2000)

The nucleic acid comprising viral genome may be either DNA or RNA; which single-stranded (ss) or double-stranded (ds) and and in a linear, circular or segmented form.RNA viral genomes may be of positive-sense of the same polarity as messenger RNA (mRNA) or negative sense of opposite polarity to mRNA or ambisense (*Sonchus yellows net virus*, a member of the family *Rhabdoviridae*), where both positive- and negative-sense regions (*Tomato spotted wilt virus*, a member of the family *Bunyaviridae*). are present on a single genome strand. The total genome size of plant viruses ranges from just over 1 kb for satellite viruses, which require helper virus for replication, and Nanoviruses (e.g., *Banana bunchy top virus*) to 28.9 kb for members of family *Reoviridae* (Sugarcane Fiji disease virus). Nearly

half of them are elongate (rigid rods or flexuous threads), and almost as many are spherical (isometric or polyhedral), with the remaining being cylindrical bacillus like rods.

The vast majority, about 90% of the known plant viruses possess a single-stranded, linear, messenger sense RNA genome. Monopartite viruses carry genomic information in a single virus particle. Multipartite virus is a a virus with a multipartite genome which is divided between two or more particles. Bipartite, tripartite and quadripartite genome viruses this information is divided among two, three and four genomic molecules, respectively. With the bipartite, tripartite and quad-partite genome viruses, the different pieces of the genome in many cases are encapsidated separately. The members of the genus *Comovirus* under family *Comovirdae* have bipartite genome with each segment encapsidated into a discrete particle. In family *Tombusvirdae*, genus *Dianthovirus*, members have bipartite genome with both segments encapsidated into a single, isometric particle. The cucumovirus and bromovirus contain tripartite genome, whereas, the alphamovirus contains quad-partite genome. Double-stranded, segmented RNA genome, contain 10-12 pieces of genomic RNA of the reoviruses (Family *Reoviridae*, eg., *Wound tumor virus*) are encapsidated within a single virion. There are DNA viruses having double-stranded and circular DNA in the family *Caulimoviridae* (CaMV), having single-stranded circular DNA molecules in the family *Geminiviridae* (MSV), and having small single-stranded circular DNA molecules in the family *Nanoviridae* (BBTV) (Hull, 2014)

An ORF can be defined as a sequence coming between an AUG initiation codon and capable of expressing a protein of 10 KDa or more. The number of genes found in plant viruses range from 1 for the satellite virus, STNV, to 12 for some closteroviruses and some reoviruses. The anatomy of a gene is that it has ORF which starts from start codon (ATG) to stop codon (TGA, TAA, TAG). Upstream region will have binding site (e.g. TATA box) and 3'terminal end with Poly (A) tail. And it will have splices and bounded by AG and GT splice signals.

DNA Sequencing

Gene cloning is the molecular method of isolation and multiplication of an individual gene sequence by its insertion into a bacterial cell, which can multiply the gene as it multiplies itself. Gene cloning is a straightforward procedure. A fragment of DNA, containing the gene to be cloned, is inserted into a circular DNA molecule called a vector, to produce a chimera or recombinant DNA molecule (Sharma *et al.*, 2011). The vector acts as a vehicle that transports the gene into a host calli, which is usually a bacterium, although other types of living cell can be used. Within the host cell the vector multiplies, producing numerous identical copies not only of itself but also of the gene that it carries. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place. After a large number of cell divisions, a \colony or clone, of identical host cells produced. Each cell in the clone contains one of more copies of the

recombinant DNA molecule; the gene carried by the recombinant molecule is now said to be cloned.

PCR amplicons can be sequenced relatively inexpensively and rapidly in automated sequencers. Amplicons can be prepared for direct sequencing relatively easily using a commercial kit for purifying directly from the PCR reaction mixture or from agarose gel. Genetic databases available on the Internet such as GenBank allow rapid comparison of one's sample sequence to extensive and growing libraries of sequences. Cloning of an amplicon is an option, if sequence quality obtained by direct sequencing is inadequate. Routine sequencing is likely to play an increasingly important role in species identification. DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases-adenine, guanine, cytosine and thymine in a DNA strand.

The advent of rapid DNA sequencing methods has greatly accelerated the basic biological and medical research. The first DNA sequences were obtained in the early 1970s by academic researchers using laborious methods based on two-dimensional chromatography. Following the development of fluorescence-based sequencing methods with a DNA sequence, DNA sequencing has become easier and orders of magnitude faster. The first full DNA genome sequenced was the genome of bacteriophage, jX174 in 1977. RNA sequencing was one of the earliest forms of nucleotide sequencing. The major landmark of RNA sequencing is the sequence of the first complete gene and the complete genome of Bacteriophage MS2 in 1972 and 1976. The first method for determining DNA sequences involved a location-specific primer extension strategy established in 1970 by Ray Wu in Cornell University. Frederick Sanger then adopted this primer-extension strategy to develop more rapid DNA sequencing methods in Cambridge, UK and published a method for "DNA sequencing with chain-terminating inhibitors" in 1977. Walter Gilbert and Allan Maxam in Harvard also developed sequencing methods, including one for "DNA sequencing by chemical degradation". In 1973, Gilbert and Maxam reported the sequence of 24 base pairs using a method known as wandering-spot analysis. Advancements in sequencing were aided by the concurrent development of recombinant DNA technology, allowing DNA samples to be isolated from sources other than viruses. The knowledge of DNA sequences has become indispensable for numerous applied fields such as medical diagnosis, biotechnology, forensic biology, virology and biological systematic. DNA sequencing may be used to determine the sequence of individual genes, larger genetic regions (clusters of genes) full chromosomes or entire genomes. Sequencing provides the order of individual nucleotides present in molecules of DNA or RNA isolated from animals, plants, bacteria and virtually any other source of genetic information. This information is useful to various fields of biology and other sciences, medicine, forensics, and other areas of studies like molecular biology, evolutionary biology, metagenomics, medicine and for forensic identification and paternity testing.

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Use of genome sequences for detection of multiple virus infection by multiplex PCR

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Multiplex polymerase chain reaction (PCR) is a variant of PCR technique in which multiple (two or more) target sequences can be amplified by USING more than one pair of primers simultaneously in the same reaction in a single tube. This technique has the potential to offer considerable savings of time, costly input material and labour effort in the laboratory. multiplex-PCR was first described in 1988 (Chamberlain *et al.,* 1988) as a method to detect deletions in the human Duchenne muscular dystrophy (DMD) gene. Since then, this method has been successfully applied in many areas of DNA analyses, such as, gene deletion (Chamberlain *et al.,* 1988), mutation and polymorphism (Rithidech *et al.,* 1997; Shuber *et al.,* 1993), quantification (Zimmermann, 1996), and reverse-transcription (RT)-PCR (Crisan 1994). In the field of infectious diseases, multiplex PCR has been shown to be a valuable tool for identification of viruses (Heredia *et al.,* 1996; Casa *et al.,* 1999; Markoulatos *et al.,* 1999; 2000a; 2000b), bacteria (Hendolin) *et al.,* 1997), and parasites (Harris *et al.,* 1998).

Designing highly efficient primers is the most critical task in this method so as to achieve specific and sensitive detection of all the target templates present in the single reaction mixture simultaneously. the important primer design considerations described below are a key to specific and sensitive amplification with high yield.

Primer Design Parameters for Multiplex PCR

Primer length

In general, for PCR, the lenghts of oligos ranging 18–30 nucleotides are good. however, multiplex PCR assays involve designing of large number of primers, hence it is required that the designed primer should be of appropriate length. usually, primers of short length, in the range of 18-22 bases are used.

Melting temperature

Primers with similar Tm are most preferred (between 55°C-60°C). A Tm variation of between 3°-5°C is acceptable for primers used in a pool. Aim for the GC content to be between 40 and 60%.

GC content

To ensure the preferred GC content, try to find a sequence with more GC content, or extend the length of the primer a littleconsidering the acceptable range. Presence of C or G residues (1-3) at the 3' end of the primer promotes efficient initiation of primer binding to the template.

Specificity

It is important to consider the specificity of designed primers to the target sequences by selecting the unique sequences to each target template. Perform the primer blast and ensure that the primers have no off targets.

Avoid primer dimer/hairpin formation

Ensure that the designed primers are not having any tendency to form the dimers or hairpin loops. Following are the tips avoid this problem:

- Avoid regions of secondary structures, and consider the regions with a balanced distribution of GC-rich and AT-rich domains.
- Avoid the stretches of 4 or more of same single base, or dinucleotide repeats (for example, GTTTTTTT or GTGTGTGTGT).
- Avoid intra-primer homology (more than 3 bases that complement within the primer) or inter-primer homology (forward and reverse primers having complementary sequences).

There are many primer design tools available online and offline which help designing the good primers. Some of the popular free online tools are as follows:

Free Online Tools for Primer Designing

- **Oligo Calc:** Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html)
- **Primer3web** version 4.1.0 (http://bioinfo.ut.ee/primer3/)
- **GenScript Online PCR Primers Designs Tool** (https://www.genscript.com/tools/pcr-primers-designer)
- **PCR Primer Design Tool - Eurofins Genomics** (https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/)

Types of Multiplex PCR

Multiplexing reactions can be broadly divided in two categories based on the type or number of templates involved/targeted in a single reaction mix.

Single Template PCR Reaction

In this technique a single DNA template will be used for simultaneous amplification of multiple specific regions using several pairs of forward and reverse primers in a single tube.

Multiple Template PCR Reaction

It uses multiple templates possessing the specific regions will be targeted for amplification in a single reaction tube using several primer sets designed for specific regions.

Problems Associated with Multiplex PCR Reaction

Optimization of multiplex PCR is very essential to achieve successful amplification of multiple target DNA sequences simultaneously in a single reaction tube at a particular annealing temperature. This can pose several difficulties, including poor sensitivity and specificity, and/or preferential amplification of certain specific targets.

The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers. One of the most important concepts in PCR is that of the optimal primer-to-template ratio. If the ratio is too high, primer-dimers are formed, as also occurs in conditions of very dilute template or excess primer. Primers must usually be in a10^7 molar excess with respect to template. For most applications, regardless of template concentration, the primer concentration cannot be raised much higher than 0.5 µM because of primer-dimer formation. Therefore, what determines the primer-to-template ratio is the amount and complexity of the template provided to the reaction. If the primer-to-template ratio is too low, product will not accumulate exponentially, since newly synthesized target strands will renature after denaturation (which reduces the yield considerably), or inhibit the formation of PCR product. Thus, primer dimers may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension (Ruano *et al.*, 1989). Theoptimization of multiplex PCR should aim to minimize suchnonspecific interactions. Hence, a pecial attention to primer designparameters, such as homology of primers with their targetnucleic acid sequences, length, GC content, and concentration, have to be considered (Brownie *et al.*, 1997).

Ideally, all theprimer pairs in a multiplex PCR should enable similar amplificationefficiencies for their respective target. This may beachieved through the utilization of primers with nearly identicaloptimum annealing temperatures and should not display either intra- or inter primer pairhomology significantly (Markoulatos *et al.*, 1999; Henegariu *et al.*, 1997).

Preferential amplification of one target sequence over anotheris a known phenomenon in multiplex PCRs. Twomajor classes of processes that induce this bias have beenidentified: PCR drift and PCR selection (18). PCR drift is abias assumed to be due to fluctuation in the interactions ofPCR reagents, particularly in the early cycles, which couldarise in the presence of very low template concentration. PCR selection, on the other hand, is defined as a mechanismwhich favors the amplification of certain templates due to theproperties of the target, the target's flanking sequences, orthe entire target genome. These properties include inter-regiondifferences in GC content, leading to preferential accessibilityof targets within genomes due to secondary structures andthe gene copy number within a genome (Mutter and Boyton 1995; Wagner *et al.*, 1994).

The alteration of other PCR components, such as PCR buffer constituents, dNTPs, MgCl2, and enzyme concentrations in multiplex PCR greater than those reported for most uniplex PCRs usually results in considerable improvement in the sensitivity and/or specificity of the test (Markoulatos *et al.*, 1999). In contrast, increasing the concentration of these factors may increase the likelihood of mispriming also, with subsequent production of nonspecific amplification products. However, optimization of those components in multiplex PCRs is usually crucial. Prior to application in a laboratory setting, multiplex PCRs must be evaluated for their sensitivity and specificity as compared with their corresponding uniplex PCRs (Markoulatos *et al.*, 1999; 2000a; 2000b). Sensitivity indicates ability of the optimized PCR protocol (including recipe and the amplification conditions) to detect the lowest possible concentration of target template DNA to be amplified successfully by PCR to produce visible amplicon on the gel. Specificity indicates the ability of the optimized PCR protocol that can yield the amplification of specific target with the help of that target specific primers even when the reaction mixture contains several sets of primer & template combinations in a single tube without any off-target/non-specific amplifications.

Wherever possible, multiplex PCRs should avoid the use of nested primers requiring a second round of amplification. The latter is a major contribution to false-positive results dueto carryover contamination, although protocols against contamination, including PCR controls (reaction and specimens' extraction controls), must be implemented in all PCR protocols. Likewise, precautions and methodologies to avoid false-negative results due to reaction failure have to be considered.

Annealing temperature is one of the most important parameters. Although many individual loci could be specifically amplified at 56–60^{o}C, it is evident that lowering the annealing temperature by 4–6^{o}C was required for the same loci to be co-amplified in multiplex mixtures. When many specific loci are simultaneously amplified, the more efficiently amplified loci will negatively influence the yield of product from the less efficient loci. This

is due to the fact that PCR has a limited supply of enzyme and nucleotides, and all products compete for the same pool of supplies (Markoulatos *et al.*, 2002).

Optimization of Reaction Components

To overcome the problems discussed above, it is necessary to optimized the components of PCR reaction such as primer, dNTPs, $MgCl_2$, amount of template, DNA polymerase enzyme and some adjuvants.

Amount of Primer

Initially, equimolar primer concentrations of 0.1–0.5µM each are used in the multiplex PCR. When there is uneven amplification, with some of the products barely visible even after the reaction was optimized for the cycling conditions, changing the proportions of various primers in the reaction isrequired, with an increase in the amount of primers for the "weak" loci and a decrease in the amount for the "strong "loci. The final concentration of the primers (0.04–0.5 µM) may vary considerably among the loci and is established empirically (8). For low copy number or high-complexityDNA, the primer concentration should be 0.3–0.5 µM. For high copy number or low-complexity DNA, the primer concentration should be 0.04–0.4 µM (Markoulatos *et al.*, 2002).

dNTP and $MgCl_2$ Concentrations

dNTP

The $MgCl_2$ concentration is kept constant (2 mM), while the dNTP concentration is increased stepwise from 0.5–1.6 mM. The best results are between 200 and 400 µM each dNTP values, above which the amplification is rapidly inhibited. Lower dNTP concentration (100 □M each dNTP) leads to PCR amplification but with visibly lower amounts of products (Markoulatos *et al.*, 1999). The dNTP stocks are sensitive to thawing/freezing cycles. After three to five such cycles, multiplex PCRs often do not work well. To avoid such problems, it is recommended to have multiple aliquots of small quantities of dNTPs and kept frozen at –20^{o}C. This "low stability" of dNTP is not so obvious when single loci are amplified (Markoulatos *et al.*, 2002).

$MgCl_2$

Optimization of Mg^{2+} is critical since Taq DNA polymerase is a magnesium-dependent enzyme. In addition to Taq DNA polymerase, the template DNA primers and dNTPs bind to the Mg^{2+}. Therefore, the optimal Mg^{2+} concentration will depend on the dNTP concentration, specific template DNA, and samplebuffer composition. If primers and/or template DNA contain chelators such as EDTA or EGTA, the apparent Mg^{2+} optimum may be shifted. Excessive Mg^{2+} concentration stabilizes the DNA double strand and prevents complete

denaturation of DNA, which reduces yield. Excessive Mg^{2+} can also stabilize spurious annealing of primer to incorrect template sites, decreasing specificity. On the other hand, inadequate Mg^{2+} concentration reduces the amount of product (Markoulatos *et al.*, 2002).

dNTP/$MgCl_2$ Balance

To work properly, Taq DNA polymerase requires free magnesium (besides the magnesium bound by the dNTP and the DNA). This is probably why increases in the dNTP concentrations can rapidly inhibit the PCR, whereas increases in magnesium concentration often have positive effects. By combining various amounts of dNTP and $MgCl_2$, it was foundthat 200 µM of each dNTP work well in 1.5–2 mM $MgCl_2$. The threshold for the reaction was roughly 0.5–1 mM $MgCl_2$ over the total dNTP concentration, with reduced PCR amplification below this $MgCl_2$ concentration (Markoulatos *et al.*, 1999; 2000a; 2000b).

PCR Buffer Concentration

Raising the buffer concentration to 2X improves the efficiencyof the multiplex reaction. This was more effective than any of the adjuvants tested [dimethyl sulfoxide (DMSO), glycerol, and bovine serum albumin (BSA)]. Primer pairs withlonger amplification products work better at lower salt concentrations, whereas primer pairs with short amplification productswork better at higher salt concentrations (Henegariu *et al.*, 1997).

Amount of Template DNA and DNA Polymerase

When the amount of template DNA is very low, efficient and specific amplification can be obtained by further lowering the annealing temperature. The most efficient enzyme concentration seems to be around 2.5 Units/50 µl reaction volumes. Excessive enzyme quantity, possibly due to the high glycerol concentration in the stock solution, results in an unbalanced amplification of various loci and a slight increase in the background. The extension rate of specific primer-target hybrids depends on the activity of the enzyme, availability of essential components such as $MgCl_2$ and dNTPs, and the nature of the target DNA. Thus, the majority of modifications to improve PCR performance must be directed to the factors affecting annealing and/or extension rates (Markoulatos *et al.*, 2002).

Use of Adjuvants: DMSO, Glycerol, BSA

The most difficult multiplex PCR reactions can be significantly improved by using a PCR additive, such as DMSO, glycerol, formamide, and betaine, which relax DNA, thus making template denaturation easier. In the multiplex PCR, DMSO and glycerol gave conflicting results. Therefore, the usefulness of these adjuvants needs to be tested and optimized in each case. BSA, in concentrations up to 0.8µg/µl increased the efficiency of the PCR much more than did DMSO or glycerol (Henegariu *et al.*, 1997).

General Considerations for Multiplex PCR Development

Development of multiplex PCRs should follow a rational approach for the inclusion or exclusion of specific pathogens in the assay. These pathogens can be organ system specific or symptom specific with respect to the age of the patient and the epidemiological characteristics of these pathogens. PCR conditions, such as compatibility among the primers within thereaction mixture such that there is no interference, are of great technical importance. The primer pairs must be inclusive for as many strains of the target pathogen as possible, and depending on the amplicon detection method, their targets are easily resolvable. The latter may be achieved by using primer pairs that result in PCR products that can be separated and clearly visualized using gel electrophoresis or hybridization probes with maximum specificity. Prior to application in a laboratory setting, multiplex PCRs must be evaluated for their sensitivity as compared with their corresponding uniplex PCRs using both serial dilutions of the target DNA and clinical specimens. Wherever possible, multiplex PCRs should avoid the use of nested primers requiring a second round of amplification. Likewise, precautions and methodologies to avoid false-negative results due to reaction failure have to be considered (Elnifro *et al.*, 2000).

The optimal combination of annealing temperature and salt (buffer) concentration is essential in any PCR to obtain highly specific amplification products. Magnesium chloride concentration needs only to be proportional to the amount of dNTP, and these values can be constant for any reaction. Although gradually increasing magnesium chloride concentrations may further influence the reaction, the other two parameters mentioned seem to be much more important in obtaining specific, high yields of PCR products. In multiplex PCR, adjusting primer amount for each locus is also essential. The list of factors that can influence the reaction is by no means complete. Nevertheless, optimization of the parameters should provide a basic approach to some of the common problems of multiplex PCR (such as spurious amplification products, uneven or no amplification of some target sequences, and difficulties in reproducing some results). While there is no clear theoretical limit to the number of sequences that can be amplified simultaneously, the constraints on establishing conditions for specific and interpretable reactions generally limit the useful number of target sequences. A multiplex PCR reaction to amplify as much as nine segments of the human dystrophin gene has been reported (Chamberlain *et al.*, 1988; Henegariu *et al.*, 1997). Thorough evaluation and validation of new multiplex PCR procedures is essential. The sensitivity and specificity must be thoroughly evaluated using standardized, purified nucleic acids. Where available, full use should be made of external and internal quality controls, which must be rigorously applied (Markoulatos *et al.*, 2000a; 2000b).As the number of microbial agents detectable by PCR increases, it will become highly desirable for practical purposes to achieve simultaneous detection of multiple agents that cause similar or identical disease symptoms and/or share similar epidemiological features.

Conclusion

PCR has revolutionized the field of infectious disease diagnosis. To overcome the inherent disadvantage of cost and to improve the diagnostic capacity of the test, multiplex PCRs for the detection of viral, bacterial, fungal, and/or other infectious agents in one reaction tube have been developed. Efforts to enhance sensitivity and specificity, and to facilitate automation have resulted in numerous publications regarding the application of multiplex PCR in the diagnosis of infectious agents, especially those which target viral nucleic acids.

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Protein based diagnosis and its applications in plant virus diagnosis

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Plant viruses can cause devastating diseases leading to losses in agriculture and are therefore a major threat to food security. It has been estimated that plant viruses can cause as much as 50 billion euros loss worldwide, per year. This situation may be worsened by recent climate change events and the associated changes in disease epidemiology. They pose a particular risk because they are difficult to detect and identify (Hadidi *et al.*, 2004). Unlike other plant pathogens, there are no direct methods available yet to control viruses and, consequently, the current measures rely on indirect tactics to manage the diseases. Hence, methods for detection and identification of viruses play a critical role in the management disease (Naidu and Hughes, 2001). Accurate diagnosis of diseases is a first important step for any crop management system. There are several methods of detection of plant viruses are available. But the laboratory based virus detection methods target either the viral genome or the coat protein. Viral genome can be either RNA or DNA and there are several methods of detecting the viral genomes using the polymerase chain reaction and its variants. Here in this chapter we are going to deal about detection methods that target the protein, especially the coat protein which gives the structure to the viruses.

The virus structure and its components

Before, dealing about the protein targeted detection methods let us look at virus morphology, terminologies and viral components. A fully assembled infectious virus is called a virion. The simplest virions consist of two basic components: nucleic acid (single- or double-stranded RNA or DNA) and a protein coat, the capsid, which functions as a shell to protect the viral genome from nucleases and which during infection attaches the virion to specific receptors exposed on the prospective host cell. Virions consist of 60-90% protein and 5-40% nucleic acid. A capsid is almost always made up of repeating structural subunits that are arranged in one of two symmetrical structures, a helix or an icosahedrons. These structural subunits (also called protomers) are made up of several polypeptides. Proteins are composed of about 150-600 or more residues of some 20 amino acid and provide the basis for serological differentiation of plant viruses. TMV is most widely studied virus. The entire TMV particle is very stable due to interaction between protein sub unit and RNA. The particle measures about 300 nm x 15 nm. The molecular weight of particle is 39.4 X 10^6 daltons. It consists of about 2100 helically arranged identical protein sub units along with axial canal. There are many viruses which has glycoproteins which are thermolabile. Viroids which lacks the structural

coat proteins and they are naked RNAs. Hence serological methods cannot be applied for the detection of viroids

Need of diagnosis and detection of viruses

With virus diseases, plant treatment after infection often does not lead to an effective control. Accordingly, the diseases are managed most effectively if control measures are applied before infection occurs. The use of healthy plant propagation material is among the most effective approaches to adopt by farmers. One of the elements essential for successful certification programs to produce such propagation material is the availability of sensitive diagnostic methods (Makkouk and Kumari, 2006). Reliable and early detection methods are still one of the main and most effective actions to develop control strategies for plant viral diseases. Serology-based methods for virus detection have contributed significantly to evaluation of the sanitary status of these crops during the last 30 years and are still the methods of choice for many laboratories involved in certification schemes (López *et al.*, 2003; Boonham *et al.*, 2014).

Serological based detection of viruses

Laboratory-based techniques have been developed over the last few decades, are now being used routinely in many laboratories for accurate detection and diagnosis of diseases caused by plant viruses. These lab techniques involved are physical, biological, cytological, serological, and molecular properties of viruses. Among these techniques serology is one of the most specific and easiest methods to obtain a rapid and precise identification (Astier *et al.*, 2007; Naidu & Hughes, 2001). Among the serological techniques, Enzyme-linked immune-sorbent assay (ELISA) methods were used extensively. Other serological techniques that were used included tissue blot immunoassays, immune-electron microscopy (trapping and decoration), Western blots, dot blots, lateral flow rapid tests, immune-capture PCR, and double diffusion tests. The great value of serological methods for plant virus identification is based on the specific reaction between the viral antigens and their specific antibodies. An antigen is a molecule that when injected into a vertebrate animal (usually a mammal or a bird), it can trigger an immune response in the animal which results in production of specific antibodies that can combine with the foreign antigen. Virus particles themselves and their proteins have many antigenic determinants (epitopes) which vary in their amino acid sequence and have the properties of inducing the production of specific antibodies. Antibodies are also proteins of the immunoglobulin group (Ig) produced against specific antigenic determinants and are present in the animal blood. The immunoglobulin G (IgG) is the most common type of Ig produced and, consequently most commonly involved in the serological tests for plant virus identification. Generally, the methods that involve the antigen antibody reactions in vitro are simple and do not require sophisticated and expensive apparatus. The most serious limitation to using serology for plant virus identification and

detection is the difficulty in producing a good virus specific antiserum. Most antisera used for plant virus identification and detection are usually prepared by immunizing mammals or birds with purified plant virus or their different types of proteins. However, several other methods have been used to produce very specific antibodies, including monoclonal antibody (Mab) which consists of a single type of antibody that reacts with only one specific epitope of a virus protein. The production of Mab consists of a series of steps, including mouse immunization, collection of antibody producing cells, fusion with myeloma cells to produce a hybridoma, screening and selection process of hybridoma specific for the desired virus epitope.

Many serological techniques have been developed for identification and characterization of plant viruses and the advent of the enzyme-linked immunosorbent assay (ELISA) has facilitated the use of serology for virus identification in large scale. The serological methods can be subdivided into two broader categories involving liquid and solid phase methods. The liquid phase methods can be represented by the double immune diffusion techniques in which the antigen and the antibodies react in agar media producing visible precipitates. In the solid phase methods, one of the reagents, usually the antibody, is trapped on a solid surface that could be nitrocellulose membrane, a microtitre plate, polystyrene or polyvinyl chloride plates. In this case the antigen-antibody reaction is detected by a labeled antibody as in the ELISA and its variations. In addition the virus particles are detected by direct visualization with serologically specific electron microscopy (SSEM). Detection range of various diagnostic methods given in Table 1.

Table 1: Detection limits of various virus detection methods (Matthews, 1993)

Method	Detection range
Gel double immunodiffusion	2-20 µg/ml
Liquid precipitin tests	1-10 µg/ml
Radial immuno-diffusion	0.5-1.0 µg/ml
Rocket immunoelectrophoresis	0.2 µg/ml -100ng/ml
Immuno-osmophoresis	50-100 ng/ml
Passive hemaglutination	20-50 ng/ml
Latex test	5-20 ng/ml
ELISA	1-10 ng/ml
Immunoelectron microscope	1-10 ng/ml
Western blotting	1-10 ng/ml

Precipitation test

Precipitin tests rely on the formation of a visible precipitate when adequate quantity of virus and specific antibodies are in contact with each other (Van Regenmortel 1982). Precipitin and microprecipitin tests are routinely used by some investigators, but agglutination and double diffusion tests are more commonly used. In double diffusion tests, the antibodies and antigen diffuse through a gel matrix and a visible precipitin line is formed where the two diffusing reactants meet in the gel. The Ouchterlony double diffusion method can be used to distinguish related, but distinct, strains of a virus or even different but serologically related viruses. However, disadvantages of this method include a lack of sensitivity in detecting viruses that occur in low concentration the need to dissociate filamentous or rod-shaped viruses to allow them to diffuse through the gelmatrix, and the need for large quantities of antibodies.

Agglutination test

In an agglutination test, the antibody is coated on the surface of an inert carrier particle and a positive antigen– antibody reaction results in clumping/agglutination of the carrier particles which can be visualized by the naked eye or under a microscope. Agglutination tests are more sensitive than other precipitin tests and can be carried out with lower concentrations of reactants than are necessary for precipitation tests (Walkey *et al.* 1992; Hughes and Ollennu 1993). Although the precipitation and agglutination tests lack the sensitivity of other serological assays, they are excellent methods for detecting viruses that occur in a reasonable concentration in plants. Tests can be conducted simply by squeezing out a drop of plant sap and testing it with the appropriate antisera. These techniques can be performed with minimum facilities and expertise and, therefore, are suitable for many laboratories with limited facilities but which have an adequate supply of antiserum (Naidu and Hughes, 2001).

Enzyme linked immunosorbent assay (ELISA)

ELISA is not a new technique, although it is widely used throughout the world because of its accuracy, simplicity and low cost. The technique utilizes the ability of antibodies raised in animals to recognize proteins, usually the coat protein, of the virus of interest. It is a very sensitive and specific serological technique introduced for identification of plant viruses in the 1970s (Clark & Adams, 1977). This technique can detect virus particles in very low concentrations and can be used with viruses of different particle morphology. Because of its adaptability, high sensitivity, and economy in the use of reagents, ELISA is used in a wide range of situations, especially for indexing many samples in a relatively short period of time. The ELISA technique is based on the basic principle in which the virus antigens are recognized by their specific antibodies (IgG) in association with colorimetric properties. The ELISA method is commonly accomplished in a 96-well polystyrene plate by adding the antigens and antibodies into the wells in an established sequence, involving several stages. In

the final stage, the positive reactions are detected when a colorless substrate, usually p nitrophenyl phosphate, undergoes a chemical change resulting in a yellow colored product as the result of exposure to the enzyme alkaline phosphate linked to the antibody. The degree of color change indicates the degree of reactivity that is read by an ELISA plate reader apparatus. It is always recommended to include a homologous antigen for the specific virus antibody and extracts from healthy plants to compare the absorption readings and to obtain a correct interpretation of the results. In addition, immunoassay sensitivity can be enhanced using different amplification systems, with avidin-biotin being the most common. The principle of ELISA techniques consists of detecting the antigen antibody interactions by enzyme induced color reaction rather than by observing their precipitation. Although different variations of this serological technique have been developed i.e., direct and the indirect ELISA.

Indirect ELISA

Indirect ELISA or plate-trapped antigen (PTA- ELISA) was developed to avoid the inconveniences and difficulties of conjugating the enzyme with the IgG specific for each virus species to be used in the second layer of antibodies in direct ELISA. For this reason, the indirect ELISA or PTA-ELISA requires antibodies produced in two different animal species and the virus particles are trapped in the wells of the ELISA plate (Fig. 1A). The indirect ELISA also requires the use of a universal IgG enzyme conjugate which can be used with the antibodies of all virus species. The so-called universal conjugate is composed of an IgG produced against the IgGs from the animal in which virus antibodies are raised linked to the enzyme alkaline phosphate. If the virus antibodies are produced in rabbits (e.g.), an antirabbit IgGs are produced in a second animal species such as goats or mice. So, the detecting antibody conjugate binds specifically to the primary virus specific antibody. In this method, the wells of the ELISA plate are, initially, covered with extracts from infected plants and healthy plant samples. Later the virus particles are covered with a layer of virus specific antibodies produced in a rabbit. The complex antigen antibodies are covered with a universal conjugate that could be an anti-rabbit IgG produced in goats or mice linked to the enzyme alkaline phosphate. The linked anti-IgG-enzyme that react with the virus antibodies (IgG) which had reacted with the virus particles adsorbed to the bottom of the ELISA plate wells will be detected by colorimetric changes of a specific substrate that is added into the wells. Nevertheless, this method has certain disadvantages such as competition between plant sap and virus particles for sites on the plate wells and, consequently, high background reactions.

Direct competitive ELISA

This assay is used to detect or quantitate soluble antigens and is most useful when both a specific antibody and milligram quantities of purified or semi purified antigen are available (Fig. 1B). To detect soluble antigens, plates are coated with antigen and the binding of

specific antibody-enzyme conjugates to antigen-coated plates is inhibited by test solutions containing soluble antigen. After incubation with mixtures of the conjugate and inhibitor in antigen-coated wells, unbound conjugate is washed away and substrate is added. The amount of antigen in the test solutions is proportional to the inhibition of substrate hydrolysis and can be quantitated by interpolation onto an inhibition curve generated with serial dilutions of a standard antigen solution.

Antibody-sandwich ELISA

Antibody-sandwich ELISAs may be the most useful of the immunosorbent assays for detecting antigen because they are frequently between 2 and 5 times more sensitive than those in which antigen is directly bound to the solid phase (Fig. IC). To detect antigen, the wells of microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and a different antigen-specific antibody conjugated to enzyme (i.e., developing reagent) is added, followed by another incubation. Unbound conjugate is washed out and substrate is added. After another incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution.

Double antibody–sandwich ELISA to detect specific antibodies

This assay is especially useful when screening for specific antibodies in cases when a small amount of specific antibody is available and purified antigen is unavailable (Fig.1D). Additionally, this method can be used for epitope mapping of different monoclonal antibodies that are directed against the same antigen. Plates are coated with capture antibodies specific for immunoglobulin from the immunized species. The test antibody solution is incubated on the plates coated with the capture antibodies. Plates are then washed, incubated with antigen, washed again, and incubated with specific antibody conjugated to an enzyme. After incubation, unbound conjugate is washed out and substrate is added. Wells that are positive for hydrolysis may contain antibodies specific for the antigen.

Triple Antibody Sandwich (TAS- ELISA)

Another widely used ELISA variation is the triple antibody sandwich i.e., TAS- ELISA, which is like the direct ELISA (DAS- ELISA), except for an additional antibody produced in another animal is used. First, the bottom of the ELISA plate wells are coated with a virus antibody produced in a species of animal (e.g., rabbit) and the virus antigen is linked in the trapped antibodies. The virus antigen is covered with a second layer of virus specific antibody produced in another animal species (e.g., mouse or goat) and the presence of this antibody is detected by adding an enzyme-conjugated specific antibody (e.g., rabbit anti-mouse IgG), that does not react with the plate well trapped antibody, followed by colorimetric changes of a specific substrate that is added into the wells (Fig. 1E).

Protein A-Sandwich (PAS- ELISA)

This ELISA variation is based on the property of protein A combining specifically with the Fc portion of the IgG. The protein A is obtained from the cell wall of Staphylococcus aureus and has a molecular weight of approximately 42 – 56 Kd (Almeida, 2001). This protein is very stable at a broad pH range and it is produced commercially, including a protein A-enzyme conjugate to be used in plant virology. It is prepared by direct dilution in pure water (1 mg/ml) and diluted in ELISA buffer to determine its adequate concentration for good results in PAS-ELISA. In the PAS-ELISA the antibody–virus–antibody layers which occur in the direct ELISA are sandwiched between two layers of protein A. The method consists of coating the bottom of the ELISA plate wells with a layer of protein A before the addition of the trapped virus antibody. Since the Fc region from the antibodies (IgG) has affinity to protein A, the added antibodies link specifically with the protein A trapped at the bottom of the wells keeping the virus antibodies in a specific orientation so that the F(ab´)2 portion of the antibodies will be free to trap the virus particles. The F(ab´)2 portion of the virus antibody orientation will increase the sensitivity of the PAS- ELISA by increasing the proportion of appropriately aligned antibody molecules. The exposed virus particles will link to the F(ab´)2 portion of a second added layer of the same antibodies which will be detected by an enzyme-conjugated protein A followed by colorimetric changes of a specific substrate that is added into the wells (Fig. 1F).

Immune Precipitation ELISA (IP- ELISA)

Considering the problems with plant viruses whose particles are not well adsorbed in the ELISA plate wells, a new ELISA technique involving the immune virus particle precipitation (IP- ELISA) was developed and validated for detection of plant viruses from different families and genera, especially those from the genus Comovirus (Lima *et al.*, 2011b). As for the other ELISA procedures, approximately 0.5 g of virus infected plant tissues are ground in ELISA extraction buffer and 0.5 ml from the obtained extract is mixed with an equal volume of specific antiserum diluted to 1:100 to 1:1000 (v/v). The mixture of infected plant extract and the antiserum is incubated at 37 °C for 3 h or overnight at 4 °C and centrifuged at 5,000 g for 10 min. The pellet containing the virus particles linked to the antibodies are resuspended in ELISA extraction buffer and used as for conventional indirect ELISA (Fig.1G). The IP-ELISA showed efficiency for detection of virus from different families and genera in different kinds of infected tissues. The immune virus precipitation followed by ELISA (IP-ELISA) for detection of viruses was shown to be a sensitive and practical diagnostic technique for plant viruses, especially for Cowpea severe mosaic virus (CPSMV) and Squash mosaic virus (SQMV), family Comoviridae, genus Comovirus (Lima *et al.*, 2011b), whose virus particles do not adsorb well in the bottom of the plate wells (personal observation).This method increases the efficiency of the indirect ELISA (Fig) without the necessity of using protein A.

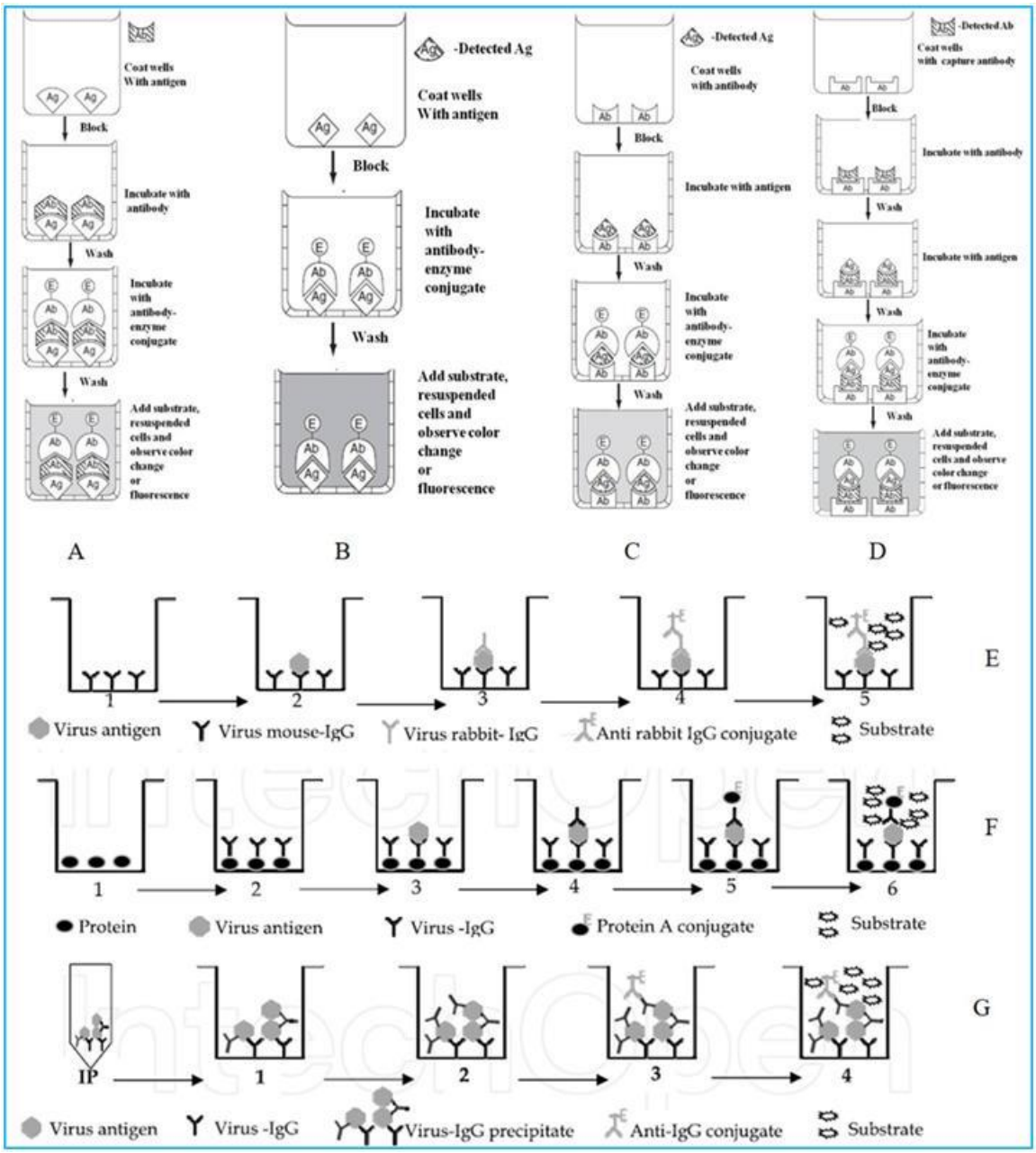

Fig. 1. Diagrammatic drawings of various ELISA formats for detection of plant viruses. **A.** Indirect ELISA to detect specific antibodies: **B.** Direct competitive ELISA to detect soluble antigen; **C.** Antibody-sandwich ELISA to detect antigen; **D.** Double antibody–Sandwich ELISA to detect specific antibodies; **E.** Triple antibody sandwich ELISA; **F.** Protein-A –sandwich ELISA; **G.** Immuno precipitation-ELISA; Ag-Antigen; Ab-Antibody; E- Enzyme. (Source: Albersio *et al.*, 2012; Ausubel *et al.*, 2003).

A modification of ELISA named voltametric enzyme immunoassay, detects the change in electrical conductivity of the substrate, rather than a colour change, when acted upon by an enzyme attached to a secondary antibody. This method is claimed to be an order of

magnitude more sensitive than ELISA. It was used to detect Cucumber mosaic virus. This method can be used for testing multiple plants for a single virus using one well per plant sample, or alternatively a single plant can be simultaneously tested for many viruses on a single plate with different antibodies coated to each well in duplicate or triplicate for reproducibility. The major constraint of the method is the requirement for polyclonal or monoclonal antibody sera specific for each virus of interest that does not cross-react with plant proteins, but cross absorption with plant sap avoids this problem substantially.

Immunoblotting

Serological solid support matrix methods like ELISA techniques were developed in which the virus antigens are trapped onto a membrane rather than in a microtitre plate. Like indirect ELISA, virus particles or their proteins are immobilized on nitrocellulose or nylon membranes. As distinguished from indirect ELISA, it is not necessary to use an ELISA reader for detecting the virus antibodies interactions and for this reason it is not possible to quantify the results by numerical absorbance values. According to the process by which the virus antigens are applied in the membranes these methods can be divided into three a) Dot blot or dot immuno binding assay (DIBA) b) Tissue blot immuno assay (TIBA) and c) Western blot

Dot Blot or Dot Immuno Binding Assay (DIBA)

This technique is a simple and easier method to prepare and apply the samples on nitrocellulose or nylon membranes. The samples containingthe virus antigens are prepared by grinding tissues in Tris-buffered saline and the extracts are applied directly on the membrane. The sample application on the membrane is usually accomplished using a plastic mold with 96 wells which presses the membrane marking the places where the samples should be applied. Usually the spaces not occupied by the antigens on the membrane are blocked with neutral protein solution. The addition of virus IgG produced in rabbit and the anti-rabbit IgG produced in mouse follow protocols similar to indirect ELISA or PTA-ELISA, except that the positive reactions in DIBA are recorded as colored dots on the membrane. Unlike in ELISA, where a soluble substrate is used for color development, a precipitating (chromogenic) substrate is used for virus detection in the DIBA (ig.2A). Hydrolysis of chromogenic substrates results in a visible colored precipitate at the reaction site on the membrane. Chemiluminescent substrates, which emit light upon hydrolysis, can also be used and the light signal detected with X-ray film as with radiolabelled probes (Leong *et al.*, 1986). Considering that DIBA is a simple, less laborious and quick test, it can be used routinely for plant virus indexing and survey programs.

Tissue immunoblotting assay (TIBA)

This assay is a variation of DIBA in which a freshly-cut edge of a leaf blade, stem, leaf,

tuber, root or an insect is blotted on the membrane, followed by detection with labelled antibodies (Fig 2B) as described above (Makkouk *et al.*, 1993). The procedure is less labour-intensive than ELISA, rapid, sensitive, simple, inexpensive, suitable for surveys of 1000 to 2000 samples per day, and the samples can be taken in the field and processed some time later (Webster *et al.*, 2004).

The disadvantages of DIBA and TIBA are possible interference of sap components with the subsequent diagnostic reactions. Sometimes the color of the sap will prevent weak positive reactions from being observed and the results cannot be readily quantified. Nevertheless, their sensitivity, the relatively short time required to assay large numbers of samples, the need for minimum laboratory facilities for the assay, the ability to store blotted membranes for extended periods, and low costs favor TIBA and DIBA as useful diagnostic techniques. The other advantage is that the samples can be blotted onto the membranes right in the field and such membranes can be carried or shipped by mail for further processing at a central location either within the country or in a different country (Naidu and Hughes, 2001).

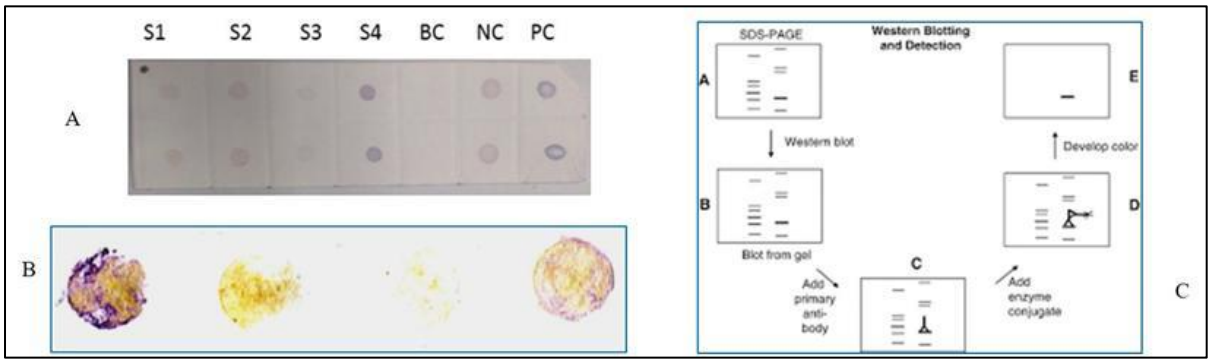

Fig. 2. Diagrammatic drawings of Dot blot, Tissue blot and western blotting formats for detection of plant viruses. **A.** Dot immuno binding assay (DIBA) for detection of banana bract mosaic virus. Samples used: PC-Positive Control; NC-Negative Control; BC-Buffer Control; Test sample: 1; Streak like: 2; Mosaic like-3; Non-symptomatic: 4; BBrMV infected **B.** Source: Selvarajan *et al.*, 2018. Tissue Blot immunosorbent assay with antibody to Tobacco eth virus (TEV). Purple colour indicates and a positive reaction and location of antigen. Leaves blotted on the membrane were infected with TEV; SMV and CMV (Left to right) Source: Chang *et al.*, 2011; C. Western blotting

Western blotting /Electroblot Immuno Assay (EBIA)

EBIA involves transferring of separated protein bands from an acrylamide slab gel into a stable solid immobilizing medium like nitrocellulose paper followed by immunological detection of immobilized proteins (Fig. 2C). Burnet (1981) used the term "Western blotting 'in anology to' Southern blotting' (transfer of DNA from gel to the nitrocellulose paper) and 'Northern blotting' (transfer of RNA from gel to the nitrocellulose membrane). Sodium dodecyl sulphate poly acrylamide gel electrophoresis of samples is primary step in EBIA.

Under the influence of an electric field charged molecules like proteins, nucleic acids and polysaccharides will migrate either to the cathode or to the anode, depending on their net charge. This phenomenon is called electrophoresis. Electrophoresis is usually carried out in gels formed in a glass tubes, slabs or on a flat bed. A slab gel is formed in glass sandwich made of two flat glass plates separated by two spacer strips at the edges and clamped together to make a watertight seal. Both tubes and slab gels are mounted vertically. In most electrophoresis units, the gel is mounted between buffer chambers containing separate electrodes so that the only electrical connection between the two chambers is through the gel. Generally, the sample is run in agarose or polyacrylamide or agarose-acryl amide composite gel. At the end of a run the gel can be stained and used for scanning or visual recording of results. The gels can also be dried and stored permanently. The major usefulness of SDS-PAGE system is to determine the molecular weights of polypeptides, proteins and nucleic acids. EBIA is more sensitive in detection of virus coat proteins (at nanograms level) in the infected plants with antiserum. Purity of the antiserum can be assessed. Mixed viral infection can be detected using two or more antiserum. Serological relationship among structural and nonstructural virus-coded proteins can be assessed.

Quartz crystal microbalance immunosensors

In this novel technique for plant virus detection, a quartz crystal disk is coated with virus-specific antibodies. Voltage is applied across the disk, making the disk warp slightly via a piezoelectric effect. Adsorption of virus particles to the crystal surface changes its resonance oscillation frequency in a concentration-dependent manner. It is therefore qualitative and quantitative. The developers of the technique claim that it is as sensitive but more rapid than ELISA, and economical (Webster *et al.*, 2004).

Immunosorbent electron microscopy

A technique known as serologically specific electron microscopy (SSEM) was introduced by Derrick (1973) and it has become widely used in plant virology. This technique combines the specificity of serological properties with the morphology of the virus particles visualized in the electron microscope. Virus particles are selectively trapped on antibody-coated grids with little contaminating host-plant material. The antibody coated grids are washed with buffer and floated on drops of extracts from virus infected plant tissue at room temperature for 3 – 4 h. After washing again for three times, the grids are stained with 1.0% uranyl acetate in 50% ethanol, dried and examined in the electron microscope. It has a great advantage of requiring only very small amounts of antiserum and antigens, and does not require the use of labeled antibodies. For those reason, the SSEM technique is more sensitive for detecting virus particles in leaf extracts and shows little contaminating host-plant material (Derrick & Brlansky, 1976).

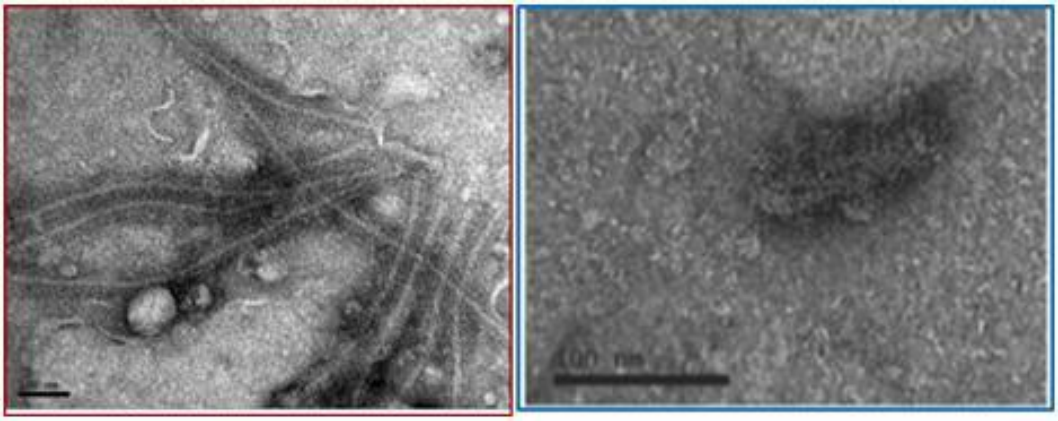

Fig. 3. ISEM for detection of BBrMV and BSMYV using BBrMV and BSMYV-VAP polyclonal antiserum (Left to right: BBrMV -BSMYV) (*Source:* Selvarajan *et al.*, 2016)

Immune Capture Polymerase Chain Reaction (IC-PCR)

This technique i.e., immune capture polymerase chain reaction (IC-PCR), combines the technical advantages of PCR with the practical advantages of serology. It was developed for the detection of several plant viruses. In this test, microtiter tubes are coated with specific virus antibodies and incubated at 37°C for 2 h. After washing, the microtiter tubes coated with the antibodies will trap the virus particles which will be disrupted followed by the release of viral nucleic acid. The virus nucleic acid is amplified by polymerase chain reaction (PCR) or reverse transcription PCR (RT- PCR), and the entire procedure is carried out in a single microtiter tube (Fig.4A). The IC-PCR has been shown to be a very useful alternative in virus detection from plant material and insect vectors (Mulholland, 2009).

Immune Precipitation Polymerase Chain Reaction (IP-PCR)

A new PCR technology involving virus particles immune precipitation (IP-PCR) was developed for identification and molecular characterization of plant viruses from different families and genera (Lima *et al.*, 2011a). This technique is very practical, specific and minimizes problems with RNA extraction combining the serological properties and the technical advantages of virus nucleic acid amplification. Approximately 0.5 g of plant tissues infected with virus is grind in 1.0 ml of extraction buffer (0.15 M Na_2CO_3, 0.035 M of $NaHCO_3$ and 0.007 M of sodium diethyldithiocarbamate, pH 9.6). The extract is obtained by straining through triple cheesecloth, and 0.5 ml was mixed with an equal volume of specific virus polyclonal antiserum diluted to 1:500 (v/v) in the antiserum buffer (PBS-Tween 20 with 0.5 M polyvinylpyrrolidone, 0.2% ovalbumin, 0.03 M sodium azide, 0.17% of sodium diethyldithiocarbamate).

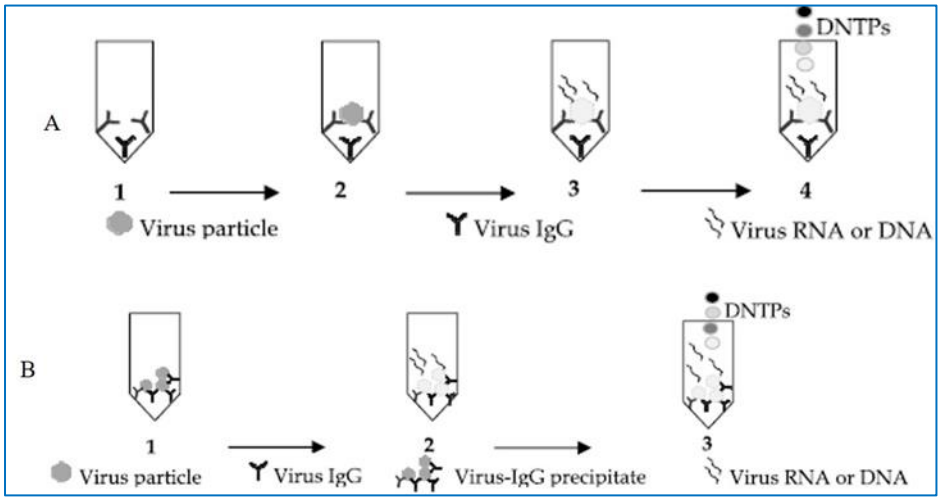

Fig. 4. Diagrammatic drawings of IC-PCR and IP-PCR formats for detection of plant viruses. A. Immuno capture PCR; B. Immune Precipitation PCR (IP- PCR)

The mixture is incubated at 37°C for 3 h or overnight at 4°C, and centrifuged at 5,000 g for 10 min. The precipitated virus particles linked with their specific antibodies are disrupted for RNA extraction using TrizolR Reagent or with a RNA isolation kit by following manufacturer's instructions. A first strand cDNA is synthesized from each virus RNA using their antisense specific primers and Reverse Transcriptase. The cDNA fragments corresponding to virus RNA are amplified by PCR (Fig.4 B). This newly developed immune virus precipitation followed by PCR or RT-PCR (IP-PCR) was shown to be a practical and sensitive technique for detection of plant viruses.

Lateral Flow Immunoassay (LFIA) Detection System

Lateral flow immunoassay (LFIA) is used for qualitative or semiquantitative detection and monitoring of viral pathogens in non-laboratory environments. LFIA is simple, efficient, and rapid which makes it possible to identify and evaluate the content of various biologically active substances in a sample in a few minutes without any special skills and equipment and could be used even under field conditions. When lateral flow assay uses antibodies raised against the target analytes then it is called lateral flow immune assay (LFIA) which most widely used in detecting the analytes. The analyts can be protein, hapten, DNA, any molecules, glycoproteins etc., If the target is not a protein then antibodies cannot be raised against them and it cannot be used for the detection. Gold nanoparticles are widely used in immuno based LFA tests. These gold nanoparticles of less than 100 nm have the capacity to resonance and scatter the light.

In colloidal gold is commonly used as a label for preparing many commercial Lateral flow devices. Both liquid and dried forms of colloidal gold are highly stable. Colloidal gold can

easily be prepared in the laboratories and it is also available from many chemical companies of repute. Use of colloidal gold in the detection of any analyte of interest is easy because it gives a highly visible colour upon functionalisation and accumulation after its reaction with specific antibodies. Latex also used as label in LFIA tests and it could be tagged with a variety of detector reagents like antibodies, fluorescent dyes and these latex beads produced in wide range of colour for multiplexing of assays.

Principle of LFIA

It is movement of a liquid sample containing the analyte through the set of membranes where specific conjugates, specific antibodies are placed. Upon dipping in the sample, the analytes present in the liquid moves by capillary action or chromatography principle upwards or laterally and it reacts / interacts with recognition molecules like antibodies conjugated with gold or latex nanoparticles and produce a coloured band/line which can be visually seen. Lateral flow immunoassay is also called immunochromatographic assay. This complex further move and reacts in a line where specific IgG are immobilised, and the excess conjugates further move and react with anti-antibodies placed in line next to test line. The liquid goes up due to pulling effect of absorption pad in the top.

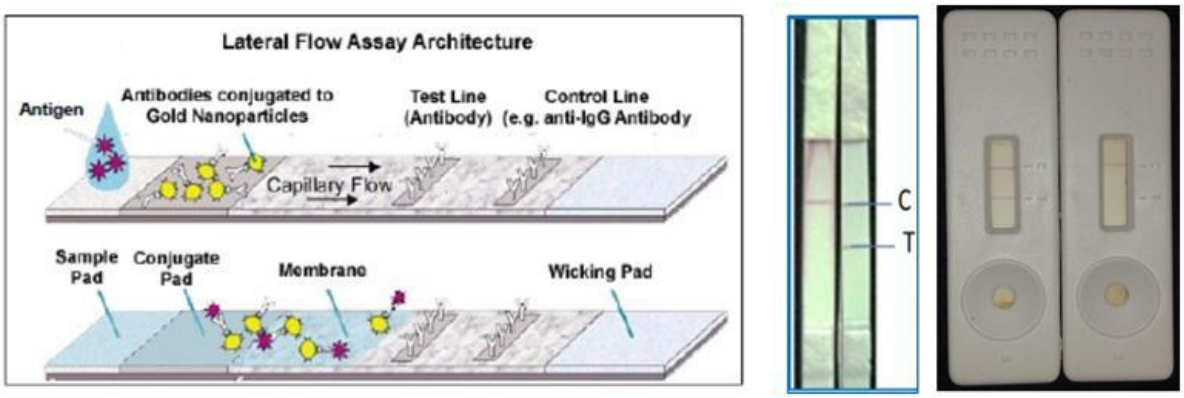

Fig. 5. Diagrammatic drawings of LFIA format for detection of plant viruses

A typical LFIA format consists of a surface layer to carry the sample from the sample application pad via the conjugate release pad along the strip encountering the detection zone up to the absorbent pad (Byzova *et al.*, 2009). The membrane is often thin and fragile, so it is pasted to a plastic layer to allow easy cutting into strips and handling. In addition, robustness is achieved by housing the strips in a plastic holder, where only the sample application window and a reading window are exposed. Current membrane strips are produced from nitrocellulose, nylon, polyethersulfone, polyethylene, or fused silica. At one end of the strip, a sample application pad is provided. The sample application pad is usually made of cellulose or cross-linked silica. Next to the sample application pad is the conjugate release pad, made of cross-linked silica, and lengthwise it is lesser than half the length of sample application

pad. Labeled analyte or recognition element(s) is applied and dried on this pad, and after the addition of the sample, this material will interact with the fluid flow; specific interactions will be initiated here and will continue during the chromatographic process.

Express immunochromatographic test strip assays were developed for detection of five plant viruses varying in shape and size of virions, viz., spherical carnation mottle virus, bean mild mosaic virus, rod-shaped tobacco mosaic virus, and filamentous potato viruses X and Y (Byzova *et al.*, 2009). Multi-membrane composites (test strips) with immobilized polyclonal antibodies against viruses and colloidal gold-conjugated antibodies were used for the analysis. These immunochromatographic test strips were shown to enable the detection of viruses both in purified preparations and in leaf extracts of infected plants with sensitivity from 0.08 to 0.5 μg/ml for 10 min. This technique can be adopted for on-site detection of these viruses under field conditions (Byzova *et al.*, 2009); Drygin *et al.* (2012) have developed immunochromatographic assay for rapid detection of potato virus X (PVX). In this assay time does not exceed 15 min, and the lower limit of the PVX detection in non-clarified leaf extract was 2 ng/ml, and a single measurement required 0.1–0.2 ml of tested solution extracted from 10 to 20 mg of potato. Plum pox virus (PPV) with specific colloidal gold-labeled antibodies using immunochromatographic assay has been developed with a detection limit of 3 ng/ml, and the test duration was just 10 min (Byzova *et al.*, 2010); Yoon *et al.*, (2014) have developed rapid immune-gold strip (RIGS) kit in a novel single strip format to detect on-site detection of tomato spotted wilt virus (TSWV), and results could be obtained in 2–5 min. Safenkova *et al.*, (2012) studied the impact of key factors influencing the analyte detection limit of the sandwich immunochromatographic assay (ICA), namely, the size of gold nanoparticles, the antibody concentration, the conjugation pH, and the characteristics of membranes for the detection of PVX. The antibody-colloidal gold conjugates synthesized at pH 9.0–9.5 at an antibody concentration of 15 μg/mL showed maximum binding with the analyte. The detection limit improved from 80 to 3 ng/mL for a series of nanoparticles with a diameter from 6.4 to 33.4 nm. In the case of larger particles (52 nm in diameter), the detection limit increased and reached 9 ng/mL. A 10 mM phosphate buffer, pH 8, and a 50 mM phosphate buffer, pH 7, were the conditions of choice for the deposition of reactants. The maximum detection limit was 2–3 ng/ mL by the standardized method. Recently LFIA have been developed for the on-farm detection of six different plant viruses utilizing specific monoclonal and polyclonal antibodies against cucumber mosaic virus (CMV), groundnut bud necrosis virus (GBNV), large cardamom chirke virus, papaya ring spot virus, peanut mottle virus (PeMoV), and potato virus Y (Bikash Mandal, Personal Communication). Yogita *et al* (2017) reported a LFIA strips for the detection of citrus triteza virus in the orchards itself.

Many commercial firms are producing these LFIA strips for detection of various pathogens including plant viruses e.g., at Agdia, Bioreba, or Forsite Diagnostics (Pocket Diagnostic).The LFIA strip technique has been reported for the detection of viruses (Danks

and Barker 2000; Salomone and Roggero 2002; Salomone *et al.*, 2002, 2004; Kusano *et al.*, 2007; Drygin *et al.*, 2009). The use of nanoparticles as labels has led to the improvements in sensitivity and multiplexing capabilities (Jain 2005; Rosi and Mirkin 2005). Metallic nanoparticles composed of gold or silver have many optical and electronic properties, based on their size and composition (Nath *et al.*, 2008). When coupled to affinity ligands, these nanoparticle materials have found important applications as chemical sensor. For example, gold nanoparticles conjugated with specific oligonucleotides can sense complementary DNA strands, detectable by color changes (Mirkin *et al.*, 1996). Other nanoparticles including fluorescent quantum dots and carbon nanotubes have been used in various applications including DNA detection and the development of immunoassays for the detection of pathogens (Bruchez *et al.*, 1998; Edgar *et al.*, 2006; Baptista *et al.*, 2006; Alivisatos *et al.*, 2005).

Advantages / disadvantages of LFIA

- Low cost, portable, suitable for on-site / in-field detection and very quick results can be obtained
- Easy to use and even a non-technical / illiterate person can also handle as it does not require specific skills
- It is specific, sensitive, efficient and more reliable
- Simultaneous detection of multiple viruses/ pathogens/ analytes is possible
- The results can be qualitative or semi-quantitative
- Once the strip is dipped in any liquid/ sample then it cannot be used

Microsphere Immunoassay

Among the immunoassays, ELISA has been exploited widely in life sciences for the detection of viral pathogens or antibodies to viral proteins or biomarkers for cancer detection. Recently, a breakthrough has been the use of microspheres in the immunoassay (xMAP technology) which has emerged as an alternative for microbial detection (Charlermroj *et al.*, 2013). The basic principle of the technology is that it employs different sets of fluorescence-coded microspheres; each bead set is filled with a combination of dyes which are conjugated with capture antibodies specific to target pathogens, and the detecting antibodies are linked with another fluorophore. There have been several reports using this microsphere technology to detect multiple analytes or biomarkers across a number of fields including human diagnostics (Kellar and Douglass 2003), food microbiology (Dunbar *et al.*, 2003), and plant pathogen detection (Bergervoet *et al.*, 2008). For simultaneous detection of potato-infecting viruses, this bead-based technology has been used (Bergervoet *et al.*, 2008). The use of paramagnetic beads in place of conventional beads in the MIA procedure allows efficient removal of excess sample compounds and reagents which has resulted in lower background values and a higher specificity than a non-wash MIA procedure. They have used MIA technology to detect PVY,

PVX, and PLRV in potato leaf extracts which were detected with equal sensitivity and specificity. In MIA, procedure takes longer time due to the fact that the results are not visible by the eye, and therefore all samples have to be analyzed resulting in longer measuring times (Bergervoet *et al.,* 2008); Charlermroj *et al.,* (2013) have developed microsphere immunoassays to simultaneously detect four important plant pathogens: a fruit blotch bacterium Acidovorax avenae subsp. Citrulli (Aac), chili vein-banding mottle virus (CVbMV, potyvirus), watermelon silver mottle virus (WSMoV, tospo virus serogroup IV), and melon yellow spot virus (MYSV, tospovirus). This assay was able to detect all four plant pathogens precisely and accurately with substantially higher sensitivity than ELISA, and if the same antibody sets were used, its assay time is also shorter (Fig. 6).

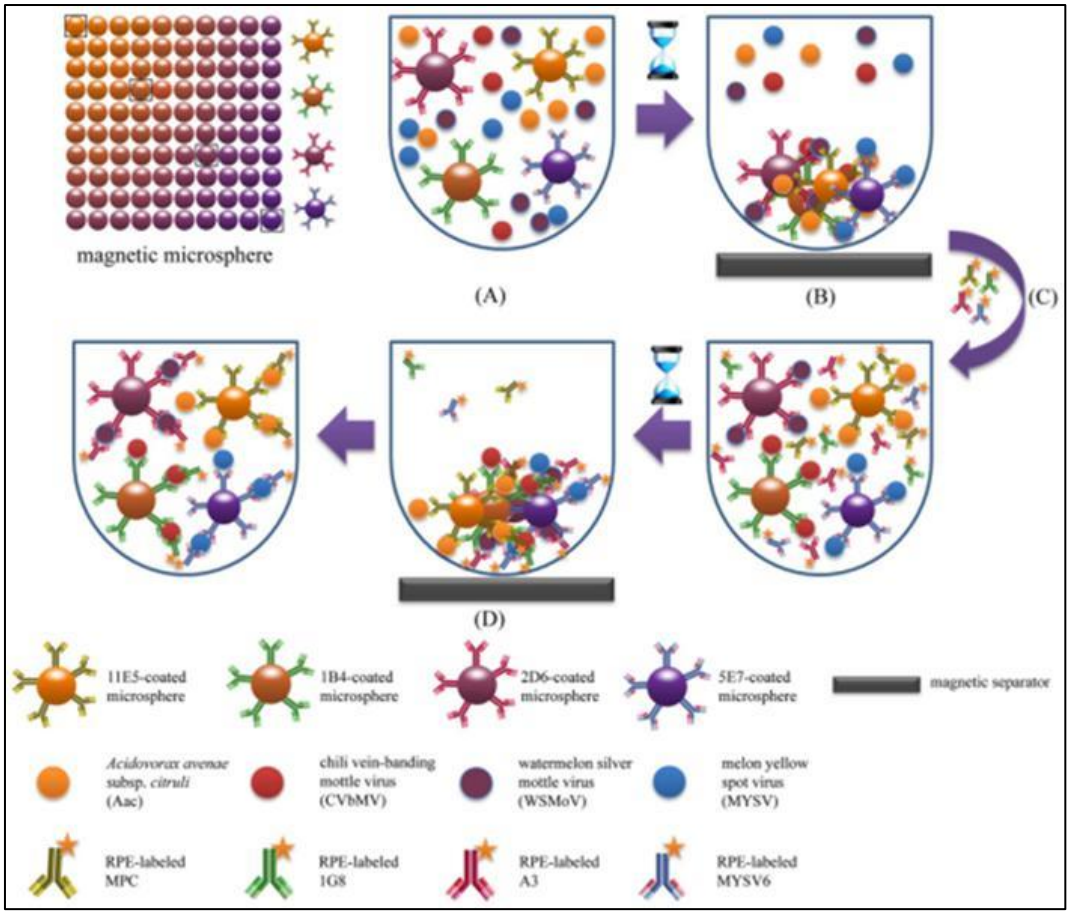

Fig 6. (A) The specific antibody-coated microspheres were mixed samples and incubated. **(B)** The unbound antigens were washed and removed by using magnetic separator. **(C)** The cocktail of RPE-labeled antibodies was added and incubated. **(D)** The unbound RPE-labeled antibodies were washed and removed by using magnetic separator before signals acquired by Luminex machine.(Source: Charlermroj *et al.,* (2013).

MIA has been employed to simultaneously detect multiple plant pathogens (potyviruses, Watermelon silver mottle virus, Melon yellow spot virus, and Acidovorax avenae subsp.

citrulli) in cucurbits (Charlermroj *et al.*, (2017) (Fig. 7). The MIA showed 98-99% relative accuracy, 97-100% relative specificity and 92-100% relative sensitivity when compared to commercial ELISA kits and reverse transcription PCR (Charlermroj *et al.*, 2017).

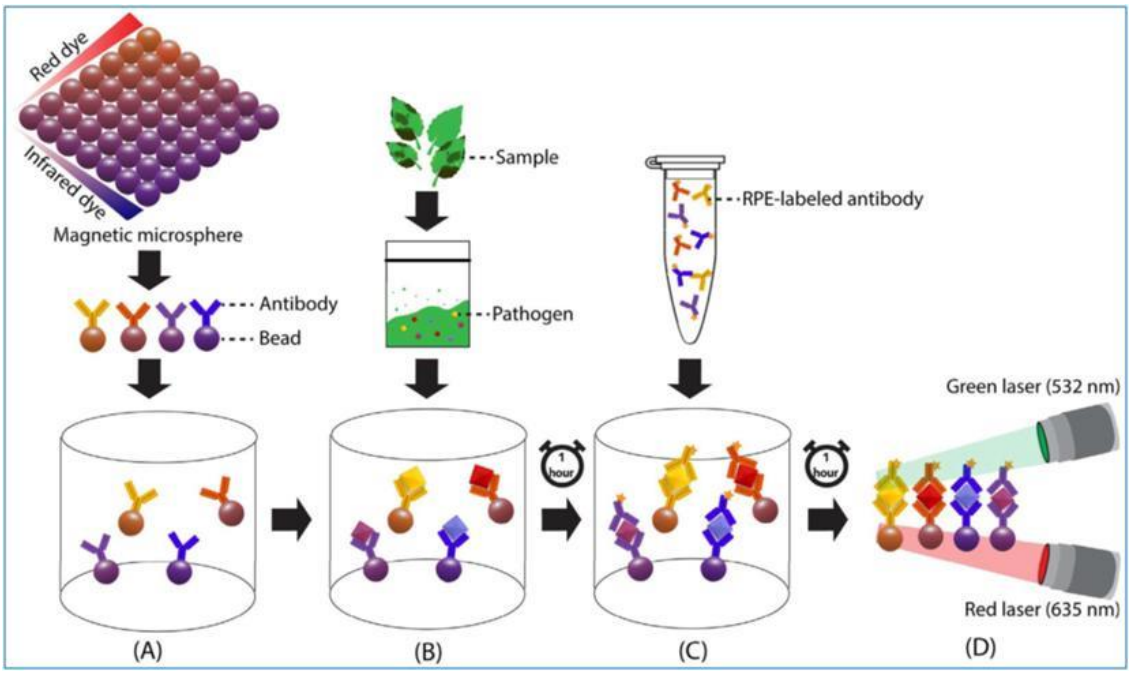

Fig. 7: Scheme of a microsphere immunoassay (MIA). **(A)** Specific antibody-coated microspheres were mixed. **(B)** Plant sample was extracted using the optimized extraction buffer and incubated with the antibody-coated microspheres. **(C)** A cocktail of R-Phycoerythrin (RPE)-labeled antibodies was added and incubated. **(D)** Each bead set was identified using a red laser (635 nm) and RPE signals were acquired using a green laser (532 nm) by a Luminex machine. (Source: Charlermroj *et al.*, 2017)

Detection of banana viruses using serological protein targeted methods

Various forms of enzyme linked immunosorbent assay (ELISA) with monoclonal and polyclonal antibodies are applied for detecting BBTV in field-grown plants, TC plants and viruliferous aphids (Wu and Su, 1990; Thomas *et al.*, 1991; Geering *et al.*, 1996; Thiribhuvanamala *et al.*, 2005; Selvarajan *et al.*, 2010). ELISA is convenient but limited in detection sensitivity, especially when very low concentrations of BBTV occur in samples (Su *et al.*, 2003). Serological detection of BSV can be achieved by different techniques, such as immunosorbent electron microscopy (ISEM) (Nodowora, 1998) and Double Antibody Sandwich (DAS) ELISA (Nodowora, 1998; Thottappilly *et al.*, 1998; Harper *et al.*, 2004; Meyer, 2005). The viral genome integrated in host chromosome is often amplified in standard PCR, which leads to false positives. To allow detection of episomal virus DNA, IC-PCR has been successfully applied (Harper *et al.*, 1997; Le Provost *et al.*, 2006). IEM sensitivity was proved to be similar to TAS-ELISA by sap dilution end point analyses (Agindotan *et al.*, 2006). Success of IC-PCR is dependent on the availability of specific antiserum of the virus

to be detected. Polyclonal antisera have been raised for a mixture of BSVs (Nodowora, 1998, Lockhart, 1986; Agindotan *et al.*, 2003) for use in IC-PCR. Chen *et al.,* (2013) reported the production of antibodies to the recombinant coat protein domain of BSV-Guangdon isolate which was effective at 1: 600 to 1: 6400 (v/v) dilutions when detecting BSV in infected plants. Sharma *et al.* (2014) developed recombinant antibodies to a putative coat protein gene of BSMYV and detected the virus from leaf samples having typical streak symptoms. Detection of episomal Banana streak Mysore virus using polyclonal antibodies raised against recombinant viral associated protein was developed (Selvarajan *et al.*, 2016). In direct antigen-coated (DAC) -ELISA, antibodies reacted specifically to BSMYV in crude sap, up to 1: 8000 dilutions, but not with healthy leaf extracts. Serological (Espino *et al.*, 1990) and combined techniques like IC-RT–PCR have been reported for detecting BBrMV (Rodoni *et al.*, 1999; Iskra-Caruana *et al.*, 2008). IC one-step RT–PCR assay enabled detection of BBrMV in leaf extract diluted up to 1X1010 (Iskra-Caruana *et al.*, 2008). Serological methods such as Direct Antigen Coating (DAC) ELISA, DAS-ELISA and Dot Immuno binding assay to detect CMV Banana isolate have been reported (Kawano *et al.*, 1995; Kiranmai *et al.*, 1996). Immunocapture multiplex PCR has been successfully used to detect BBrMV, CMV and BBTV (Selvarajan *et al.*, 2008; Sharman *et al.*, 2000). At present, LFIA's are not available for on-site or in-field use to the stakeholders of banana industry. Recently we have developed LFIA (Fig.1) for two banana viruses namely BBrMV and CMV (communicated, Selvarajan). The result could be evaluated visually by seeing the pink bands developed on the LFIA strips within 5-10 min. The detection limit of the test was up to 10 ng of expressed viral coat protein and 1:20 dilution of crude extract). These tests are highly accurate, when compared with traditional microplate enzyme-linked immunosorbent assays.

Conclusion

Plant virus detection become very important in eliminating the infected materials. Serological based methods are very robust and easily adopted in any environment. With little technical knowledge the protein based technique can be used for the detection of dplant viruses whereas NA based techniques which need sophisticated insturments and high skill for the adoption. For routine detection of viruses in planting material especially the clonal propagated plants serological based kits are highly useful. To ensure virus free planting material high throughput method of choice would be serological based rather NA for most of the viruses with an exception to viroids. The ELISA and LFIA dipsticks are available from many private firms in a easy to use forms. On-site detection of viruses by any illiterate growers is possible with the dipsticks which are readily available in the markets.

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Genome based developments in diagnosis of phytoplasmas

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Abstract

The impact of phytoplasmas in agriculture has become serious and early diagnosis is the best option to prevent the disease spread. Many times, the symptoms-based diagnostic is not sufficient or able to discriminate among the diverse pathogens. Until the early 1980s, phytoplasma diseases were detected by symptoms, grafting, transmission electron microscopic observation and DAPI staining. Enzyme-linked immunosorbent assay (ELISA) was rarely used since the antisera were developed only for a few phytoplasma-associated diseases. Around 1990, advances in molecular biology enabled direct detection of phytoplasma DNA by hybridization and polymerase chain reaction technologies. PCR amplification of the 16S rRNA genes of phytoplasmas has become the key in phytoplasma diseases detection and now, several variants of PCR like nested PCR, quantitative PCR, microarrays, and NGS are used for detection of phytoplasmas. The approach using RFLP analyses of PCR amplified 16S rDNA fragments provides a simple, reliable and rapid means for differentiation and identification of known phytoplasma strains. Recently isothermal amplification methods like LAMP and RPA have been developed for specific and sensitive diagnosis of phytoplasamas which are used withour thermal cyclers.

Introduction

Phytoplasmas are cell wall-less bacteria having very small size ranging from 200 to 800 nm (Fig. 1) and associated with diseases of several plant species. These disorders are characterized by unique symptoms of phyllody, witches'broom, flat stem, leaf yellows, decline, flower malformation, growth aberrationsn (Fig. 2). They are strictly host-dependent and can survive and multiply only in plant phloem tissues or insect emolymph. The phytoplasma genome is very small (600 to 1,200 kbp) and its phylogenetic studies propose that the common ancestor for phytoplasmas is *Acholeplasma laidlawii*. They can transmit by dodder, grafting and through leafhoppers (Bertaccini and Lee 2018).

The development of a reliable method for the detection of phytoplasmas in infected tissues is still one of the biggest challenges for the study of phytoplasmas. The long-time absence of a proper culture media for these bacteria has a direct impact in the available information of universal molecular targets for the development of improved detection methods. This information is of high concern regarding the impact of the disease in agriculture worldwide, because the control of this pathogen is based mainly in the prevention of the infection, due to

the absence of a direct field control of the pathogen (Bertaccini *et al.,* 2014). Up to now, 36 ribosomal groups and more than 200 subgroups of phytoplasmas have been identified, that are able to infect more than 800 species of plants. All these groups were defined on the bases of 16SrRNA gene sequences, although other conserved genes have also been used to strengthen, cross confirm this classification. The phytoplasma identification relied for more than two decades on the basis of diagnostic techniques such as observation of symptoms, insect or dodder/graft transmission to host plant.

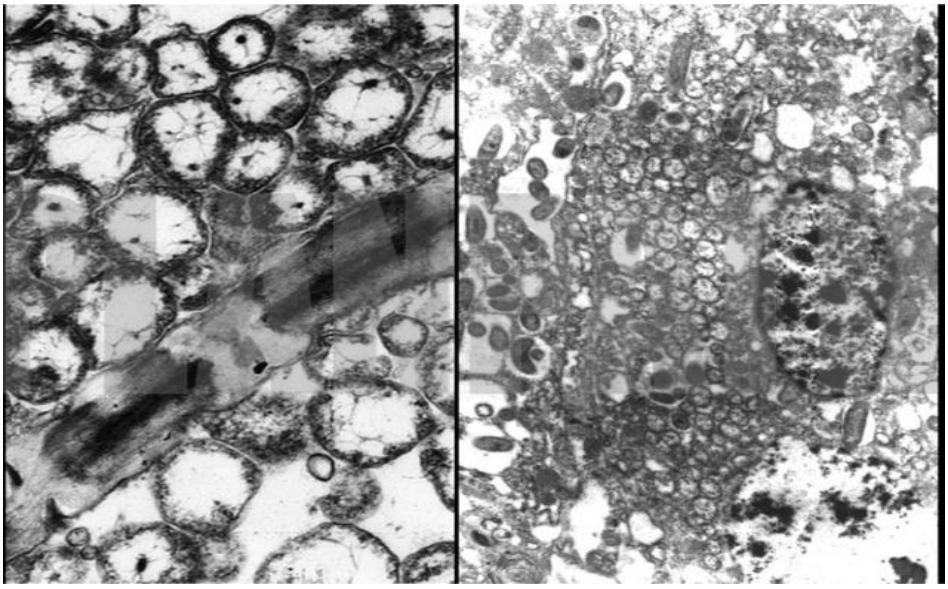

Fig. 1: Polymorphic phytoplasmas bodies inside phloem tissues of infected plants

DAPI staining protein together with electron microscopy observation of ultra-thin sections of the phloem tissue. Phytoplasma strains were differentiated and identified by their biological properties, such as the similarity in symptoms induced in infected plants, plant hosts and insect vector ranges (Lee and Davis 1992) but this was laborious and time-consuming, and often the results were inconsistent. Serological diagnostic techniques for the detection of phytoplasmas began to emerge in the 1980's when polyclonal and monoclonal antisera were produced and tested for the phytoplasma detection using fast and handling methods such as ELISA and immunofluorescence (Lee *et al.,* 1993a; Chen *et al.,* 1994). However, in the last 20 years, the applications of DNA-based methods allowed to distinguish different molecular clusters inside these prokaryotes. Molecular detection methods include dot and Southern blot hybridization and PCR technology. Dot and Southern blot hybridization assays were also used in phytoplasma detection for some years; however, both are currently completely replaced by PCR assays. More recently, different variants of PCR (nested PCR, quantitative PCR) techniques have been developed that have been shown to be effective methods of phytoplasmas diagnosis (Christensen *et al.,* 2004; Rao *et al.,* 2019). PCR assays using

universal primers are now routine assays for the detection of phytoplasmas in symptomatic plants.

Fig. 2: (a) Sesame phyllody; (b) Sugarcane white leaf; (c) Chrysanthemum phyllody; (d) Petunia flat stem; (e) Brinjal little leaf; (f) Toona witches'broom; (g) Rose flower

Several universal and many phytoplasma group-specific primers have been designed for detection of phytoplasmas (Table 1). Nested PCR assay, designed to increase both sensitivity and specificity, was performed by using a group-specific primer. Therefore, nested-PCR is capable of detection of dual or multiple phytoplasmas present in the infected tissues in case of mixed infection (Lee *et al.*, 1994, 1995). The majority of primers used in PCR are derived from 16S ribosomal gene, that due to the conservation and the presence also of variable regions is ideal for phylogenetic analysis that are the basis for the classification (Lee *et al.*, 1993, 1998).

DNA extraction

The quality of the DNA extraction is the first high-relevance step that must be considered in phytoplasmas detection. Since phytoplasmas reside almost exclusively in sieve tubes, the starting material for DNA extraction should include as much phloem tissue as possible. Plants contain also many secondary metabolites, such as polyphenols and polysaccharides, which may inhibit the polymerase reaction and they are significantly increased in phytoplasma infected plants (Choi *et al.*, 2004). PCR inhibition can be overcome by diluting the DNA extracts (Padovan and Gibb 2001; Heinrich *et al.*, 2001; Brzin *et al.*, 2003). It is therefore essential to remove inhibitory substances during the DNA extraction step. The polymerase chain reaction (PCR) technology is the widely used method of choice for phytoplasma detection and requires a quality template DNA extracted from diseased plants of suitable quality and concentration. The resulting phytoplasma-enriched pellet is then processed using CTAB-based buffer followed by chloroform/isoamyl alcohol extraction prior to isopropanol precipitation. This procedure is effective in producing highly purified DNA from fresh tissues from a wide variety of herbaceous and woody plants (Ahrens and Seemuller 1992; Doyle and Doyle 1990). Commercially available kits have also been employed for DNA extraction from diseased plants for detection of phytoplasmas by PCR assays.

Target selection

Phytoplasma differentiation is routinely based on amplification of 16S rRNA gene sequences and allowed to distinguish 34 ribosomal groups (16Sr) and more than 200 subgroups (Bertaccini and Lee, 2018). The use of additional genetic markers could enhance the discrimination resolving power for finer phytoplasma strain distinction. During the last years, a constant increase of the available genetic information on phytoplasma strains have led to the identification of several potential molecular markers: despite this, the 16SrRNA gene is the most used marker for the development of new protocols (Lee *et al.*, 1998; IRPCM, 2004).

Since the first phytoplasma specific primers were developed in 1991 (Deng and Hiruki 1991), several primer pairs have been tested for phytoplasma DNA amplification (Table 1). The P1/P7 primer pairs are the external primers that amplify the longest fragment that includes the almost entire sequence of 16S ribosomal RNA gene, 16S-23S ribosomal RNA intergenic

spacer, the complete tRNA-Ile gene and a small portion of 23S ribosomal RNA gene (Fig. 3). P1 matches a highly conserved region in the beginning of 16S gene, being present in most of the *Mollicutes* species. In the other extreme of the gene, P7 primer shows high conservation among phytoplasmas, but when is compared against other *Mollicutes*, two or three nucleotide polymorphisms are present in 3' region, depending on the species. This generates a high specificity of the PCR for phytoplasma amplification. The following primer pair that is widely used for phytoplasma detection is R16F2n/R16R2, being also the most useful for obtain the amplification product mainly used for phytoplasmas classification. The resulting 1,244 bp amplicon is widely used for phylogeny studies and also for rapid identification in association with RFLP techniques (Zhao *et al.*, 2009).

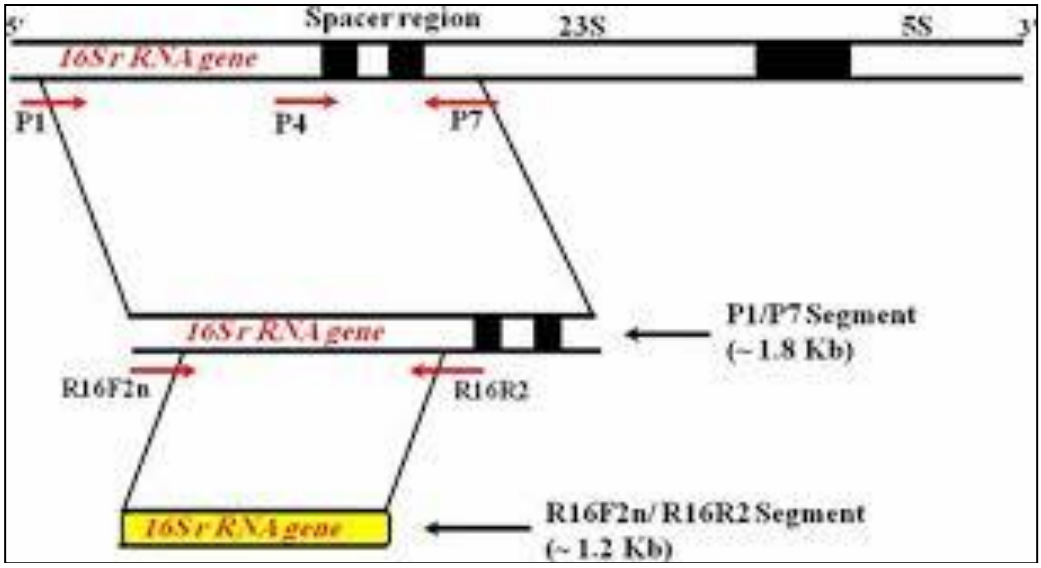

Fig. 3: Location of primer region in phytoplasma

Multilocos gene specific detection

Some additional genetic tools for phylogenetic analyses and finer strain differentiation procedures have been developed. More variable single copygenes, such as ribosomal proteins (*rpl22* and *rpS3), SecY, SecA, tuf,* gyrA, B, and *GroEL* have been reported to be suitable for differentiation of some phytoplasmas (Betaccoini *et al.*, 2014; Bertaccini *et al.*, 2019) . Classification of phytoplasma strains can, however, become more refined and specific if moderately conserved genes, such as the ribosomal protein (*rp*) genes, are used as genetic markers. The use of additional genetic markers enhances the resolving power of phytoplasma classification. RFLP analysis of amplicons obtained by group- or subgroup-specific *rp* gene-based primers is used for finer differentiation of phytoplasma strains within a given group or subgroup. The *rp* gene-based classification not only readily resolves 16Sr subgroups within a given 16Sr group, but also provides finer differentiation of closely related phytoplasma strains. Ribosomal protein (*rp*) genes are more variable than 16S rRNA genes and have more

phylogenetically informative characters, which substantially enhances the resolving power of classifying distinct phytoplasma strains within a given 16Sr group.

In recent years, interest focused on conserved and less-conserved nonribosomal genes, such as *secA, tuf, map, secY, pnp* and ribosomal protein genes, or variable genes encoding surface proteins such as *vmp1*, *imp*, *amp*, *stamp*, and *hfl*B. Genetic and phylogenetic analyses of these genes have led to precise phytoplasma strain classification and differentiation, with discoveries in population genetics such as the existence of interspecies recombination in group specific detection.

Table 1. List of universal primers for amplification of 16SrRNA gene of phytoplasmas.

Name	Sequence 5'-3'	Gene	Reference
P1	AAGAATTTGATCCTGGCTCAGGATT	16Sr	Deng and Hiruki 1991
P7	CGTCCTTCATCGGCTCTT	23Sr	Schneider *et al.*, 1995
R16F2n	GAAACGACTGCTAAGACTGG	16Sr	Gundersen and Lee 1996
R16R2	TGACGGGCGGTGTGTACAAACCCCG	16Sr	Lee *et al.*, 1995
F1	AAGACGAGGATAACAGTTGG	16Sr	Davis and Lee 1993
B6	TAGTGCCAAGGCATCCACTGTG	16Sr	Padovan *et al.*, 1995
R16mF2	CATGCAAGTCGAACGGA	16Sr	Gundersen and Lee 1996
R16mR2	CTTAACCCCAATCATCGA	16Sr	
P1A	AACGCTGGCGGCGCGCCTAATAC	16Sr	Lee *et al.*, 2003
P7A	CCTTCATCGGCTCTTAGTGC	23Sr	
U5	CGGCAATGGAGGAAACT	16Sr	Lorenz *et al.*, 1995
U3	TTCAGCTACTCTTTGTAACA	16Sr	
M1(758f)	GTCTTTACTGACGC	16Sr	Gibbs *et al.*, 1995
M2(1232r)	CTTCAGCTACCCTTTGTAAC	16Sr	
R0	GAATACCTTGTTACGACTTAACCCC	16Sr	Lee *et al.*, 1995
P3	GGATGGATCACCTCCTT	16Sr	Schneider *et al.*, 1995
P4	GAAGTCTGCAACTCGACTTC	16Sr	
P5	CGGCAATGGAGGAAACT	16Sr	
16Sr-SR	GGTCTGTCAAAACTGAAGATG	IS	Lee *et al.*, 2006
Pc399	AACGCCGCGTGAACGATGAA	16Sr	Skrzeczkowski *et al.*, 2001
Pc1694	ATCAGGCGTGTGCTCTAACC	IS	
PA2f	GCCCCGGCTAACTATGTGC	16Sr	Heinrich *et al.*, 2001
PA2r	TTGGTGGGCCTAAATGGACTC	IS	
SN910601	GTTTGATCCTGGCTCAGGATT	16Sr	Namba *et al.*, 1993
SN910502	AACCCCGAGAACGTATTCACC	16Sr	
1F7	AGTGCTTAACACTGTCCTGCTA	16Sr	Manimekalai *et al.*, 2010
7R3	TTGTAGCCCAGATCATAAGGGGCA	16Sr	
3Fwd	ACCTGCCTTTAAGACGAGGA	16Sr	
3rev	AAAGGAGGTGATCCATCCCCACCT	16Sr	
7R2	GACAAGGGTTGCGCTCGTTTT	16Sr	
5Rev	ACCCCGAGAACGTATTCACCGCGA	16Sr	

Source: Bertaccini *et al.*, (2019)

Restriction fragment length polymorphism analysis

Since the collective RFLP pattern characteristic of each phytoplasma is unique (Lee *et al.*, 1998) the PCR/RFLP analyses on 16Sr RNA gene ideally allow detection and differentiation of all phytoplasmas. This system allocates the worldwide detected phytoplasmas into 36 groups and more than 200 subgroups (Bertaccini and Lee 2018). Moreover, this system is more flexible for epidemiological studies than the use of the '*Candidatus*' taxa designation (IRPCM 2004) adopted until now for 43 phytoplasmas. RFLP analysis of randomly cloned chromosomal DNA fragments has also been employed, mainly to differentiate closely related phytoplasmas (Daire *et al.,* 1997; Botti and Bertaccini 2003). Streamlined computer-assisted RFLP analysis for rapid identification and classification of phytoplasmas was developed (Wei *et al.,* 2007; Zhao *et al.,* 2009). This work resulted in an expanded classification scheme in which ten possible new phytoplasma 16S rRNA groups and numerous sub-groups were identified (Lee *et al.*, 2007) and are available in the *i*PhyClassifier a web-based research tool for quick identification of phytoplasma strains.

Cloning and sequencing of PCR products

The cloning of the amplified product followed by sequencing is another complementary technique that can be used for phytoplasma identification. Amplified products are purified by gel elution/purification and sequenced directly or cloned prior to sequencing. For cloning, DNA fragments are ligated into plasmid vector e.g. pGEM-T and recombinant plasmid used to transform *Escherichia coli* strains. Plasmid DNA is purified and sequencing of both strands is performed by Sanger sequencing method. Primers for sequencing PCR products are the same as for PCR amplification whereas the standard primers are used for sequencing the cloned fragments. The sequences are then compared with known phytoplasma sequences in GenBank using the Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nlm.nih.gov/blast).

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) also known as real-time polymerase chain reaction offers several advantages over the conventional PCR. It is a fast, sensitive, and reliable detection technique amenable to high throughput. Two fluorescent chemistries are available, intercalating dyes or hybridization probes. Intercalating dyes are relatively less expensive than TaqMan® hybridization probes, but the TaqMan® hybridization probes is the most commonly used for phytoplasma detection. The qPCR may be designed for universal detection of phytoplasmas, group or subgroup specific detection, or for simultaneous detection of several phytoplasma strains. qPCR plays an important role in phytoplasma detection as well as in host-pathogen interaction and in epidemiological studies. The technology represents an advanced variant of the polymerase chain reaction, in which the accumulation of amplified fragments of nucleic acid is measured as the increase of fluorescent signal during each reaction cycle.

Microarrays

This technology offers a generic assay that can potentially detect and differentiate all phytoplasmas in one assay (Nicolaisen *et al.,* 2013). DNA microarray is a powerful tool for identification and differentiation of phytoplasma strains and detection is based on oligonucleotide probes spotted on glass slides, allowing one sample to be analyzed on each slide. However, formats for higher throughput have also been developed, such as multiple arrays on each slide (Nicolaisen and Bertaccini 2007), and arrays in microtitre plates. The key factor in microarray development is the probe design that starts with identification of the most suitable target region. Probes of different lengths can be used on microarrays depending on the application. Short probes (15–25 nt) exhibit reduced sensitivity but show better discrimination of minor sequence differences, whereas longer probes (>50 nt) show increased sensitivity but do not discriminate between target sequences with only few sequence differences.

Loop-Mediated Isothermal Amplification (LAMP)

The LAMP method has been widely used due to its high efficiency, specificity and simplicity (Notomi *et al.,* 2000). It requires two long outer primers and two short inner primers that recognize six specific sequences in the target DNA. The first inner primer containing sense and antisense sequences in the DNA which hybridize the target sequence and initiate DNA synthesis. Next, the outer primer carries out the strand-displacement DNA synthesis and produces a single stranded DNA which works as a template for the second inner and outer primers producing a DNA molecule with a loop structure (Nagamine *et al.,* 2002). The unremitting cycling reaction accumulates products with repeated sequences of target DNA of different sizes. The LAMP method is considered superior to the PCR and microarray-based methods due to its cost-effectiveness, high specificity, better sensitivity, and convenient procedure (conducted at constant temperature without the need for a thermal cycler) and evaluation. LAMP amplicons can be easily visualized by color indicators, the turbidity of magnesium pyrophosphate formed during the reaction (precipitate) or by agarose gel electrophoresis (Goto *et al.,* 2009). LAMP assays have been successfully used to detect phytoplasmas in several crops (Dickinson 2015).

Recombinase polymerase amplification (RPA) assays

The RPA is another rapid, isothermal amplification method with high specificity and sensitivity and does not require an initial heating step to denature the target DNA as it relies on an enzymatic activity to separate the dsDNA in order to the assist primer binding to the target sequences (Yan *et al.,* 2014; Lobato and O'Sullivan 2018). In RPA, the isothermal amplification of specific DNA fragments is achieved by the combination of enzymes and proteins, *viz.* the recombinase, single-stranded binding proteins (SSB) and strand displacing polymerase, used at a constant low temperature. The displaced strand is further stabilized by

SSB and the polymerase initiates synthesis. RPA products can be visualized on gel after purification, although alternate methods, such as fluorescence and/or hybridization. The reaction begins with the integration of a recombinase protein with the primers prior to their annealing to specific sequences in the target (Zhang *et al.*, 2014). Following primer annealing, the recombinase dissociates from the primers and leaves their 3' end accessible to the DNA polymerase to initiate the amplification. This creates a d-loop which is stabilized by a single stranded binding protein (SSB) to keep the DNA open as a DNA polymerase with strand displacement activity continues the amplification Using RPA, billions of DNA copies can be generated efficiently in 60 min with an incubation temperature between 37^{o}C and 42^{o}C (Yan *et al.*, 2014). The low incubation temperature and short reaction time make RPA a suitable assay for use in point-of-care diagnostic applications. RPA is highly sensitive with a detection limit as low as 6.25 fg of genomic DNA input with a specificity >95% (Boyle *et al.*, 2014).

Conclusion

The continuous effort to improve the phytoplasma diagnostic procedures aims at quicker and cheap and robust methods. Sensitivity is not an issue per se, as the current nested PCR protocols are extremely sensitive, but the achievement of high levels of sensitivity without the risk of false positive results that can be associated with nested PCR is highly desirable. The recent introduction of diagnostic assays based on quantitative PCR reduces the risk of amplicon contamination. Several other procedures like microarray, next generation sequencing, and isothermal amplifications methods are gaining importance for specific and senstivivcevdetection of leage indexing iof phytoplasma samples in less time, thus improving the efficiency or ability to detect multiple phytoplasmas in a single step. It should be noted, however, that the major limitation to the development of high throughput, robust diagnostic assays for phytoplasmas remains the difficulty in developing a rapid and cost/labour effective preparation of representative nucleic acids extracts. Due to the intrinsic characteristics of phytoplasma diseases, i.e. the low concentration and irregular distribution of the pathogens, it is unlikely that the field of diagnostics will see another boost such as that given by the introduction of the isothermal amplification techniques like LAMP and RPA, which needs further validation and confirmation with diverse plant samples. The efforts for the obtaining more efficient detection phytoplasma methods are still undergoing.

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Genome Assisted Diagnosis of Plant Pathogenic Phytoplasmas

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Introduction

Phytoplasmas, formerly known as mycoplasma-like organisms (MLOs), are a large group of obligate, endophytic, cell wall-less bacterial parasites classified within the class Mollicutes (Lee *et al.*, 2000). Phytoplasmas are known to infect more than thousand plants species including many economically important plants and crop species (Rao *et al.*, 2017). The typical symptoms shown by phytoplasma infected plants include: whitening, yellowing or reddening of the leaves indicating chlorosis, shortening of the internodes leading to stunted growth, smaller leaves and excessive proliferation of shoots resulting in a 'broom' phenotype, loss of apical dominance and phyllody (Lee *et al.*, 2000). Phytoplasmas were thought to be of viral origin since it could not be cultured in artificial media and could pass through a bacteria-proof filter. In 1994, the name 'Phytoplasma' was adopted by the Phytoplasma Working Team at the 10th Congress of International Organization of Mycoplasmology, replacing the term MLO (ICSB- Mollicutes, 1994, 1996). Reviews published from time to time gives good insight of phytoplasma studies including its taxonomy, etiology, transmission and interaction with insect and plant hosts (Lee *et al.*, 2000; Weintraub *et al.*, 2006; Hogenhout *et al.*, 2008; Sugio *et al.*, 2011; Rao *et al.*, 2017).

Timely detection and accurate pathogen characterization are one of the necessities for effective disease management and control. At the same time, assessment of imported product for the presence of pathogens can be unreliable when using methods based on visual inspection for the pathogen(s) or symptoms of infection, DNA hybridization (e.g. PCR-based assays) or antibody affinity (e.g. ELISA). Hence, effective plant pathogen diagnosis is an essential tool which will help deliver world food security. With a rising international trade in seeds and stock plants and agricultural intensification, there is an increasing likelihood of new and emerging diseases becoming established (Massart *et al.*, 2017). In view of this, the detection of '*Candidatus* Phytoplasma' is difficult due to the lack of disease symptoms in the early stages of plant life cycle and during the post-acquisition period of insect life cycle. The phytoplasma related disease management is multi-layered, complex task which include quarantine, surveillance and eradication effortswhere success of each of the task count on efficiency and cost-effectiveness of protocols used. The rapid detection using various forms of PCR help screening of large number of planting material in short period of time for the phytoplasma presence but cannot assign the taxonomic status, resulting into incomplete and precise diagnosis.

Existing Methods of Phytoplasma Detection &Diagnosis

The 16S rRNA genes have served as the primary character for phytoplasma molecular taxonomy and classification as it contains information for differentiation of a wide array of phytoplasma strains. Currently, there are two widely accepted phytoplasma classification schemes; one is based on phylogenetic analysis of 16S rRNA gene sequences (Kirkpatrick *et al.*, 1989; Namba *et al.*, 1993; Schneider *et al.*, 1993; Gundersen *et al.*, 1996; Smart *et al.*, 1996) and another is based on Restriction Fragment Length Polymorphism (RFLP) analysis of a 1245 bp PCR amplified 16S rRNA gene fragment (Lee *et al.*, 2000; Zhao *et al.*, 2009). Further, to automate the RFLP analysis, Zhao *et al.* (2009) designed an interactive online tool, *iPhyClassifier* to expand the efficacy and capacity of the current 16S rRNA gene sequence-based phytoplasma classification system. The *iPhyClassifier* performs sequence similarity analysis, simulates laboratory restriction enzyme digestions, subsequent gel electrophoresis and generates virtual restriction fragment length polymorphism (RFLP) profiles. Based on calculated RFLP pattern similarity coefficients and overall sequence similarity scores, *iPhyClassifier* makes suggestions on tentative phytoplasma 16Srgroup/subgroup classification status and '*Candidatus* Phytoplasma' species assignment. While both schemes can reliably classify diverse phytoplasmas into groups, the latter offers a faster mechanism, by distinguishing subtle RFLP pattern differences, to identify and differentiate distinct subgroup lineages among phytoplasmas within individual groups.However, both schemes require time consuming, multi-steps protocols to be followed including end-point PCR and Sanger Sequencing to generate near full-length sequence of single fragment of 16S rRNA gene. The *iPhyClassifier* requires a full or near full-length (~1245 bp), good quality 16S rRNA gene sequence (Gundersen *et al.*, 1996; Zhao *et al.*, 2009) for the accurate assignment of phytoplasma group and subgroup.

In addition to sequence information of 16S rRNA gene, several phytoplasma housekeeping genes were employed (known as' Multi-Locus Sequence Analysis', MLSA) for the better taxonomic resolution up to strain level. The phytoplasma 16S-23S rRNA intergenic spacer (IGS) region which is about 232 bp (varies in different species), contains a portion that codes for the highly conserved $tRNA^{Ile}$. The IGS region is comparable to the 16S rRNA gene sequence in its capacity for use in delineating distinct phytoplasma lineages (Smart *et al.*, 1996). Combined analysis of the entire16S rRNA gene plus IGS region sequence proved to be useful in several cases for differentiating distinct type of strains within a given 16S rRNA subgroup (Marcone *et al.*, 2000; Andersen *et al.*, 2006).The tuf gene, encoding the elongation factor, EF-Tu, is another highly conserved gene that has been frequently used to distinguish and classify phytoplasmas. It was found that tuf gene, like 16S rRNA gene, emerged as a potential marker for classification of phytoplasma (Makarova *et al.*, 2012). The resolving efficacy for separation of distinct lineages among phytoplasmas was found to be lower than that of the 16S rRNA gene (Marcone *et al.*, 2000). Further, DNA sequence of SecA and 23S

rRNA gene (Hodgetts *et al.*, 2008) and SecY (Lee *et al.*, 2005) were employed for classification of phytoplasmas. The sequence similarity for 480bp amplicon of SecA ranged from 69.7 to 84.4% for phytoplasma strains representing 12 16SrRNA groups. Several molecular markers, other than the 16S rRNA gene, identified, have thus shown much-improved resolving power in delineation of these ecological strains. It has to be noted that the phytoplasma MLSA system to diagnose the phytoplasma strains rely on highly accurate gene sequences obtained from individually run end-point PCR assays and dideoxy sequencing method.

Next Generation Sequencing (NGS)Assisted Diagnosis of Plant Pathogens

Next generation sequencing (NGS) is a technology with rapidly expanding possibilities including potential applications for the detection of plant pathogens including viruses, fungal, bacterial pathogens and phytoplasmas. The availability of genome sequences obtained using next-generation sequencing (NGS) has revolutionized the field of infectious diseases. The tremendous amounts of data have not only enabled advances in fundamental biology, helping to understand the pathogenesis of microorganisms and their genomic evolution, but have also had implications for overall microbiology. NGS technologies allow a generic approach to pathogen identification that does not necessarily require prior knowledge on the targeted pathogens and can deliver a species/strain specific result (Adams *et al.*, 2009; Pecman *et al.*, 2017). However, the genome assisted diagnosis of plant pathogens including bacteria and virus is still in the infant stage. More than 38,000 bacterial and 5,000 viral genomes have been sequenced to date, however most of them are representatives of significant human pathogens. The pathogen detection bioinformatic workflow requires to be standardized for the routine detection and diagnosis of plant pathogens including viruses and phytoplasmas including the possibility of providing a method for the identification of novel viruses and/or strains. The genomes of many crops are still unknown or incomplete, and plant virus and phytoplasma sequences are poorly represented in databases.

Genome assisted diagnosis of Sugarcane Grassy Shoot (SCGS) phytoplasma, a case study

In order to develop reliable genome assisted diagnosis of SCGS phytoplasma, we genome sequenced the 16SrXI-B subgroup strain SCGS phytoplasma. The 16SrXI group phytoplasma conventionally belongs to the Rice Yellow Dwarf (RYD) group which consist of three phytoplasma species viz. '*Ca*. Phytoplasma oryzae' (Jung *et al.*, 2003), '*Ca*. Phytoplasma cynodontis' (Marcone *et al.*, 2004) and '*Ca*. Phytoplasma cirsii' (Safarova *et al.*, 2016). The SCGS phytoplasma shows 98.07 % and 97.67 % similarity with *Ca*. Phytoplasma cynodontis and *Ca*. Phytoplasma oryzae, respectively leaving researcher confused about its species delineation. In order to achieve the clear taxonomic status of SCGS phytoplasma, which in turn proved to be useful in its accurate diagnosis, we genome sequenced SCGS phytoplasma

along with the strain of 16SrXIV-A (a representative of *Ca*. Phytoplasma cynodontis). The genome sequence of two strains related to *Ca*. Phytoplasma oryzae were already present in the GenBank database which further helped in accurate data analyses. TheBacterial Pan Genome Analysis Pipeline, BPGA (Chaudhary *et al.*, 2016) lead to genome-based phylogeny of phytoplasma strains with 45 core sequences whose genome sequences were available. This work further lead to shortlisting of key phytoplasma genes viz. Secretary Protein 'A' subunit (secA), Secretary Protein 'E' subunit (secE), Secretary Protein 'Y' subunit (secY), LeucinetRNA ligase (leuS), Tu Elongation Factor (tuf), Molecular Chaperons (GroEL), ribosomal proteins (rp) and 16S rRNA gene sequences from the obtained genomes. This will prove beneficial in upcoming designing of primers for genome assisted diagnosis of SCGS phytoplasma along with its closest taxonomic neighbor strains related to '*Ca*. Phytoplasma oryzae' and '*Ca*. Phytoplasma cynodontis'.

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Exploring the relationship between thrips and tospoviruses

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Thrips (Thysanoptera: Thripidae) are economically important insect pests of numerous crops grown all over the world (Childers and Achor 1995). The name of the order was derived based on the characteristic fringed wings. In the Greek 'thysanos' means 'fringe' and 'pteron' means 'wing'. Thrips are mostly yellow, orange, black, black or whitish-yellow in colour. They crawl leisurely or fast when disturbed. The apex of the abdomen is flexed upward to leap from the plant surface. The life cycle of thrips consists of eggs, first and second in star larvae, pre-pupa, pupa, and adults. The developmental period varies depending on the temperature. The pre-pupae and pupae do not feed and remain mostly inactive. They move very slowly only if disturbed. The adult female makes a slit with their ovipositor in tender plant parts and eggs are embedded in the upper epidermal layer of plant tissues. The reproduction of thrips is haplodiploid and capable of parthenogenesis. The thrips damage leaf tissue by probing into cells and imbibing the sap. Feeding of thrips causes damage to the leaves, flowers or fruits and sometimes induces galls.

Besides being important insect pests, thrips act as vectors of tospoviruses (genus *Orthotospovirus*, family *Tospoviridae*). Thrips transmit tospoviruses in a persistent and propagative manner (Ullman *et al.*, 1992, 1997; Whitfield *et al.*, 2005). Thrips acquire the tospoviruses only during their first instar larval stage and generally transmit the virus to plants after becoming adults (van de Wetering *et al.*, 1996). The studies on thrips and tospoviruses indicate a strong co-evolutionary relationship between them (Whitfield *et al.*, 2015). Sixteen species of thrips are known as the vectors of tospoviruses and more than 29 tospoviruses are recorded throughout the world (Rotenberg *et al.*, 2015; Turina *et al.*, 2016). *Frankliniellaschultzei*, *Scirtothripsdorsalis*, *Thrips palmi*, and *T. tabaci* have been reported to transmit five tospoviruses in India (Ghosh *et al.*, 2017; Mandal *et al.*, 2012). Recently, *Frankliniellaoccidentalis* and tomato spotted wilt virus (TSWV) have recorded in India (Renukadevi *et al.*, 2015; Tyagi and Kumar 2015; Suganthy *et al.*, 2016).

Tospoviruses affect both yield and quality of the crops, leading to reduced returns to the growers. Among the tospoviruses, groundnut bud necrosis virus (GBNV) is widely distributed in India and endemic in Andhra Pradesh, Gujarat, Haryana, Himachal Pradesh, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Orissa, Punjab, Rajasthan, Tamil Nadu, Uttar Pradesh, and West Bengal. GBNV alone is responsible for US$ 89 million per annum crop losses in Asia (Reddy *et al.*, 1995).

Table 1: Tospoviruses and their thrips vectors

Tospovirus	Thrips vector
Groundnut bud necrosis virus	*Thripspalmi, Frankliniellashultzei*
Watermelon bud necrosis virus, watermelon silver motile virus, melon yellow spot virus, Calla lily chlorotic spot virus	*Thrips palmi*
Capsicum chlorosis virus, tomato necrotic ringspot virus	*Thripspalmi, Ceratothripoidesclaratis*
Tomato spotted wilt virus	*Thripspalmi, Frankliniellashultzei, Frankliniellabispinosa, Frankliniellafusa, Frankliniellaoccidentalis, Frankliniellaintonsa, Thripstabaci, Thripssetosus*
Tomato chlorotic spot virus, chrysanthemum stem necrosis virus	*Frankliniellaintonsa, Frankliniellashultzei, Frankliniellaoccidentalis*
Groundnut ringspot virus	*Frankliniellashultzei, Frankliniellaoccidentalis, Franklinielllagemina*
Zucchini lethal chlorosis virus	*Frankliniella zucchini*
Impatiens necrotic spot virus	*Frankliniellafusa, Frankliniellaoccidentalis, Frankliniellaintonsa*
Iris yellow spot virus	*Thripstabaci, Frankliniellafusa*
Groundnut yellow spot virus, groundnut chlorotic fan-spot virus, Peanut chlorotic fan-spot virus, Peanut yellow spot virus	*Scirtothrips dorsalis*
Polygonum ringspot virus	*Dictyothripsbetae*
Soybean vein necrosis virus	*Neohydatothripsvariabilis*
Alstroemeria necrotic streak virus, tomato zonate spot virus	*Frankliniellaoccidentalis*
Tomato yellow (fruit) ring virus	*Thrips tabaci*

A 70-90% loss in groundnut due to GBNV was recorded in India (Singh and Srivastava 1995). *T. palmi* is a known vector for transmission of GBNV (Lakshmi *et al.*, 1995; Ghosh *et al.*, 2019). It feeds on more than 200 plant species (Walker, 1992, Cermeliand Montagne, 1993, Loomans *et al.*, 1999) belonging to family Cucurbitaceae, Solanaceae, Fabaceae, and Asteraceae. Losses of about 15% in potato and 90% in cucumber are reported due to infestation of *T. palmi* (Vercambre, 1989, Cooper, 1991, MacLeod *et al.*, 2004). It was considered to be restricted to Southeast Asia for many years, but it has been

widely introduced across the globe in recent decades (Capinera, 2008, Seal *et al.*, 2013). Some interactions have been reported based on *in vivo* study, like in the case of TSWV glycoproteins with an unknown midgut protein of *F. occidentalis* (Bandla *et al.*, 1998; Medeiros *et al.*, 2000). Gc protein is thought to facilitate entry of tospoviruses into thrips tissues, but this remains to be functionally demonstrated (Garry and Garry 2004; Whitfield *et al.*, 2005b). The present understanding of thrips-tospovirus interaction is largely based on F. occidentalis and TSWV. Not much is known about the relationships of thrips and tospoviruses. Based on the well-studied tomato spotted wilt virus (TSWV)-*Frankliniella occidentalis* relationship, it is understood that virus propagates in the thrips body and is transported through the midgut to the salivary gland (Ullman *et al.*, 1992, 1997; Whitfield *et al.*, 2005). Virus transmission occurs exclusively by adult thrips while virus acquisition takes place during the larval stages (Sakimura 1963).

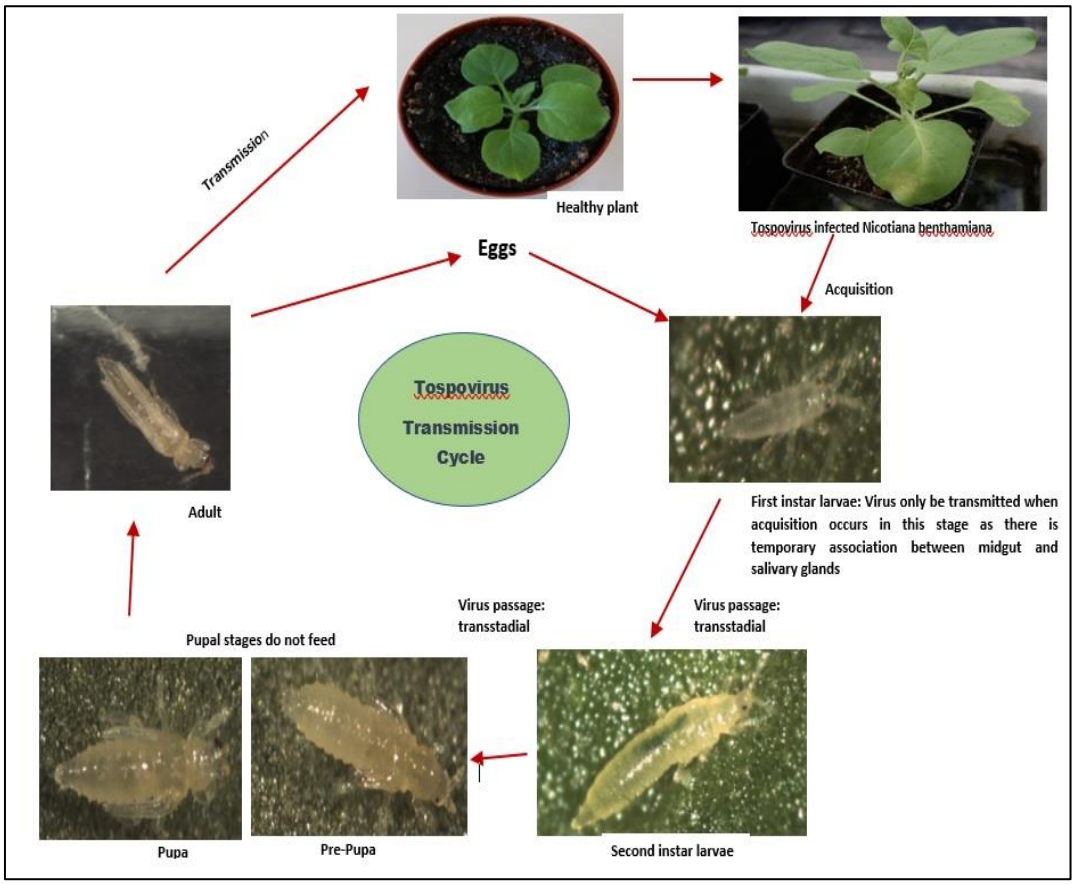

Fig. 1. Thrips-tospovirus relationship.

Differential transcriptomics and proteomics of *F. occidentalis* in response to TSWV have predicted few probable receptors for TSWV (Badillo-Vargas *et al.*, 2012; Schneweis *et al.*, 2017). The glycoprotein G_N of TSWV is involved in the primary binding to the thrips midgut epithelial cells, whereas G_C is necessary for fusion (Whitfield *et al.*, 2004, 2005; Garry and Garry 2004). Analyses of the available genome data for both *T. palmi* and GBNV show*T.*

palmi proteins including cathepsin, C-type lectin, enolase, clathrin, and vATPase subunit E are involved in direct interaction with GBNV glycoproteins to facilitate entry into thrips cells (Jagdale and Ghosh, 2019). C type lectin likely is the first cellular receptor with which GBNV-GN interacts to attach to thrips gut cells and then is internalized by endocytosis mediated by clathrin and vATPase. Infection of tospoviruses adversely affects the biological traits of *T. palmi*due to virus multiplication and movement within thrips body interfering with essential physiological processes of thrips (Ghosh *et al.,* 2019a). The developmental time of L1 was significantly extended post WBNV and GBNV exposure. The mean adult life span of both male and female *T. palmi* was reduced as compared to non-exposed *T. palmi*. WBNV- and GBNV-exposed females produced about 50% fewer offspring compared to non-exposed females. In case of survivability of each instar, GBNV appeared more pathogenic to *T. palmi* than WBNV. Tospovirus exposure favoured a female-biased ratio in the experimental population. Cryptic species of *T. palmi* having morphologically identical features are also common in *T. palmi*which exert differential responses to a tospovirus (Ghosh *et al.,* 2019b).

Genome assisted diagnostics of *T. palmi*

In the case of thrips, nucleotide sequences of several gene regions have been utilized for species discrimination and phylogenetic analyses (Brunner *et al.,* 2004; Asokan *et al.,* 2007; Inoue & Sakurai 2007; Hoddle *et al.,* 2009; Buckman *et al.,* 2013). Amongst them, the mitochondrial cytochrome c oxidase I gene (COI) is the most extensively used due to its wide acceptance as a universal barcode. The first molecular study of thrips based on the partial sequence of COI was reported in 1998 (Crespi *et al.,* 1998), whereas the characterization of COI of *T. palmi*was published for the first time in 2002 (Brunner *et al.,* 2002). Since then, about 250 nucleotide sequences of *T. palmi* COI have been submitted to the National Center for Biotechnology Information (NCBI) database to date. Such a high number of COI sequences has made the identification of *T. palmi* easy, irrespective of its wide geographical distribution and broad host range (Ghosh *et al.,* 2017). However, the mitochondrial genes of thrips are known for having a relatively fast evolutionary rate (Shao and Barker 2003). Based on the COI sequences, the possibility of cryptic species in *T. palmi*was studied (Ghosh *et al.,* 2019b). Based on a neighour joining (NJ) analysis of all the COI sequences of *T. palmi* available so far, the global *T. palmi* population forms three major clades. Three isolates, one each from China, Pakistan, and India branched separately to those. A high mean intraspecific distance (0.0621) was observed within the *T. palmi* population. A haplotype data file generated for all these COI sequences of *T. palmi* using DnaSP6 revealed 29 haplotypes (standard deviation of diversity 0.015) of *T. palmi* globally (Ghosh *et al.,* 2019b). The most common haplotype (n=121) has been shared among India, Pakistan, Japan, Thailand, Dominican Republic, China, USA and Taiwan. The next common haplotype (n=71) occurred in India, Pakistan and Bangladesh. There were two more low frequency haplotypes recorded

from India. All the Indonesian isolates formed a separate haplotype. Other than these, few more (~18) singleton clusters have been observed in the analyses.

Detection of tospovirus in *T. palmi*

Tospovirus infection in thrips can be detected through RT-PCR. Total mRNA from the viruliferous thrips can be isolated using TRIzol.

- cDNA can be synthesized from mRNA in a 20 µl reaction mixture containing, 4µl of 5% cDNA synthesis buffer, 2 µl of dNTP mix, 1 µl of RNA primer, 1 µl of RT enhancer, 1 µl of verso enzyme mix, 1 µl of RNA template and 9 µl of sterile distil water at following reaction conditions: 42^{o}C for 40 min and 95^{o}C for 2 min. Further, synthesized cDNA can be stored at -20^{o}C.
- Amplification of cDNA can be accomplished with GBNV-specific primers in a 25 µl reaction mixture containing, 2.5 µl 10X PCR buffer, 0.6520 µl dNTP mix, 1 µl each forward and reverse primer (FP 5' CCA TCT ACT TCA GTA GAA AAC ACT AG 3' and RP 5' AGA GCA ATC AGT GCA ACA ATT AAA TA 3'), 1 µl cDNA template, 0.15 TaqDNA polymerase and 18.7 sterile distil water. The reaction may be carried out at following conditions, initial denaturation 94^{o}C for 5 min., denaturation 94^{o}C for 30 sec., annealing 55^{o}C for 45 sec., extension 72^{o}C for 1 min., final extension 72^{o}C for 10 min.
- Amplification can be visualized on 1% agarose gel electrophoresis.

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Sequence based molecular phylogeny: basic concepts and application in understanding classification and taxonomy of plant viruses

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The development of new rapid, inexpensive next generation high-throughput technologies sequencing over the last 10 years or so is changing the ways we think about the application of sequences to plant virology. These techniques can be applied not only to partially purified and characterized viruses but also to the detection of potential new viruses in crude preparations by metagenomics. These new technologies rely on a combination of template preparation, sequencing, and imaging and on the analysis of the sequence data by assembly methods and genome alignment. They provide a vast amount of data that can be used for a wide range of purposes including virus classification, identification of new viruses, diagnostics, and ecological studies. The analysis and comparison of sequence data are playing an increasing role in virus classification. Most of these analyses and comparisons are undertaken using Bioinformatics. Bioinformatics, a new field of science includes biology, computer science, statistics and Information Technology. The sudden growth in the quantitative information in biology has resulted in realization of inherent bio-complexity issues which call for innovative tools to convert the information into knowledge. Bioinformatics, in one hand, involves computer specialists and statisticians for development of the tools and new algorithms for organizing and analyzing the data and in other hand helps biologists in understanding the structural and functional genomics, proteomics, protein engineering etc. using those tools (computational biology) in a biologically meaningful manner. In line with the theme of the "Central Dogma", bioinformatics utilizes the prediction approach to find out the sequence similarity in DNA that can lead to structural and functional similarity in protein and thus narrows down the search for understanding the functional role of a protein.

From the recognition by Ivanovski in 1892 that tobacco mosaic disease is caused and transmitted by fine pore filtrates (Lechevalier, 1972), viruses have been isolated, characterized, identified and studied from animals, plants, protists, bacteria and even other viruses (Flint *et al.,* 2008). The detection and identification of viruses are difficult due to poor resolution of the diagnostic tool used. However, with the advancement of science, technology is now providing a much better high resolution alternative method of virus detection with the development of recombinant DNA protocols and DNA sequencing methods. Due to ease of sequencing technology huge number of viruses is being sequenced every day. There are 27,091 full-length virus genomes deposited in GenBank as of 2010. The technology thus

allows a larger genomic view of viruses, particularly as populations rather than single entities. The data generated thus is needed attention for proper analysis to illustrate them in more meaningful way.

From the first virus genome sequence completed (MS2; 3.6 kb) (Fiers *et al.*, 1976) to that of the recent largest virus (mimivirus; 1.2 Mb) (Le Raoult *et al.*, 2004), the viral genome and its analysis have revealed high-resolution details of the molecular basis of a particular biological system, along with unexpected and surprising details.

As model organisms, viruses have served to increase our understanding across many fields of the life sciences, including medicine, biochemistry, genetics, cell biology, molecular biology, applied biology, biotechnology, etc. They have proven useful demonstrations of novel technical and methodological applications. And their relatively small genomes contain fascinating and often paradigm changing biological information. Viral genomes differ from other organism genomes in complexity, despite their generally smaller sizes and presumed "simplicity". For example, one problem is that the smaller size of the genome dictates a higher density of gene coding, with all six reading frames utilized. Coding regions frequently overlap.

As improved high throughput technology is available and more bioinformatic tools are developed, application of these methodologies to viruses will solve some of the outstanding biological questions of current times, and will allow new strategies to prevent outbreaks. In virology research, virus-related databases and bioinformatic analysis tools are essential for discerning relationships within complex datasets about viruses and host-virus interactions. Databases and bioinformatic tools that contain genomic, proteomic, and functional information have become indispensable for virology studies. Bioinformatic analysis on viruses involves the general tasks related to the analysis of any novel sequences, such as identification of open reading frames, gene prediction, base calling and assembly, homology searching, sequence alignment, and motif and epitope recognition. However, specific features in virus biology determine specific challenges to those general bioinformatics tasks. For example, many viruses have overlapping open reading frames or translational frameshifts. In addition, the extent to which related viruses undergo recombination often makes it impossible to achieve the success of classical phylogenetic analysis for evolutionary histories. Because of the volume and diversity of sequences available in virus databases such as strains, isolates, mutants and quasi species, it is necessary to have the required data organized and integrated in virus-specific repositories. The predictions of features such as transmembrane domains, glycosylation sites, and protein secondary and tertiary structure are important for analyzing the structure-function relationship of proteins encoded in viral genomes. Biochemical pathway analysis can help elucidate information at the biological systems level. Microarray analysis provides methods for high throughput screening and gene expression profiling. More recently, metagenomics, using direct nucleic acid extraction from uncultured

microorganisms, massive genome cloning, high-throughput DNA sequencing, advanced computational algorithm and bioinformatics, provides a new paradigm in plant virus diagnosis by direct detection of all or specific virus populations in a particular ecosystem.

The recent ICTV Reports on Virus Taxonomy "Species demarcation criteria in each genus." These criteria include nucleic acid sequence and amino acid sequence derived from the genome sequence data differentiation for many of the appropriate genera. Molecular phylogenetic analysis helps us identifying and grouping viruses.

Different terminologies those are being routinely used bioinformatics analysis are described below:

Terminologies and concept of sequence analysis

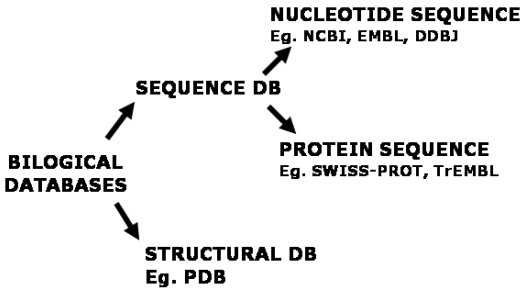
Biological Database:

Organized body of persistent data

Update

Query

Retrieve Components



Ways of submitting DNA sequence

- There are two principal ways of submitting DNA sequences to GenBank and EMBL.
- BankIt
- Sequin
- Webin-Align

Annotation

Refers to commentary or explanation of the information appended to DNA or protein sequences stored in databases.

Annotation can include:

Known information about

- Source, Country, Organism
- protein(s) sequence
- predicted protein structure
- domain(s) of the protein.

- quaternary structure of the protein.
- protein function
- common post-translational modifications of the protein

Data Retrieval

Collection of data from databases

Data Mining

Generation of information from data in databases. E.g. – primer designing, gene finding, phylogenetic relationship study etc.

Gene Finding approaches

- Content based approach: The content based approach relies upon the differences in composition of nucleotide bases between the coding exons and noncoding introns.The periodicity of repeats and compositional complexity of codon triplets differentiate the exons from introns.
- Site based approach: The gene has its own syntax. Start codon,stopcodon, donor and acceptor sequences, noncoding introns, ribosome binding sites, transcription factor binding sites, promoter sites, the poly adenylate sites etc are the specific signatures of genes
- Comparative method: The anonymous sequence is compared with cDNA sequence library.

Phylogenetic relationship study: Terminologies and Concepts

Homology:

This is a state of gene or morphological character that shares a common ancestry with a different gene or morphological character. For molecular sequence data, it is taken to mean that two sequences or even two characters within sequences are descended from a common ancestor.

This term is frequently misused as a synonym for 'similar', as in "two sequences were 70% homologous". This is totally incorrect! Sequences show a certain amount of similarity. From this similarity value, we can probably infer that the sequences are homologous or not.

Homology cannot be measured only we can say whether homology is there or not but we can measure similarity. Homologous sequence must have similarity, but if there is similarity we cannot say there is homology.

Homologous Gene Super family

A) Orthologous Gene

Same sequence and same function but found in different taxa. e.g. - DNA Polymerase of Goat, DNA Polymerase of Human. Result of lineage Transfer.

B) Paralogous Gene

Found in same taxa. Same sequence but different function. e.g. - Hemoglobin, Myoglobin. Result of a gene duplication.

Alignment

An Alignment is an computational hypothesis which identify positional similarity or identity between bases/Amino Acids. Two ways: Local and Global Alignment.

Sequence Alignment Tools

- BLAST
- FASTA
- BLITZ
- BEAUTY, a modified BLAST

BLAST (Basic Local Alignment Search Tool)

- BLAST is the algorithm used by a family of five programs that will align your query sequence against sequences in a molecular database.
- Statistical methods are applied to judge the significance of matches.
- Alignments are reported in order of significance, as estimated by the applied statistics.
- BLASTN: Compares a nucleotide query sequence against a nucleotide sequence database.
- BLASTP: Compares an amino acid query sequence against a protein sequence database.
- BLASTX: Compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.
- TBLASTN: Compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).

What We Know From BLAST

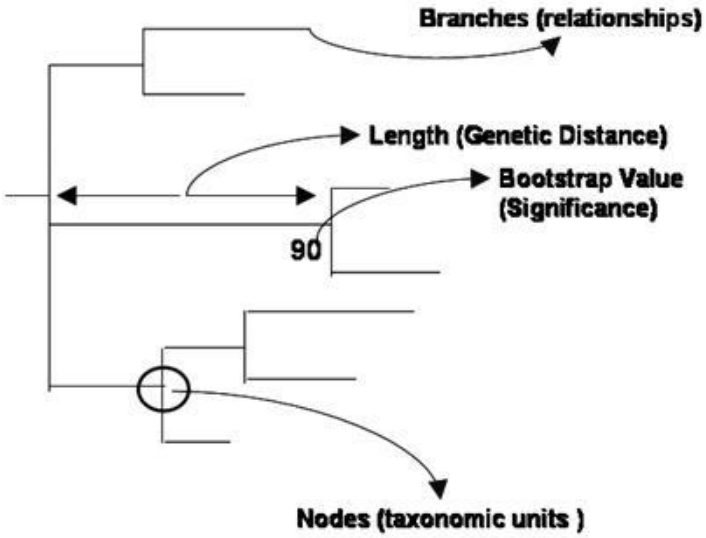
- Sequences that share similarity with query sequence
- Helps to retrieve those sequences

What We Do Not Know From BLAST

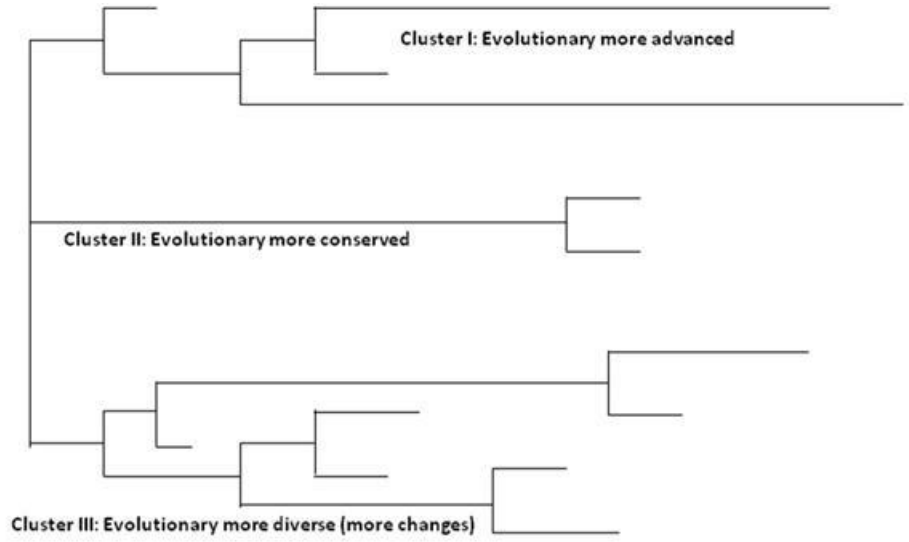
- Can not quantify the sequence similarity
- Cannot tell us about the relationship between all those sequences

Multiple Alignment: ClustalW

Quick pairwise alignment: calculate distance matrix …> guide tree…> Progressive alignment following guide tree



The branching pattern of a tree is called the TOPOLOGY



Representation of relationship through LINE: DENDROGRAM

Types of dendrogram

Phylogram

This is a phylogenetic tree that indicates the relationships between the taxa and also conveys a sense of time or rate of evolution. The temporal aspect of a phylogram is missing from a cladogram.

Cladogram

A dendrogram depicting the hypothesised branching order of a number of sequences. Cladograms do not give any indication of temporal change, but phylogram does.

Rectangular Cladogram / Phenogram – Suitable for grouping in taxonomic studies

Slanted Cladogram – Suitable for understanding convergence, divergence or parallelism in evolutionary studies

Presentation of a tree

Rooted tree: Assume that all taxa derived from a common ancestor

Unrooted tree: Assume that all taxa derived not from a common ancestor

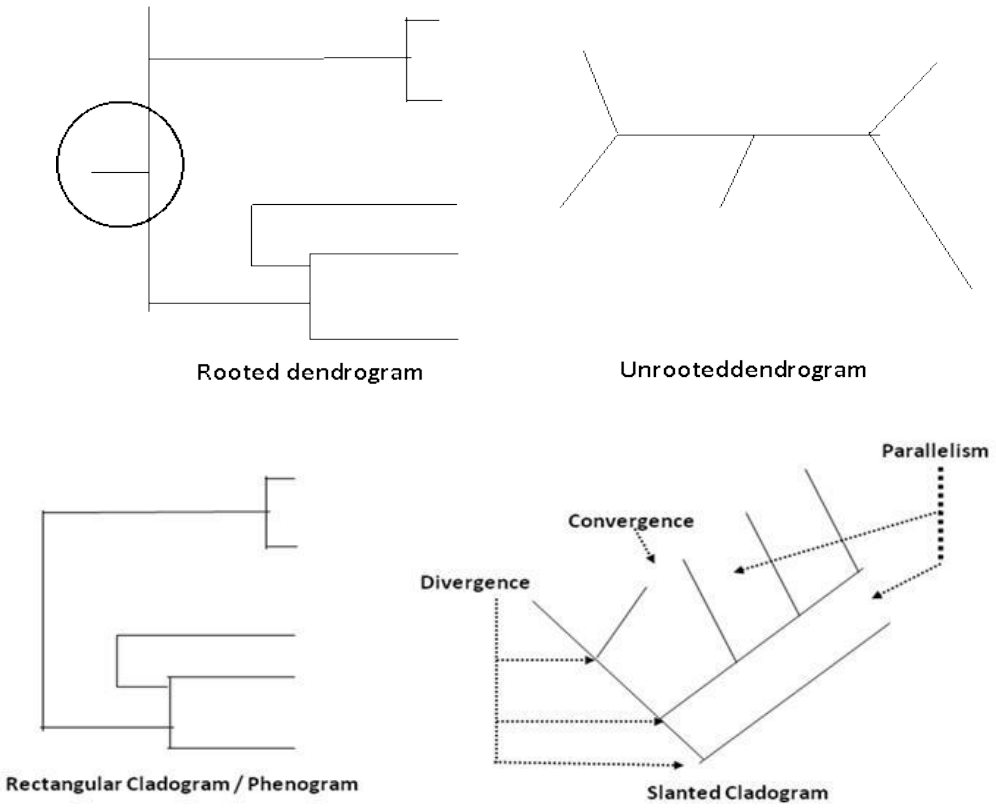


Fig. 2: Different formats of phylogenetic tree representation.

Methods for constructing phylogenetic tree:

Character based and distance based method for tree development

Neighborhood- joining tree - distance based method

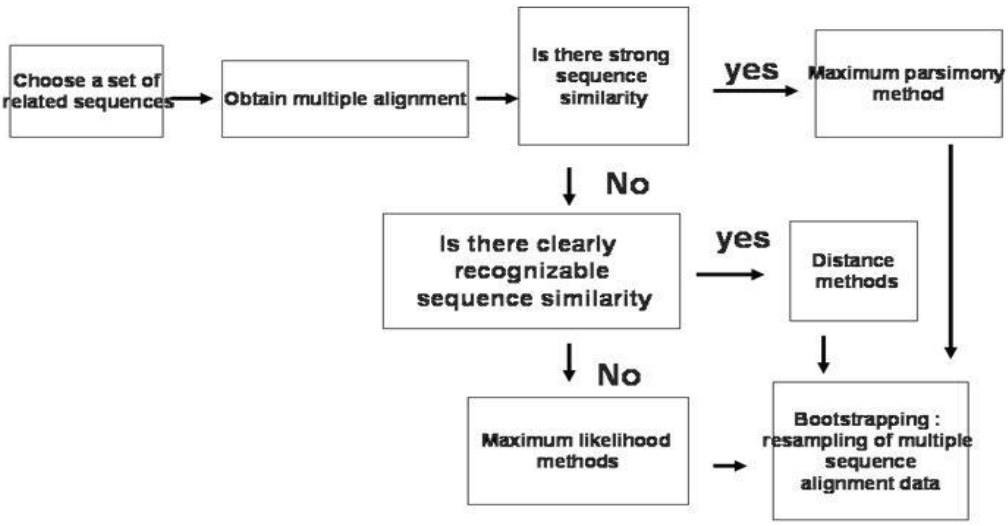
Parsimony tree – character based tree. Use when sequences are quite similar, e.g. – strains of different viruses. Use small numbers of sequences for parsimony analysis.

Bootstraping:

- The bootstrap is a method for assessing the statistical significance the positions of branches in a phylogenetic tree.
- For each aligned pair, it samples scores from random positions in the alignment, adding the scores.
- When all the pairs have been sampled, it converts the scores to distances and computes a tree.
- This whole process is repeated many times and the frequency with which particular tree features are observed is taken as a measure of the probability that the feature is correct.

When to choose what type of tree:

Different bioinformatics analysis that are being routinely used for virus genomics studies are described below:



Open reading frame (ORF) identification and gene prediction

ORF finding is the basis for further homologous search, functional analysis, and identification of viral proteins for possible utilities such as antiviral agents or vaccine targets. From genomic DNA or RNA sequences, ORFs can be identified for candidate genes.

National Center for Biotechnology Information (NCBI)'s ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) is a general ORF prediction tool. The program GeneMark (http://opal.biology.gatech.edu/GeneMark/genemarks.cgi) also provides gene prediction tools for viruses. In addition, the Gene Ontology (GO) (http://www.geneontology.org/) provides a controlled vocabulary for genome annotation.

Homology searching and sequence alignment

Homology searching against known or already annotated viral genomes, such as using the BLAST program, can also be used for predicting genes in unknown viral genomes. Homology searching is usually the next step for genome annotation and functional analysis after ORF finding in viral genome research. A high degree of homology between an ORF from an unknown genome and a known protein may suggest the new protein's similar function to the known one. A commonly used program for homology searching is BLAST. The program can be used for both nucleotide and amino acid sequence searching.

The sequence alignment program ClustalW (http://www.ebi.ac.uk/clustalw/) has been used extensively in studying viral genomes. Nucleotide and amino acid sequence alignments are important in comparing viral sequences in different species and strains. Such analysis is useful for identifying similarities, comparing conserved and non-conserved regions, establishing evolutionary relationships, and building phylogenetic trees.

A comprehensive list of programs for building phylogenetic trees is available at Phylogeny Programs (http://evolution.genetics.washington.edu/phylip/software.html). Phylogeny packages are grouped nicely at this site, according to the available methods such as maximum likelihood and Bayesian methods, or computer systems on which they work.

Basic sequence analysis steps:

1. After obtaining a sequence purify it from the contamination of vector sequence. Use online service like VecScreen (www.ncbi.nlm.nih.gov/tools/vecscreen/) for the purpose. After an initial idea from VecScreen, carefully see the border region between vector and insert using Bioedit Sequence Alignment Editor software and remove the vector sequence.
2. Join two or more sequences of a single clone obtained from primer walking by removing the overlapping sequence (use “allow end to slide” option in Bioedit to find the overlapping ends).
3. If it is circular molecule, then find the origin of the sequence (e.g. – in case of begomoviruses it is TAATATT↓ACC)
4. Go to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and choose a BLAST program to run (e.g. - nucleotide blast for searching a nucleotide database using a nucleotide

query). Paste the query sequence and analyse it using either megablast (if you expect a highly similar sequences) or discontiguous megablast (when there is more dissimilar sequences you expect in database) or blastn (when there is somewhat similar sequences you expect in database).

5. Select the sequences in database which showed high scores (low E-value) after analysis in BLAST. Retrieve those sequences from database in fasta format.
6. Find out the ORFs in the virus genome using online service ORF finder (www.ncbi.nlm.nih.gov/projects/gorf/gorf.html). If anomaly observed carefully check the sequence as there may be some sequencing error due to repetitive sequence.
7. Annotate the sequence based on their feature (ORFs, any typical feature like stem loop structure etc.).
8. Do a multiple alignment using ClustalW algorithm in Bioedit/MEGA and develop a sequence identity matrix.
9. Alternatively do the alignment using MEGA software and develop a bootstrapped consensus phylogenetic tree.
10. Draw the genome of the virus using Snap Gene Viewer software to visualize the genome organization.

PRACTICALS

Polymerase chain reaction (PCR) assay for detection of a DNA virus (A case study of banana bunchy top virus)

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Polymerase chain reaction (PCR) is one of the most widely used and one of the main stays of molecular biology. The wide adoption of the PCR is due to elegant simplicity of the reaction and ease of practical manipulation steps. This techniques is used for *in vitro* amplification of precise fragment of DNA termed as DNA template and has been developed by Kary Mullis in 1980s. It is a highly sensitive technique that can detect DNA template (in a given sample) up to the concentration of 10^{-6} ng/µl i.e. up to 1 fg. It does require prior information of the target DNA sequence from which a pair of oligonucleotides called primers each complementary to the stretch of the 3' side the target DNA is chemically synthesized. The reaction is catalyzed by thermostable enzyme DNA polymerase and consists of three stages of time and temperature termed as (i) denaturation, (ii) annealing and (iii) extension. Each of the steps is repeated for 30-40 times. During first cycle, DNA polymerase synthesizes new complementary strands that act as a template in the next round of the cycle. Furthermore, in the first cycle of PCR the DNA polymerase enzyme copies entire sequence of template DNA, contrary to the belief that product specific amplicon is copied. In the next cycle of PCR, half of the template DNA belongs to the original template DNA and other half are those of newly copied DNA strands. With completion of each cycle, percentage of original template DNA decreases whereas that of newly formed specific template increases thus producing amplicons of specific size rapidly after third cycle (Fig. 1). With just one pair of template DNA, exponential amplification of template DNA in PCR assay results in production of $10.7x10^8$ numbers of copies after 30 cycles run in a thermal cycler. The final PCR product is analysed through gel electrophoresis of agarose gel stained with ethidium bromide (EtBr) using (TAE) buffer.

Banana bunchy top virus (BBTV), a circular single stranded DNA virus of the genus *Babuvirus* in the family *Nanoviridae* infect banana and produces symptoms of bunched leaves at the top of the infected plant in severe cases. BBTV genome is multi-component and consists of six components of circular single stranded DNA *viz.*, DNA-R, DNA-U3, DNA-S, DNA-M, DNA-C and DNA-N components (formerly DNA-1 to DNA-6, respectively) each approximately 1,000 nt long (King *et al.*, 2012). DNA-R encodes a replication-associated protein (*rep*), DNA-S a capsid protein (*cp*), DNA-M a movement protein (*mp*), DNA-C a cell-cycle link protein (*Clink*), and DNA-N a nuclear shuttle protein (*nsp*) genes (Stainton *et al*., 2015) (Fig. 2). Using the nucleotide sequence of replicase initiator protein (*rep*) gene

(GenBank accession no. JX197072), primers were designed for detection of BBTV in banana plant samples (see *Appendix I*).

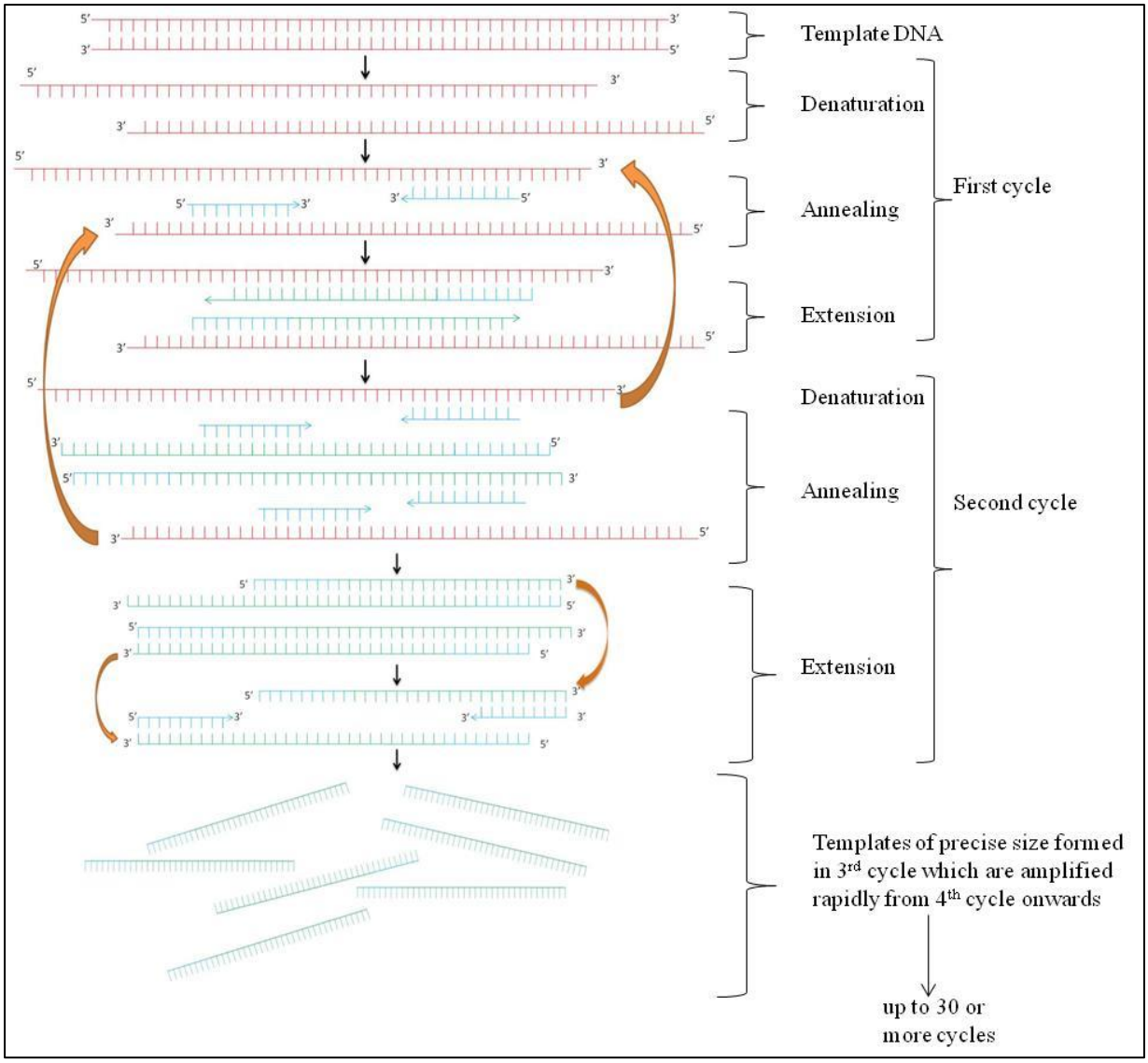

Fig. 1. Diagrammatic representation of specific DNA amplification in a polymerase chain reaction assay

Protocol

Equipments:

- Mortar and pestle
- Refrigerated centrifuge
- Nano drop spectrophotometer for quantification of DNA
- Pipettes, single channel (0.5-10 µl, 20-200 µl, 100-1000 µl)
- Thermal cycler

The first step perform a PCR assay is to isolate a high quality DNA from the plant sample either manually or through kit followed by PCR assay in a thermal cycler.

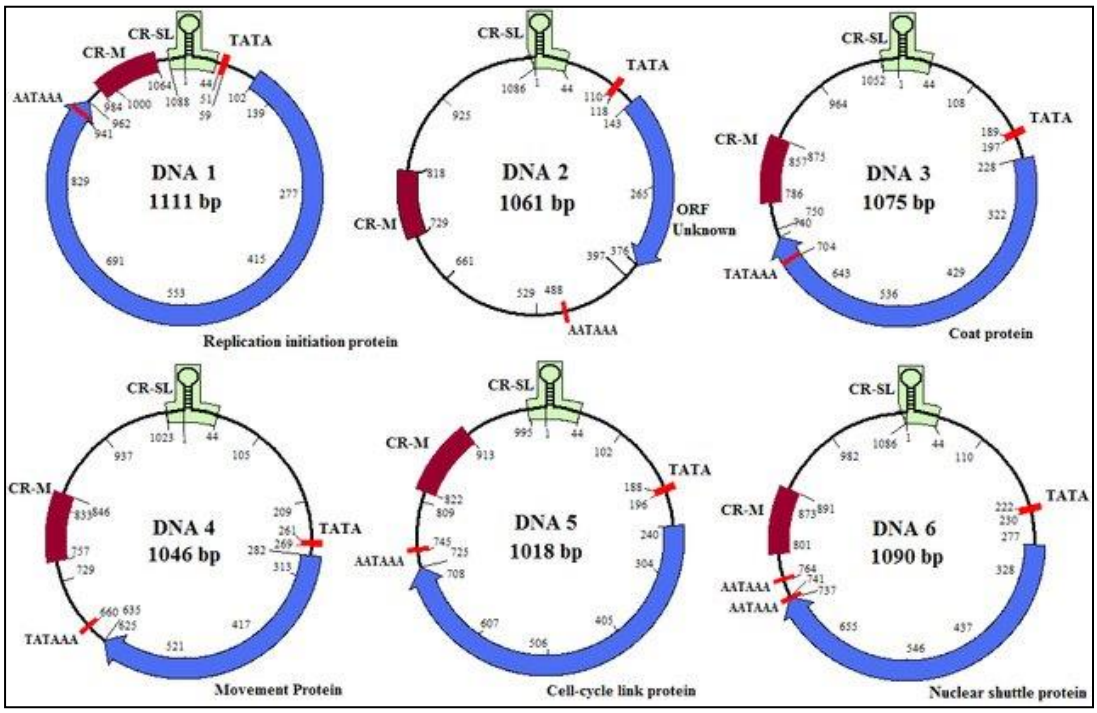

Fig. 2. Genomic organization of BBTV component (source Islam *et al.*, 2010)

DNA extraction

Obtaining high quality intact DNA is the first and the critical step in performing PCR. Isolate DNA from infected leaf sample. DNA can be isolated either manually or using any commercially available kit which yield good quality and quantity of DNA. The procedure mentioned below is for plant DNA isolation using DNeasy Plant Mini kit (Qiagen).

1. Weigh 100 mg test sample (leaf tissue) and disrupt using liquid nitrogen in an autoclaved pestle and mortar.
2. Soak the powdered sample immediately with 400 µl buffer AP1 and 4 µl RNAse A. Vortex for 10 s and incubate at 65ºC for 10 min in water bath. Invert the tubes 2-3 times during incubation.
3. Add 130 µl buffer P3. Mix and incubate for 5 min on ice followed by centrifugation at 20,000 g for 5 min at RT.
4. Transfer the clear lysate into a QIA shredder column and centrifuge for 2 min at 20,000 g.
5. Transfer the flow through to a new 1.5 ml eppendorf tube and add 1.5 volumes of buffer AW1 and mix by pipetting.

6. Transfer 650 µl of the mixture to a DNeasy Mini spin column and centrifuge at 6000 g for 1 min. Discard flow through and repeat the step with remaining sample.
7. Place the spin column into a new 2 ml collection tube and add 500 µl buffer AW2 and centrifuge for 1 min at 6000 g.
8. Discard the flow through and add another 500 µl buffer AW2 and centrifuge for 2 min at 20,000 g.
9. Transfer the spin column to a new eppendorf and add 30 µl buffer AE for elution. Incubate for 5 min at room temperature and centrifuge for 1 min at 6000 g.
10. Repeat the above step and store DNA at -20ºC until further use.

Estimation of quality and quantity of DNA

Determine the integrity of DNA using Nanodrop spectrophotometer. DNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 nm and 280 nm is used to assess the purity of DNA. Pure DNA has an A_{260}/A_{280} of 1.8. Usually, a value of 1.8-2.0 indicates good quality of DNA. A more reliable method to assess the quality of DNA is to run the DNA sample in a 0.7% agarose gel.

Requirements: Chemicals and consumables

- 10X enzyme buffer (provided with *Taq* DNA polymerase enzyme)
- $MgCl_2$ (25 mM)
- dNTP mixture (10 mM)
- Reverse primer (10 µM)
- Forward primer (10 µM)
- Template DNA (~10-50 ng)
- *Taq* DNA polymerase enzyme (commercially available)
- Sterile distilled water

The detailed composition of PCR mixture for a standard PCR is as follows:

Chemical	Stock conc.	Working conc.	Vol for 25µl
Taq pol buffer	10X	1X	2.5 µl
$MgCl_2$	25mM	1.5mM	1.5 µl
dNTP	10mM	0.2mM	0.5
Forward primer	10µM	0.2µM	0.5 µl
Reverse primer	10µM	0.2µM	0.5 µl
Taq polymerase	5U/µl	0.25 µl	0.5 µl
Template DNA	-	100-150 ng	150-250 ng (1 µl)
Distilled water		Make up to 25 µl	Make up to 25 µl

A standard PCR assay is a five step procedure: i) Initial Denaturation (94°C); ii) Denaturation (94°C); iii). Annealing* (depends on primer pair); iv) Extension (72°C); v). Final extension (72°C for 10 min) in which step ii) to iv) are repeated 25 to 35 times as per desired protocol or requirement. The final PCR product may be analyzed on a 1% agarose gel stained with ethidium bromide.

*annealing temperature may be different for different set of primers and is calculated using following formula:

Annealing temperature (T_a) = (T_m-5) degree centigrade where T_m = 4°C(No. of G/C nucleotides in primer) + 2°C(No. of A/T nucleotides in primer)

Gel electrophoresis

Gel electrophoresis is the standard lab procedure for separating DNA by it size (e.g., length in base pairs) for visualization and purification. Gel electrophoresis unit uses an electrical field to move the negatively charged DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones.

Requirements:

Equipments:

- Casting tray
- Well combs
- Voltage source
- Microwave oven
- Horizontal gel electrophoresis unit
- Gel documentation system

Reagents:

- Agarose powder
- Ethidium bromide (EtBr) of stock concentration of 10 mg/mL
- 50X Tris-acetate-EDTA (TAE) buffer (for recipe of 50X TAE stock buffer, refer to Annexure I)
- 6X loading dye for PCR products
- DNA molecular weight ladder (1 Kb or 100 bp)

Procedure for preparation of 1 % agarose gel:

1. Weigh 1 g of agarose powder
2. Mix agarose powder in 100 ml 1X TAE in a microwaveable conical flask.

3. Heat the mixture in the flask for 2-3 min in a microwave oven till the agarose powder is completely dissolved. During heating some of water in the buffer evaporates and thus alter the final percentage of agarose and constituents of TAE buffer. Therefore, it is advised to make-up the final volume using sterile distilled water.
4. Allow the agarose gel to cool down up to 50-55°C (or till you can hold the hot flask comfortably in your hand), add 2-3 µl EtBr from stock solution to a final concentration of 0.2-0.5 µg/mL.
5. Pour the gel in a gel casting tray with comb in place. Remove any bubble in the casting tray using pipette tip.

   Allow agarose gel to solidify at room temperature for 20-30 min or until it has completely solidified.

   Place the casting traiy into the Gel electrophoresis unit and remove the comb.

   Add 2 µl loading dye to 10 µl of each PCR product and load the samples carefully in the wells one-by-one. Also, load 5 µl molecular weight ladder in an empty well.

   Fix the positive and negative terminal fitted on the cover of the electrophoresis unit to the tank and run the gel at 70-80 V. The DNA is negatively charged and will run towards the positive electrode.

   After successfully running the gel for 40-60 min, turn off the power supply and take out the gel along with the tray from the electrophoresis tank. Place the gel in a gel documentation system or on a UV-transilluminator and capture images of the gel for further analysis (Fig. 3).

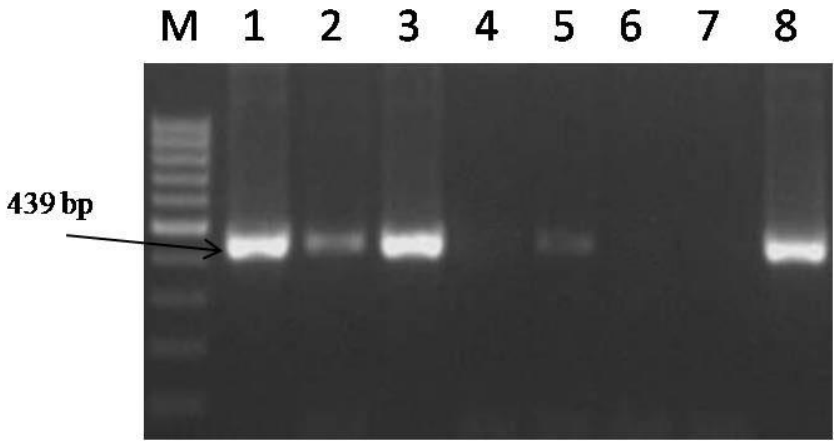

Fig. 3. Analysis of PCR products on an agarose gel through electrophoresis showing specific amplicons of ~ 439 bp with BBTV positive banana samples.

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Detection of viruses by Reverse Transcription - Polymerase Chain Reaction (RT-PCR) (A case study of Cucumber mosaic virus and Viroid)

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Reverse transcription PCR (RT-PCR) is the most commonly used method to detect RNA plant viruses. This technique utilizes RNA as the starting template. First, the enzyme reverse transcriptase produce a complementary single-stranded DNA strand called cDNA using gene specific reverse primer, or using oligo (dT) primer where poly A tail is present on the template RNA. This cDNA is used as a template in PCR assay to amplify and convert the single-stranded cDNA into double-stranded DNA using DNA polymerase.

Cucumber mosaic virus is a widespread plant virus infecting important vegetables, plantains, and horticultural crops. It is a multicomponent virus with a tripartite (+) single stranded RNA genome along with two subgenomic RNA. (Fig. 1). RNA 1 encodes the 1a protein. RNA 2 encodes the 2a protein and the 2b protein, but the 2b protein is only expressed from the subgenomic RNA 4A. RNA 3 encodes the 3a and capsid protein (CP), but the CP is only expressed from the subgenomic RNA 4. Subgenomic RNA 4 is encapsidated in virions. Subgenomic RNA 4A is only encapsidated into virions of subgroup II CMV strains. The virus induces systemic infection in host plant causing variety of symptoms depending on plant species most commonly mild to severe mosaic Various molecular and serological methods are routinely being used for its detection, RT-PCR is generally used for its sensitive detection.

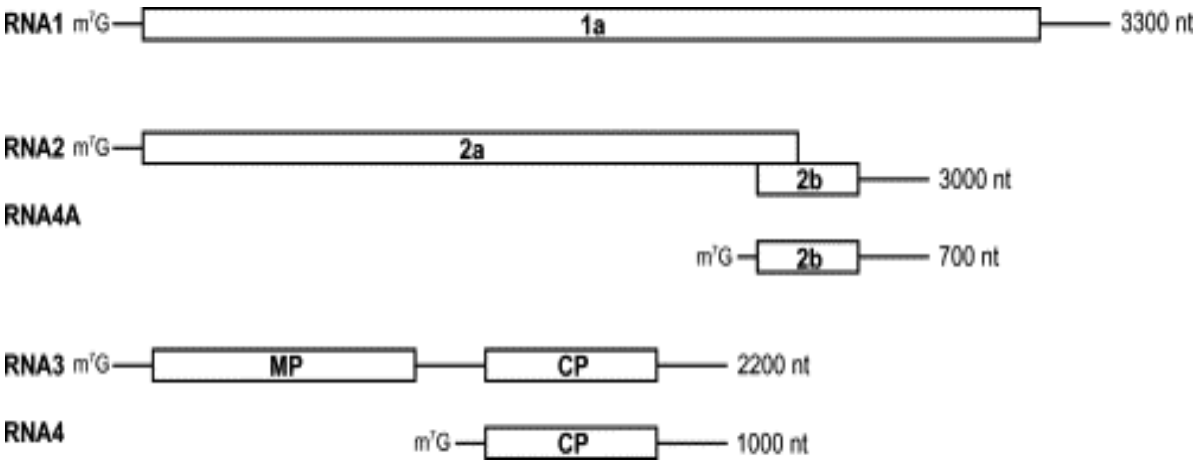

Fig. 1. The genome organization of Cucumber mosaic virus. The three genomic RNAs (1, 2 and 3), as well as the two subgenomic RNAs (RNAs 4 and 4A), are shown.

Viroids are the non-coding subviral pathogens replicating autonomously in their hosts having small (241–401nts) circular single stranded RNA (Flores *et al.*, 2005). They do not code for any proteins. Viroids are classified into two families, viz. *Pospiviroidae* and *Avsunviroidae* discriminating on the presence of a central conserved region in the secondary structure and nuclear replication (*Pospiviroidae*) or a branched secondary structure lacking the central conserved region, presence of ribozymes and plastidial replication (*Avsunviroidae*) (Fig. 2). Though most viroids cause latent infection in the plants but some produce varied symptoms like stunting, epinasty, leaf distortion, localized veinal chlorosis and necrosis. Since, viroids are non-coding single-stranded RNA particles, RT-PCR assay is most reliable method for detection of viroids.

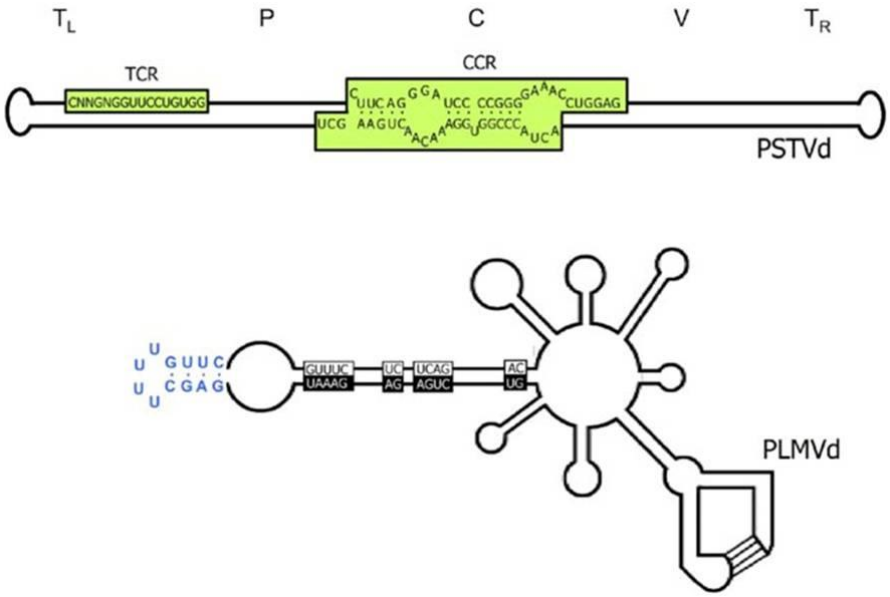

Fig. 2. The structural organization of a potato spindle tuber viroid (PSTVd) (family *Pospiviroidae*). The approximate location of the five structural domains-terminal left (T L), pathogenic (P), central (C), variable (V), and terminal right (T R)-is indicated, as well as that of the central conserved region (CCR), the terminal conserved region (TCR), and the terminal conserved hairpin (TCH) are shown. Lower panel, scheme of the multibranched (Source: Flores *et al.*, 2012)

Requirements

Equipments:

As discussed under section 1.1 for a standard PCR assay

Reagents:

- RNA isolation kit **(**commercially available Plant RNA isolation kit or a verified procedure for manual RNA isolation may be used)
- Reverse transcription-PCR kit containing following component:
  - ✓ 5X buffer

- ✓ Reverse transcriptase enzyme (160 unit/µl)
- ✓ $MgCl_2$ (25 mM)
- ✓ dNTP (10 mM)

- Forward primer (10 mM)
- Reverse primer (10 mM)
- Template RNA (~500 ng/ µl)
- DNA polymerase enzyme
- Sterile distilled water

Protocol

RT-PCR is used to amplify a segment of RNA that lies between two regions of known sequences. A standard RT-PCR is a four step procedure: (i) cDNA synthesis using reverse transcriptase at 42°C; (ii) **denaturation** at a high temperature (90° - 95°C); (iii) **annealing** of target specific primers and (iv) **primer extension** by a thermostable DNA polymerase.

RT-PCR can be performed in one or two step reaction. One-step RT-PCR combines the first-strand cDNA synthesis (reverse transcription) reaction and PCR reaction in the same tube using gene-specific primers, simplifying reaction set up. Two-step RT-PCR begins with the reverse transcription of either total RNA or poly (A)+ RNA into cDNA using a reverse transcriptase. Following cDNA synthesis step, the cDNA is used as template for the next step i.e. PCR step. It also provides the ability to save some RNA sample for further use. The protocol for two step reaction has been described in this manual.

RNA extraction

Isolate RNA from infected leaf sample either manually or using any commercially available kit which yield good quality and quantity of RNA. The procedure mentioned below is for plant RNA isolation using RNeasy Plant Mini kit (Qiagen).

1. Weigh 100 mg test sample (leaf tissue) and disrupt using liquid nitrogen in an autoclaved pestle and mortar.
2. Add 450 µl buffer RLT or buffer RLC and 5 µl β-mercaptoethanol to the powdered sample and transfer the lysate to a clean microcentrifuge tube. Incubate at 56°C for 3 min for proper cell lysis and vortex vigorously during incubation.
3. Transfer the lysate to a QIA shredder spin column (lilac) placed in a 2 ml collection tube. Centrifuge for 2 min at full speed at room temperature. Transfer the supernatant of the flow-through to a new microcentrifuge tube without disturbing the cell-debris pellet.
4. Add 0.5 volume of ethanol (96-100%) to the cleared lysate and mix immediately by pipetting.

5. Transfer the sample (usually 650 µl) with any precipitate to an RNeasy mini spin column (pink) in a 2 ml collection tube. Close the lid and centrifuge for 30 s at 10,000 rpm. Discard the flow-through.
6. Add 700 µl buffer RW1 to the RNeasy spin column. Close the lid and centrifuge for 30 s at 10,000 rpm. Discard the flow-through.
7. Add 500 µl buffer RPE to the RNeasy spin column. Close the lid and centrifuge for 30 s at 10,000 rpm. Discard the flow-through.
8. Add 500 µl buffer RPE to the RNeasy spin column. Close the lid and centrifuge for 2 min at 10,000 rpm. Discard the flow-through.
9. Place the RNeasy spin column in a new 2 ml collection tube. Centrifuge at full speed for 1 min to dry the membrane.
10. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30-50 µl RNase free water directly to the spin column membrane. Close the lid and incubate for 1 min at room temperature.
11. Centrifuge at 10,000 rpm for 1 min to elute RNA and store it at -80°C until further use.

Estimation of quality and quantity of RNA

Determine the integrity of RNA using Nanodrop spectrophotometer. RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 nm and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A_{260}/A_{280} of 2.1. Usually, a value of 1.8-2.0 indicates good quality of RNA.

Reverse transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR can be performed in 2 steps, first cDNA is synthesized using RT enzyme followed by PCR amplification using DNA polymerase in separate microfuge tube.

(i) cDNA synthesis

Assemble the RT reaction components on wet ice and prepare mix by dispensing into 200 µl microfuge tube in the order listed below [See box].

Recipe of reaction mixture

Reagents	Volume (µl)
5X Enzyme buffer	5.0
$MgCl_2$ (25 mM)	3.0
dNTP Mixture (10 mM)	1.25
Reverse primer (10 µM/µl)	1.0
Template	6.0
Reverse transcriptase	0.5
Sterile distilled water	7.25
Total volume	25.0

Note: Prior to use RNA as a template, denature RNA (6 µl) along with 1µl of reverse primer at 65°C for 5 min and then snap chill on ice for 5 min and use it as template for reverse transcription of template RNA.

Mix RT reaction assembly by inversion and place the tubes in a thermal cycler with following reaction conditions [See box].

RT Temperature profile

Steps	Temperature	Time	Cycle
Annealing	25°C	5 min	1
c-DNA synthesis	42°C	60 min	1
Inactivation	70°C	15 min	1

Note: The profile mentioned above is for Improm-II Reverse transcriptase (Promega, Maddison, USA). Parameters may vary if using RT enzyme from a different source. Annealing temperature can be optimized between 37°C - 55°C.

(ii) Polymerase Chain Reaction

Assemble the PCR reaction components on wet ice and prepare amplification mix by dispensing into a separate 200 µl microfuge tube in the order listed below [See box].

Recipe of reaction mixture

Reagents	Volume (µl)
10X enzyme buffer	2.5
$MgCl_2$	1.0
dNTP mixture (10 mM)	1.0
CMV Forward primer (10 µM/µl)	1.0
CMV Reverse primer (10 µM/µl)	1.0
c-DNA	6.0
Taq DNA polymerase (2U/µl)	0.5
Sterile distilled water	12
Total volume	25.0

Mix the PCR reaction mixture well by inversion and place the tubes in a thermal cycler with the following reaction conditions [See Box]. Details of primer sequences are given in Appendix.

PCR Temperature profile

Steps	Temperature	Time	Cycle
Initial Denaturation	94°C	4 min	1
Denaturation	94°C	30 sec	30
Annealing	50-60°C*	30 sec	
Primer extension	72°C	1 min	
Final primer extension	72°C	10 min	1

Note: Annealing temperature is specific to a particular combination of primer used for amplification and can be optimized further.

Electrophoresis analysis of amplicons

Analyze the RT-PCR products in 1% agarose gel in Tris-acetate EDTA (TAE) buffer containing ethidium bromide (0.5 µg/ml) as described in previous case study (Fig. 3).

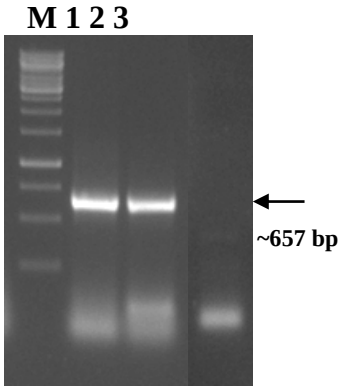

Fig. 3. Electrophoresis analysis of RT-PCR using specific primers for CMV detection. Lane 1: Test sample (~657 bp amplified fragment CMV c-DNA); Lane 2: Positive control (~657 bp amplified CMV c-DNA); Lane 3: Healthy banana leaf sample (no amplification seen); M = 1kb DNA ladder

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Detection of mixed viruses by multiplex PCR
(A case study for simultaneous detection of Banana bunchy top virus and banana streak Mysore virus)

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Polymerase chain reaction (PCR) is the most widely used nucleo-based method for the detection of plant viruses. It is a primer -mediated *in vitro* reaction involving amplification of target nucleic acid sequences (Mullis *et al*., 1986). Different variants of PCR have been developed ranging from conventional PCR, reverse transcription (RT)-PCR, immunocapture (IC-PCR), mutiplex PCR, Real-time or Quantitative PCR to improve the sensitivity and specificity for detection of plant viruses. Multiplex-PCR allows the simultaneous and sensitive detection of different DNA or RNA plant viruses in a single reaction. It is a useful tool since many times crops get infected by more than one virus and their individual detection by PCR is not only expensive but also time consuming. A multiplex PCR assay may be a duplex, triplex or tetraplex PCR depending on the number of viruses it can detect in a single assay.

Requirements: As discussed for a standard PCR assay in chapter 1.

Duplex Polymerase Chain Reaction (PCR)

Duplex PCR is the amplification and detection of two target sequences in one reaction. Primers for detection are specific for each target sequence and both targets should have amplicons of varying size so that they could be easily visualized and differentiated through gel electrophoresis. Also, each primer pair to be used for multiplexing should have same or nearly same melting temperature (T_m). The protocol for detection of Banana streak Mysore virus (BSMYV) and Banana bunchy top virus (BBTV) by duplex PCR has been described.

Protocol

DNA extraction

As described under section 1.2.

Estimation of quality and quantity of DNA

As described under section 1.2.1

Duplex Polymerase Chain Reaction

Assemble the PCR reaction components on wet ice and prepare amplification mix by dispensing into 0.2 ml PCR tube in the order listed below.

Recipe of reaction mixture

Reagents	Volume (µl)
10X Enzyme buffer	2.0
$MgCl_2$ (25 mM)	1.0
dNTP mixture (10mM)	1.0
Primer A F (10µM)	1.0
Primer A R (10µM)	1.0
Primer B F (10µM)	1.0
Primer B R (10µM)	1.0
Primer C F (10µM)	1.0
Primer C R (10 µM)	1.0
DNA template (ng/µl)	1.0
Taq DNA polymerase (2 U/µl)	0.5
Sterile distilled water	8.5
Total volume	20.0

Primers used for detection of BSMYV and BBTV by duplex-PCR assay

Primer	Specific detection	Amplicon size	Reference
A Forward	BSMYV RT/RNAse		Geering *et al.*,
A Reverse	H region of ORF	~589 bp	(2000)
B Forward	BBTV		Selvarajan *et*
B Reverse	Partial rep gene	~439 bp	*al.*, (2011)
C Forward			Lagoda *et al.*,
C Reverse	STMS	~248 bp	(1998)

Note: Primers C-Forward and Reverse are specific to *Musa* genomic DNA and used as internal control to detect amplification from *Musa* genome.

Mix the PCR reaction mixture well by inversion and place the tubes in a thermal cycler with the following reaction conditions [See Box].

PCR Temperature profile

Steps	Temperature	Time	Cycle
Initial Denaturation	94°C	4 min	1
Denaturation	94°C	30 sec	
Annealing	58°C	30 sec	35
Primer extension	72°C	1 min	
Final primer extension	72°C	10 min	1

Note: Optimum annealing temperature for all the three sets of primers is 58°C. Annealing temperature is specific to a particular combination of primer used for amplification and can be optimized further. Uniplex or Standard PCR reaction can also be performed using one set of primers for detection of specific virus.

Electrophoresis analysis of amplicons

Analyze the PCR products in 1.5% agarose gel in Tris-acetate EDTA (TAE) buffer containing ethidium bromide (0.5 µg/ ml) as described earlier (Fig. 1).

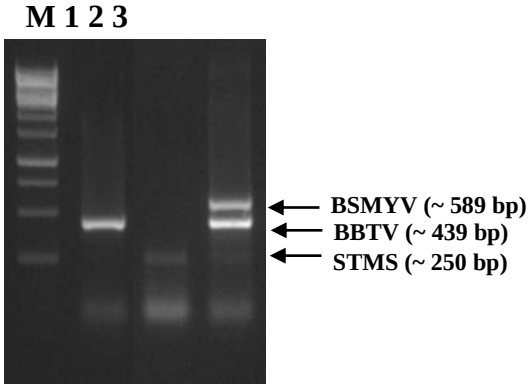

Fig. 1. Gel electrophoresis analysis of duplex PCR. Lane 1: Test sample (~439 bp amplified BBTV); Lane 2: Healthy control (~250 bp amplified fragment corresponding to *Musa* genome DNA); Lane 3: Positive control (~589 bp amplified BSMYV, ~439 bp amplified BBTV DNA & ~250 bp amplified *Musa* genome specific DNA); M = 1 kb DNA ladder (Thermo Scientific, USA)

Point to remember

In hybrid banana, a positive amplification for detection of BSMYV by PCR may be from endogenous sequesnces and therefore duplex RT-PCR should be perfomed.

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Nested Polymerase Chain Reaction (nested PCR) Assay
(A case study of phytoplasma detection in brinjal little leaf)

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Nested polymerase chain reaction (Nested PCR), a variant of PCR is being used to increase sensitivity and/or specificity. Primers of nested PCR are chosen within a DNA sequence that has been previously amplified. Nested primers are used to re-amplify the product of a PCR reaction that did not yield enough material. The first round PCR usually amplifies a larger fragment and that serves as template for the next round PCR with nested primers amplifying a smaller size fragment.

Nested PCR is most commonly used method for the detection of Phytoplasmas. Phytoplasma are economically important plant pathogens of agricultural, horticultural and ornamental crops, causing a wide variety of symptoms that range from mild yellowing to death of infected plants. Phytoplasma are specialized bacteria that are obligate parasites of plant phloem tissue and transmitted through insects (vectors). The genome size of phytoplasmas ranges between 0.5 Mb to 1.6 Mb. Due to their low concentration and erratic distribution in the sieve tubes of the in infected planting material, PCR based approach is used to confirm presence and identification of phytoplasma. Numerous universal and/ or group specific, PCR primer combinations have been devised to amplify 16SrRNA gene of phytoplasma. RFLP and sequence analysis of PCR amplified 16S rRNA gene, the 16/23S spacer region and 23S ribosomal DNA from various phytoplasmas have become a foundation for establishing Phytoplasmas taxonomy and phylogeny (Lee *et al*., 1998, Wang and Hiruki, 2001) (Fig. 1). The procedure for detection of sugarcane grassy shoot phytoplasma (SCGS) has been described in this manual. The same procedure can be followed for identification of phytoplasma affecting other crops.

Requirements

Same as that of PCR described in section 1.2.2

Reagents/Buffers

DNA Extraction: DNA can be extracted using DNA extraction kit or by manually using CTAB method)

Following stock reagents should be prepared in sterile distilled water or autoclave after preparation

a) 1M Tris
b) 0.5M EDTA
c) 5M NaCl
d) 10% CTAB

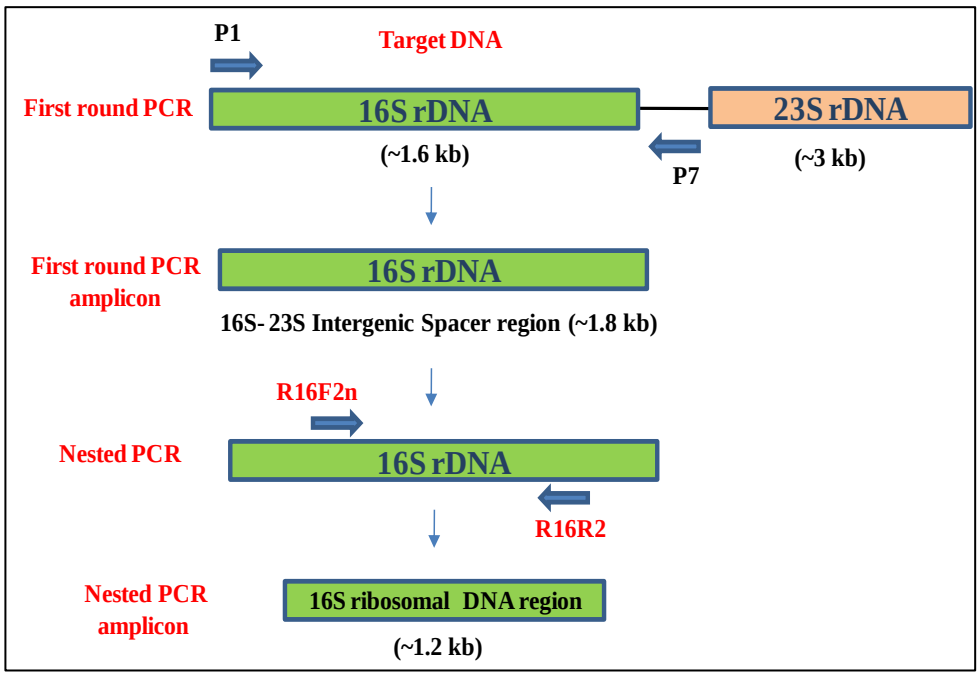

Fig. 1. Schematic diagram of target DNA for phytoplasma identification by Nested PCR

DNA Extraction Buffer

For each 1 gm of plant sample, prepare 10 ml extraction buffer as described in the box below:

Reagent	Volume	Final concentration
1 M Tris	1 ml	0.1 M
0.5 M EDTA	400 µl	0.02 M
5 M NaCl	2.7 ml	1.4 M
10% CTAB	3.5 ml	3.5%
β-mercaptoethanol	50 µl	
Distilled water	2.25 ml	

Protocol

DNA isolation using CTAB method

Total genomic DNA from symptomatic plants can be isolated by CTAB method (Ahrens and Seemüller, 1992). Phytoplasma are inhabited in phloem sieve tubes, hence the DNA

extraction is very tricky. The maximum concentration of phytoplasma can be obtained from midribs of leaves, stalks tissues of herbs and phloem containing tissues of the affected hosts. The protocol for DNA extraction from leaf midrib and/ stalk tissues has been described below.

1. Grind 1.0 gm of plant tissue in 10 ml DNA Extraction Buffer (DEB).
2. Incubate at 65ºC for 30-60 min.
3. Mix suspension with one volume of chloroform : isoamylalcohol (24:1) and centrifuge for 10 min at 6000 rpm at room temperature.
4. Precipitate the aqueous phase (nucleic acid) by adding 0.8 volume of chilled isopropanol and store at -20ºC for overnight.
5. Centrifuge for 20 min at 12000 rpm at 4ºC and discard the supernatant.
6. Resuspend the pellet in 1 ml TE buffer.
7. Add 1µl of RNAseA (10mg/ml) and incubate at 37ºC for 30 min.
8. Add 0.8 volume of chilled isopropanol and mix well.
9. Centrifuge for 15 min at 4ºC, 12000 rpm and discard the supernatant.
10. Wash the pellet with 1 ml chilled 70% ethanol and centrifuge at 12000 rpm for 20 min at 4ºC.
11. Discard supernatant and dry the pellet at 37ºC. Add TE buffer to dissolve pellet and store at 4ºC for overnight.
12. Finally, store extracted DNA at -20ºC until further use.

Estimation of quality and quantity of DNA

The quality and quantity of isolated DNA will be checked in a nanodrop spectrophotometer Nanodrop 1000C (Thermo Scientific, USA)

Polymerase Chain Reaction

a) First round PCR using P1/P7 primers

Assemble the PCR reaction components on wet ice and prepare amplification mix by dispensing into a separate 0.2 ml PCR tube in the order listed below [See box].

Recipe of reaction mixture

Reagents	Volume (µl)
10X Enzyme buffer	2.5
$MgCl_2$ (25 mM)	1.5
dNTP Mixture (10 mM)	0.5
P1 forward (10 µM/µl)	0.5
P7 reverse (10 µM/µl)	0.5
DNA template (100 ng/µl)	1.0
Taq DNA Polymerase (5 U/µl)	0.25
Sterile distilled water	19.0
Total volume	25.0

Note: Primers P1 and P7 are used in first round for amplification of phytoplasma 16S rRNA and part of Intergenic spacer region (Gundersen and Lee 1996; Deng and Hiruki 2001). Details of primer sequences are given in Appendix.

Mix the PCR reaction mixture well by inversion and place the tubes in a thermal cycler with the following reaction conditions [See Box].

PCR Temperature profile

Steps	Temperature	Time	Cycle
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	
Annealing	55°C	1 min	30
Primer extension	72°C	1 min	
Final primer extension	72°C	10 min	1

b) Nested PCR using R16F2n/R16R2 primers

Assemble the PCR reaction components on wet ice and prepare amplification mix by dispensing into a separate 0.2 ml PCR tube in the order listed below [See box].

Recipe of reaction mixture

Reagents	Volume (µl)
10X Enzyme buffer	2.5
$MgCl_2$ (25 mM)	1.5
dNTP Mixture (10 mM)	0.5
R16F2n forward (10 µM/µl)	0.5
R16R2 reverse (10 µM/µl)	0.5
P1/P7 PCR product	1.0
Taq DNA Polymerase (5 U/µl)	0.25
Sterile distilled water	19.0
Total volume	25.0

Note: Primers R16F2n and R16R2 are used in nested PCR reaction for amplification of phytoplasma 16SrDNA using P1/P7 PCR product as template (Gundersen and Lee 1996). Details of primer sequences are given in Appendix.

Mix the PCR reaction mixture well by inversion and place the tubes in a thermal cycler with the following reaction conditions [See Box].

PCR Temperature profile

Steps	Temperature	Time	Cycle
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	
Annealing	56°C	1 min	30
Primer extension	72°C	1 min	
Final primer extension	72°C	10 min	1

Electrophoresis analysis of amplicons

Analyze the PCR products in 1% agarose gel in Tris-acetate EDTA (TAE) buffer containing ethidium bromide (0.5 µg/ml) as described above (Fig. 2).

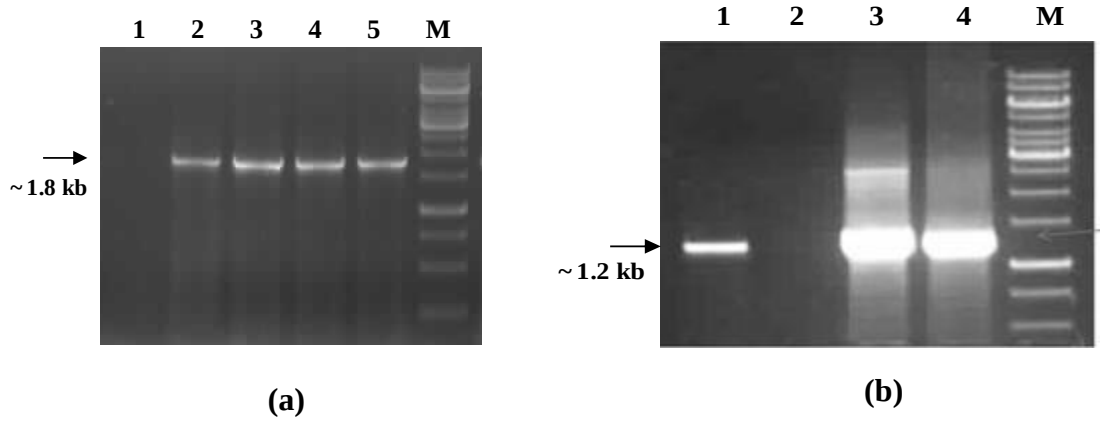

Fig. 2. Electrophoresis analysis of first round PCR using P1/P7 primers for Phytoplasma detection. (a) Lane 1: Negative control; Lane 2-5: test samples. Electrophoresis analysis of nested PCR using R16F2n/R16R2 primers (b) Lane 1, 3 & 4: Test samples, Lane 2: Negative control, M = 1 kb DNA ladder

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Nucleo-based isothermal amplification assays

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Recent advances in diagnostic assays has lead to development of various nucleic acid amplification techniques which are rapid, sensitive, specific and do not require use of high cost equipments. They are carried out at room temperature or at a constant single temperature and do not require the reiterative cycling of a reaction cocktail between different temperatures to achieve amplification. Isothermal amplification assays are suitable for devising assays for on-spot disease detection. Various isothermal amplification assays such as rolling circle amplification (RCA), recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP) etc. have been developed recently and are employed in several diagnostic devices and kits. However, these assays are suitable for amplification of short stretches of DNA. Isothermal amplification assays also utilizes set of one or more primer pairs which may be complementary to the target strand or may be random hexamer primers, which are further extended into 3' direction with the help of one or more polymerase or strand-displacement enzymes. In the present manual, we will describe protocol for RCA and RPA assay for detection of banana streak Mysore virus (BSMYV) and banana bunchy top virus (BBTV), respectively.

Diagnostics and characterization of circular DNA viruses using Rolling Circle Amplification (RCA)
(A case study of Banana streak Mysore virus detection in banana)

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Rolling circle amplification (RCA) is an isothermal amplification where a short DNA primer is amplified to form a long single stranded DNA using a circular DNA template and special DNA polymerases. Rolling circle amplification (RCA) technique was discovered at the end of 20th century, and since then it revolutionised the diagnosis and genomics of DNA viruses. It allows reliable detection for all circular DNA viruses from their natural or experimental host plant sources. The advantages of this technique are (i) no expensive devices are required, (ii) simple handling, (iii) amplification of the entire circular DNA component from the infected sample without any prior knowledge of sequences in a single step, and (iv) low cost per reaction.

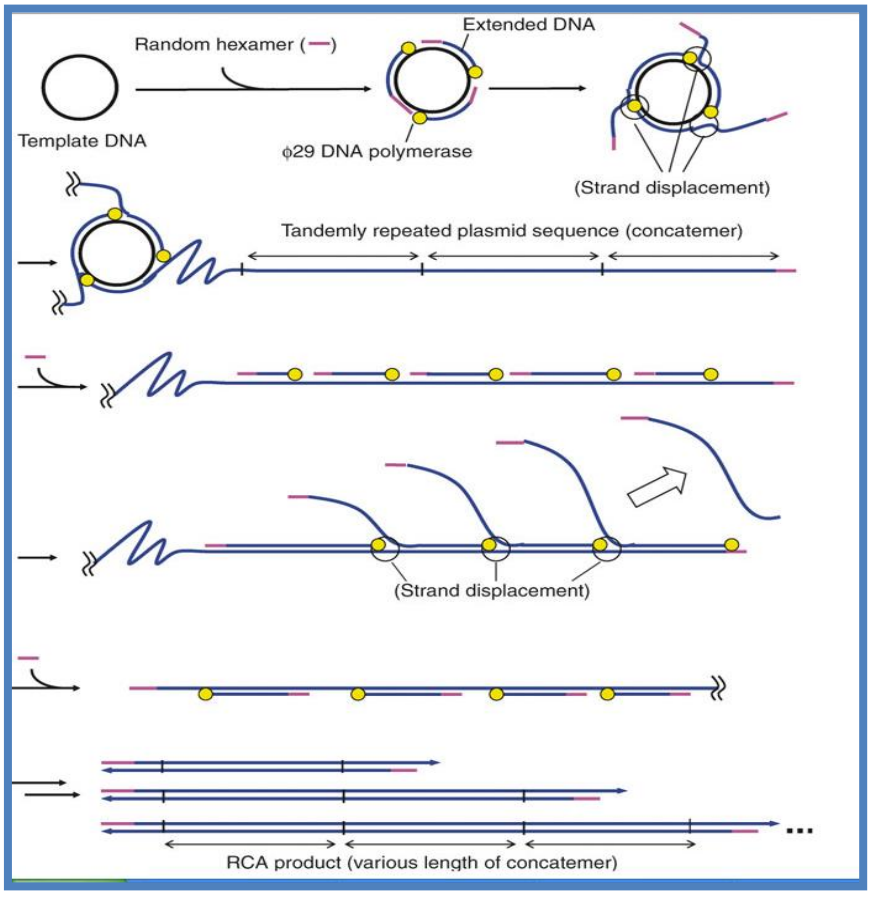

Fig. 1. Steps Involved in Rolling Circle Amplification (RCA)

In RCA, Random hexamers (NNNNNN) are hybridized with circular DNA, resulting double-strand segments which function as oligonucleotides in the polymerization reaction carried out by Φ29 DNA polymerase. The Φ29 DNA polymerase is a highly processive polymerase (more than 70 kb template) featuring strand displacement activity which allows for highly efficient isothermal DNA amplification. By this means it allows circular DNA to be replicated by enormous times. Φ29 DNA polymerase also possesses a 3'→5' exonuclease (proof-reading) activity. Replicative form (RF) of viruses is the best substrate for this enzyme (Fig. 2). The RCA product after restriction digestion needs to be sequenced for confirmation of viral origin. In the study at this centre, genome sequences of three episomal Banana streak MY virus (BSMYV) isolates and shorter Banana streak OL virus variants were reported by sequence-independent improved rolling circle amplification (RCA) (Baranwal *et al.*, 2014, Sharma *et al.*, 2015).

Requirements

Equipments

- Distilled/RO/Millipore Unit
- Microcentrifuge tubes (1.5 ml)
- Waterbath with Thermostat Incubator
- Horizontal gel electrophoresis unit with power pack
- Gel documentation unit with computer and printer
- Micropipettes (1-10 µl, 20-200 µl),
- Infected plant tissue
- DDW (autoclaved double distilled water)

Reagents (procured commercially)/ Buffers

Exo resistant Random Hexamers, Pyrophosphatase, 10x Reaction Buffer ,Φ29 DNA polymerase, dNTPs (10mM), Ethidium Bromide, 0.5 M EDTA, Running Buffer.

Protocol

DNA extraction

Total DNA was extracted from 100 mg of fresh and frozen leaves with Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit following the manufacturer's protocol.

The detailed method of DNA isolation is given below:

- 100 mg of tissue is ground into find powder with the help of liquid nitrogen in pre cooled pestle mortar. Immediately the tissue powder was transferred into an

autoclaved 1.5 ml micro centrifuge tube containing 350 µl of Lysis Buffer A. Vortexed the solution for 10-30 s to mix it thoroughly.

- 50 µl of Lysis Buffer B is added along with 20 µl RNase A.
- The mixtures are incubated for 10 min at 65 °C, and have been vortexed occasionally.
- To this lysate, 130 µl of Precipitation Solution was added and mixed thoroughly by inverting the tube 2-3 times. The same mixture was incubated on ice for 5 min.
- The lysate is centrifuged for 5 min at ≥20,000xg (≥14,000 rpm)
- The supernatant is collected, and transferred to the clean microcentrifuge tube (not provided). 400 µl of Plant gDNA Binding Solution and 400 µl of 96% ethanol are added to that microcentrifuge tube and mixed well.
- The half of the prepared mixture is added to the spin column and centrifuged for 1 min at 6000xg (~8,000 rpm). The flow-through was discarded and remaining mixture was added onto the same column. The column is kept for centrifugation for 1 min at 6,000xg (~8,000 rpm).
- 500 µl of Wash Buffer I is added to the spin column (ethanol must be pre added to the Wash Buffer I). Centrifuged the same for 1 min at 8,000xg (~10,000 rpm). The flow-through is discarded and placed back into the collection tube.
- 500 µl of Wash buffer II is added to the column (ethanol must be pre added to the Wash Buffer II) to the spin column for second wash and centrifuged for 3 min at maximum speed ≥20,00xg (≥14,000 rpm).
- The genomic DNA is eluted using 50 ≥ of Elution Buffer.

Integrity of DNA is checked by agarose gel electrophoresis and quantity was determined using Nanodrop spectrophotometer. A ration of absorbance of 1.8 at 260 nm to that of 280 nm will indicate that the sample is free from protein contamination.

Rolling Circle Amplification

RCA is a sequence independent amplification, carried out overnight at isothermal temperature 30 °C in water bath. The reaction mixture contained 10-50 ng of DNA, 2 µl of reaction buffer (10X), 2 µl of exo resistant random hexamer primers (Thermo Scientific, Lithuania), 2 µl of dNTPs (10 mM), and double distilled water to make the final volume of 20 µl. The mixture is denatured at 94 °C and cooled down to room temperature for 5 min, after which 4 µl of pyrophosphates (0.1 U/ µl) and 1 µl of Φ29 DNA polymerase (0.1 U/ µl) are added. The reaction mixture is incubated for 19 h at 30 °C in circulation water bath and later inactivated at 65 °C for 10 min.

Restriction digestion and gel electrophoresis

RCA concatamers were digested using set of restriction enzymes having unique site on viral genome under study (Fig. 2a). 3 µl of RCA product (corresponding to 300-400 ng of DNA)

were digested with 10 U of restriction enzymes (Fig 2b). The amplified RCA product following digestion with restriction enzymes is analyzed on 1-1.2 % agarose gel stained with ethidium bromide.

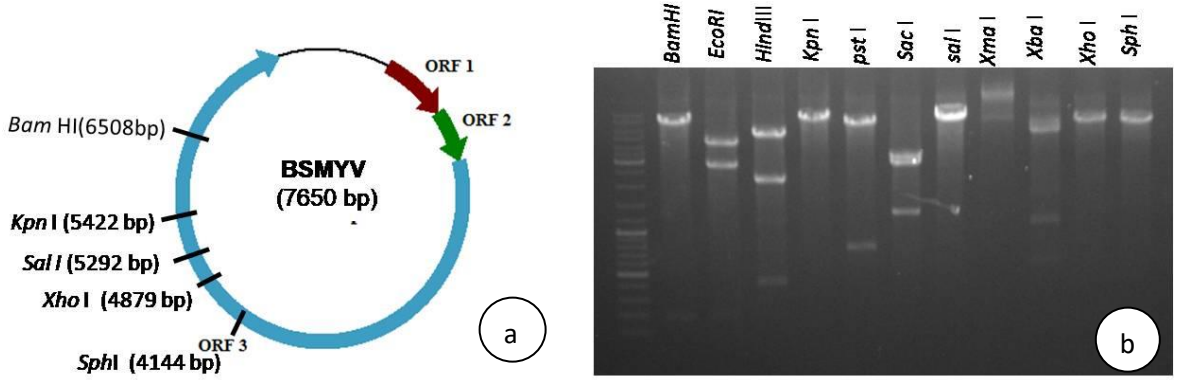

Fig. 2. Viral Genome (Banana Streak Mysore Virus) with unique restriction site **(a)** and its restriction pattern **(b)** using eleven enzymes *viz*, *Kpn* I, *Hind* III, *BamH* I, *EcoR* I, *Sal* I, *Pst*I, *Sac* I, *Xma*l, *Xho*I, *Xba*I and *Sph*I

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Recombinase polymerase amplification (RPA) assay
(A case study of BBTV detection in crude banana extract)

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Recombinase polymerase amplification (RPA) is a rapid, isothermal amplification method with high specificity and sensitivity. In RPA, the isothermal amplification of specific DNA fragments is achieved by the combination of enzymes and proteins, *viz.*, the recombinase, single-stranded binding proteins (SSB) and strand displacing polymerase, used at room temperature (i.e. 37-40˚C). This technique can produce sufficient quantity of amplicons in 25-30 minute which a normal polymerase chain reaction (PCR) could produce in 1.5 to 2 hour reaction and the final product could be easily visualized on an agarose gel. RPA employs two sets of primers which are combined with the recombinase that targets the dsDNA and facilitates strand displacement. The displaced single stranded DNA strand is stabilized by SSB proteins and the polymerase initiates synthesis (Fig. 1). RPA products can be visualized on gel after purification, although alternate methods, such as fluorescence and/or hybridization. Crude sap or purified DNA samples may be utilized as template in RPA assay (Kapoor *et al*., 2017).

Requirements

Recombinase Polymerase Amplification (RPA) kit: TwistAmp® Basic Kit is commercially produced and sold by TwistDX, England. The kit contains following ingredients:

i. Rehydration buffer
ii. Freeze dried pellet (containing polymerase and SSB protein) (provided in 200µl microtubes)
iii. Magnesium acetate (280 nM)

Equipments required:

- Mortar pestle
- Centrifuge
- Dry bath
- Gel documentation system or UV transilluminator
- Electrophoresis unit

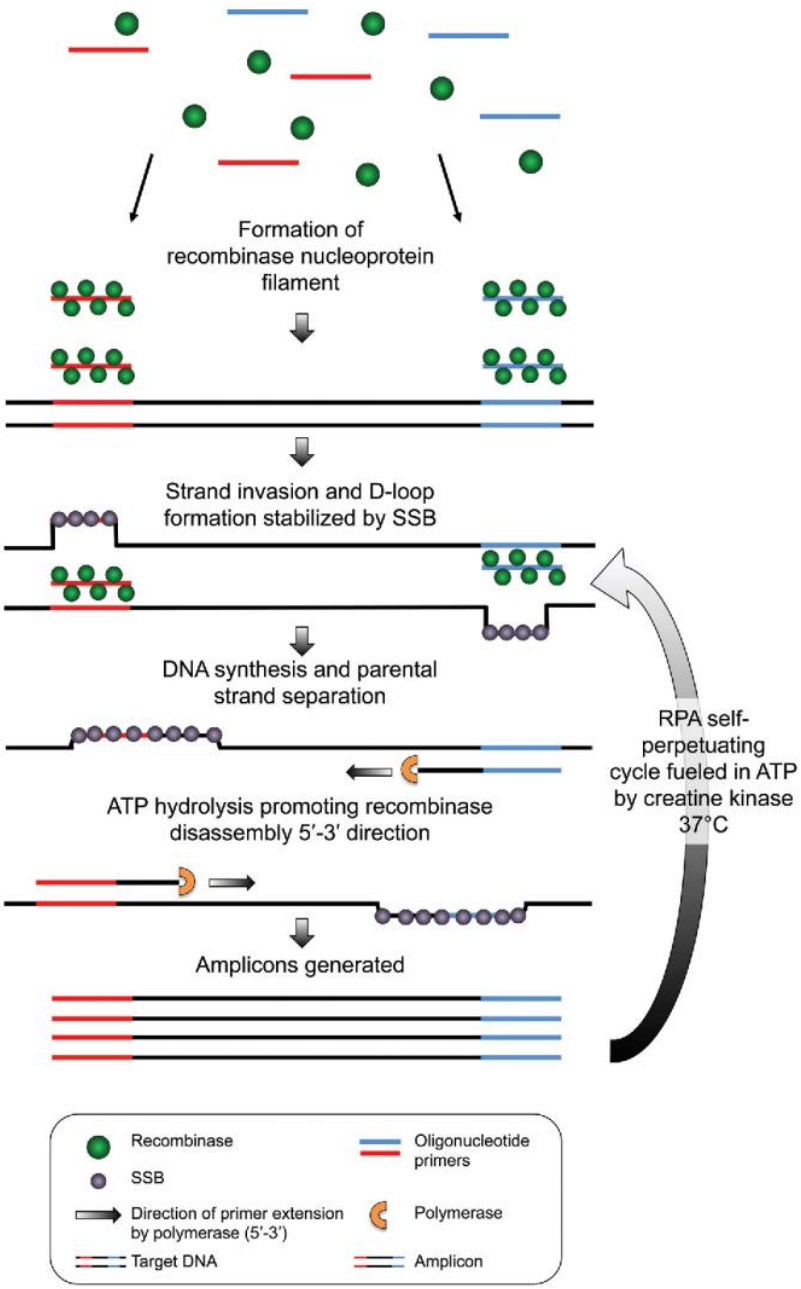

Fig. 1. Diagrammatic representation of recombinase polymerase amplification reaction
(*Source:* Daher *et al*., 2016)

Protocol

Template preparation:

100 mg of plant leave sample were ground in 500µl 0.5M NaOH. The homogenate were transferred to 1.5ml eppendorf tube and centrifuge at 12000 rpm at room temperature to get the supernatant. This supernatant/crude extract will be used as template for RPA assay or

alternatively DNA of desired sample may be isolated using commercial plant DNA isolation kit or through manually.

RPA reaction:

A Master mix for RPA assay were prepared containing 12.2 µl distilled water, 2.4 µl of each primer (F and R), 29.5 µl of rehydration buffer, and 1 µl template (crude extract).This reaction mixture were added to freeze dried pellet and mixed with pipetting. In the same mixture, 2.5 µl of 280 nM MgAc were added and mixed well. The same mixture was incubated at 37˚C for 30 min.

Note: Template should be added to the reaction mixture at the last step and the 50 µl master mix can be utilized for two reactions of 25 µl each.

Gel electrophoresis of RPA products

The final products of RPA assay can be analysed by running the RPA products through 2% agarose gel stained with ethidium bromide (0.5 µg/ml) in 0.5M Tris-acetate EDTA (TAE) buffer (Fig. 2).

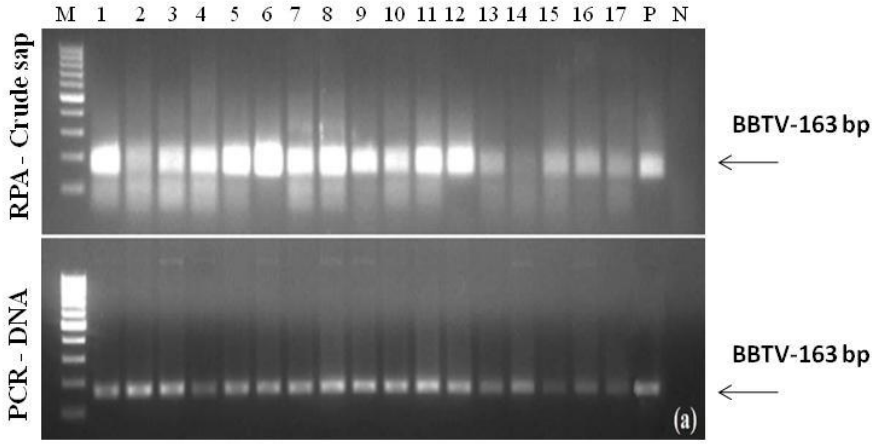

Fig. 2. Comparison of BBTV detection in banana samples using RPA assay with crude sap and PCR assay with purified DNA.

Reference

Daher, R.K., Stewart, G., Boissinot, M. and Bergeron, M.G. 2016. Recombinase Polymerase Amplification for Diagnostic Applications. *Clinical Chemistry*, DOI:10.1373/clinchem.2015.245829

Kapoor, R., Srivastava, N., Kumar, S., Saritha, R.K., Sharma, S.K., Jain, R.K., Baranwal, V.K., 2017. Development of a recombinase polymerase amplification assay for the diagnosis of Banana bunchy top virus in different banana cultivars. Arch. Virol. 162, 2791–2796.

Enzyme linked immunosorbent assay (ELISA) and its application in plant virus diagnosis

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Enzyme-linked immunosorbent assay (ELISA) also known as enzyme immunoassay (EIA), is a large group of enzyme-amplified immunoassays which were introduced into plant virology in the mid 1970s. These assays are still the most robust assays for routine detection of plant viruses in plant samples especially in crude extracts, owing to its simplicity, sensitivity (detection limits of 1-10 ng/ml) and adaptability. These are serological assays based on the detection of virus particles (antigen) complexed with enzyme-labelled antibodies. The amount of virus present is proportional to the amount of enzyme-labelled antibody and is detected by a colorimetric reaction with the enzyme's substrate (Clark and Adams, 1977). ELISA techniques are mainly divided into direct and indirect ELISA. When antigen is detected directly by the enzyme labelled virus specific antibody (i.e. primary antibody), it is known as direct ELISA. However, when antigen is detected indirectly by binding of unlabeled primary antibody-antigen complex with enzyme labelled conjugated antibody (secondary antibody), it is known as indirect ELISA. Since the labelled secondary antibody is directed against all antibodies of a given species, it can be used with a wide variety of primary antibodies (Clark and Bar-Joseph, 1984).

The key step, immobilization of antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (labelled primary antibody, direct ELISA) or indirectly (labelled secondary antibody, indirect ELISA) (Fig. 1).

In the direct ELISA, the antigen is trapped onto walls of the wells of a microtiter plate which are detected by virus specific antibodies (primary antibodies) labelled with enzyme. The direct antigen coating ELISA (DAC-ELISA) and double-antibody sandwich ELISA (DAS-ELISA) can be performed via direct or indirect ELISA. In DAS-ELISA, antigen is sandwiched between primary antibody and enzyme labelled conjugate antibody (secondary antibody) whereas in DAC-ELISA antigen is directly coated onto wells of microtitre plate bounded by specific primary antibody which is identified by another enzyme-labelled probe (anti-immunoglobulin molecules, secondary antibody).

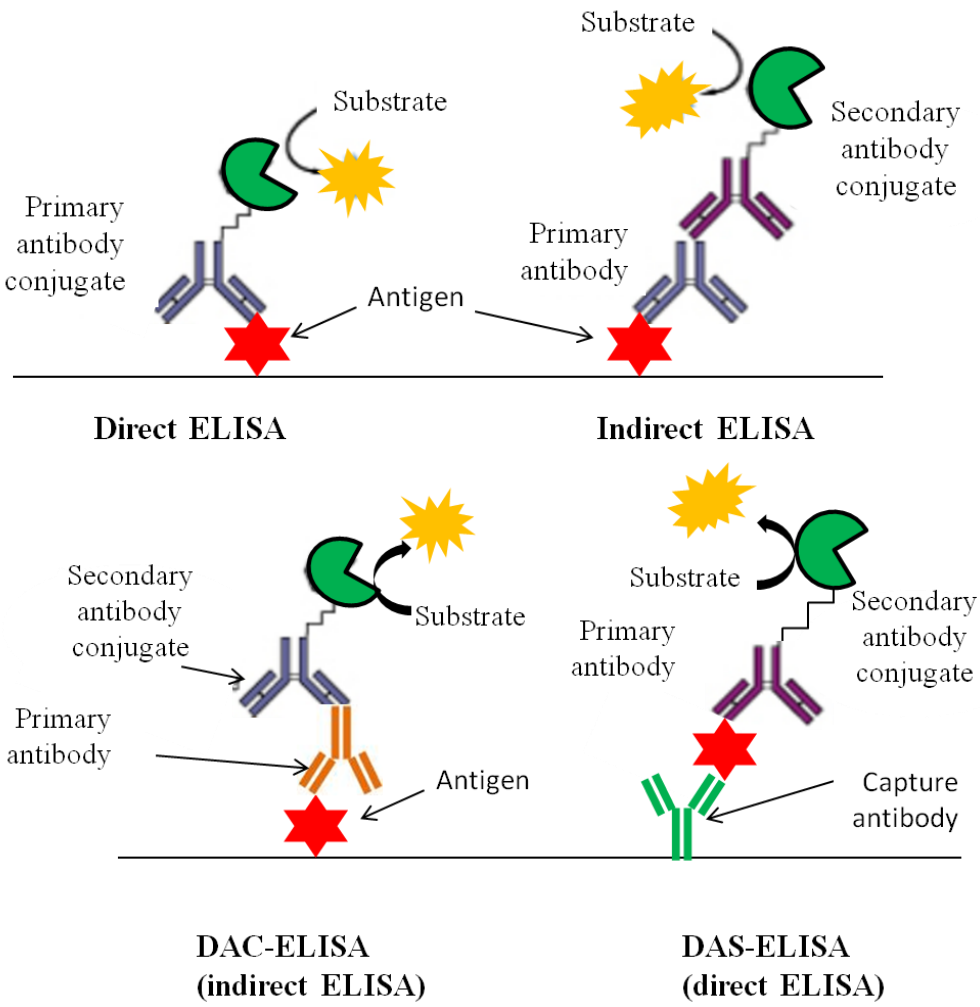

Fig. 1. Schematic representation of various forms of ELISA.

Direct antigen-coated ELISA (DAC-ELISA)

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DAC-ELISA is most commonly used in laboratories for routine virus detection of large number of samples wherein the immunoprobe (specific/primary antibody) is not enzyme-labelled but is itself identified by another universal enzyme-labelled probe (anti-immunoglobulin molecules, secondary antibody). It is a five step procedure: (i) coating the microtitre plate wells with antigen; (ii) blocking unbound sites with heterologous protein; (iii) addition of specific primary antibody; (iv) addition of enzyme-labelled anti-immunoglobulin molecules; (v) addition of enzyme substrate and determination of enzyme-mediated colour reaction (Fig. 1).

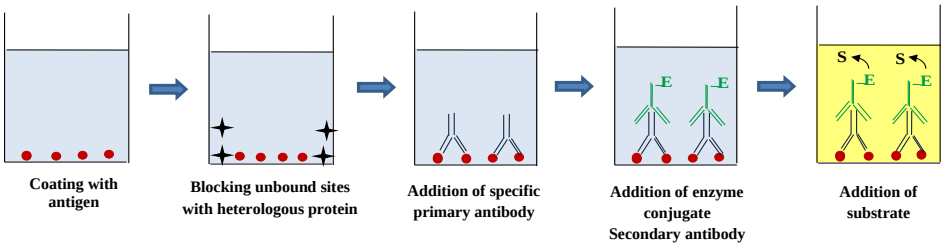

Fig. 1. Basic steps involved in DAC-ELISA

Requirements

(i) General laboratory equipments for serological studies

- Weighing Balance
- ELISA reader with printer
- Single channel pipette (1-10 µl, 20-200 µl, 200-1000 µl)
- Multichannel pipette (30-300 µl)
- Distilled/RO/Millipore Unit
- Hot Plate/Magnetic Stirrer
- Autoclave
- pH Meter
- Refrigerator (4°C)
- Tissue Grinder/ Pestle & Mortar
- Incubator
- Microcentrifuge/Minicentrifuge


Multichannel pipette

(ii) Consumables and Supplies for sero diagnosis


Reagent reservoir

- Polystyrene microplates (96 wells)
- Microtips (10 µl, 200 µl, 1000 µl)
- Microcentrifuge tubes (1.5 ml, 2 ml)
- Reagent reservoir
- Virus specific antisera
- Antigen (virus infected plant material)
- Goat anti-rabbit enzyme conjugate (universal conjugate)
- Substrate (p-nitrophenyl phosphate - PNPP)
- Disposable Gloves

Protocol for DAC-ELISA

1. Weigh 500 mg healthy and infected samples and grind using autoclaved mortar and pestle in coating buffer containing 2% (w/v) PVP at a ratio of 1;3 or 1:5 (w/v) at room temperature. Centrifuge at 12,000 rpm for 2 min and separate supernatant.
2. Dispense 100 µl extract from test as well as healthy samples to each well of the microtitre plate (as per ELISA plan) (See below). Cover the plate and incubate at 37°C for 1 h.
3. Decant and wash the plate by flooding the wells with PBS-T for about 3 min. Repeat wash and soak operations thrice and drain out residual liquid on a paper towel.
4. Dispense 100 µl blocking solution (Bovine Serum Albumin, 1 %, w/v) to each well. Incubate at 37°C for 1 h to block polystyrene well reactive surfaces.
5. Decant and wash the plate thrice as described above.
6. Prepare dilution of virus specific antibody in PBS-TPO and dispense 100 µl to each well and incubate at 37°C for 1 h. (Antiserum dilutions have to be prepared after carefully reading the manufacturer's instructions)
7. Decant and wash the plate thrice as described above.
8. Dispense 100 µl anti-rabbit immunoglobulin alkaline phosphatase (1:30,000, universal conjugate, Sigma, USA) diluted in PBS-TPO to each well. Incubate at 37°C for 1 h.
9. Decant and wash the plate thrice as described above.
10. Dispense 100 µl freshly prepared substrate (p-nitrophenyl phosphate-PNPP, Sigma, USA) solution in substrate buffer (5 mg PNPP tablet in 10 ml substrate buffer) to each well. Incubate at 37°C for 1 h.

11. Measure the intensity of color in each well at 405 nm by using ELISA reader after 15, 30 and 60 min. Stop the reaction by adding 50 µl of 3 M NaOH (Stop solution) to the wells.
12. Compare the absorbance values of the test samples with healthy control. Consider samples showing absorbance (OD_{405}) values more than two times of healthy control as positive (Fig. 2).

Template for the testing of virus infected samples

Ag→		**1:5**				**1:10**				
Ab↓	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**12**
A										
B		Buffer	Buffer			Buffer	Buffer			
C		Healthy	Healthy			Healthy	Healthy			
D		+ve Ctrl	+ve Ctrl			+ve Ctrl	+ve Ctrl			
E		Sample	Sample			Sample	Sample			
F										
G										
H										

Note: Plan of ELISA will vary depending on the number of samples to be tested. It is suggested to keep the border wells of the plate empty.

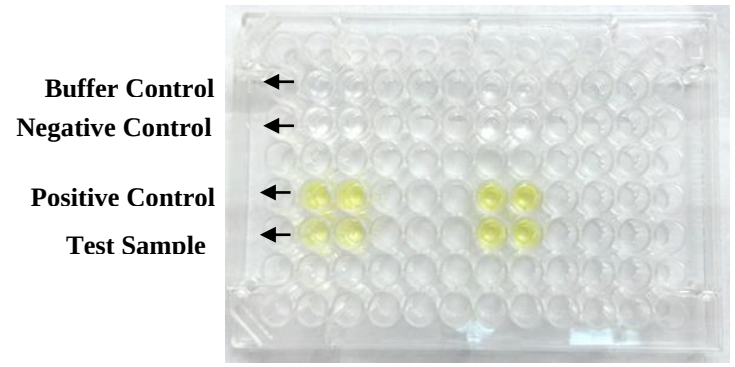

Fig. 2. DAC-ELISA analysis for Cucumber mosaic virus detection after 1 h of PNPP substrate addition. Healthy leaf of banana was used as negative control. CMV infected chilli leaf was used as positive control.

Double antibody sandwich (DAS-ELISA)

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The double antibody sandwich-ELISA (DAS-ELISA, direct procedures) has been the most popular ELISA procedure for plant virus detection. It is a virus-specific and requires each detecting antibody to be conjugated to an enzyme. It is a four step procedure: (i) coating the microtitre plate wells with antigen specific coating antibody (IgG); (ii) addition of antigen containing sample; (iv) addition of enzyme - labelled antibody (E); (iv) addition of enzyme substrate (S) and determination of enzyme - mediated colour reaction (Fig. 1).

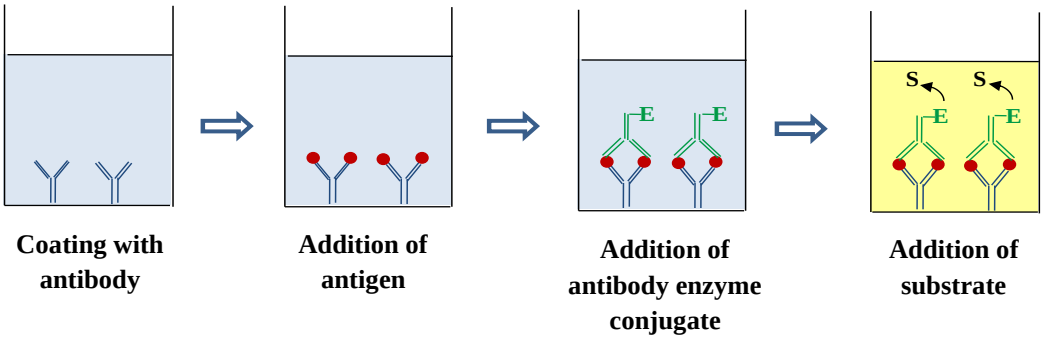

Fig. 1. Basic steps involved in the DAS-ELISA.

Protocol for DAS-ELISA

1. Dilute coating antibody in coating buffer as recommended by the manufacturer and dispense 100 µl of the diluted antibody to the required number of wells (as per ELISA Plan). Cover the plate with aluminium foil and incubate at 37°C for 4 h or 4°C overnight.
2. Decant and wash the plate by flooding the wells with PBS-T for about 3 min. Repeat wash and soak operations thrice and drain out residual liquid on a paper towel.
3. Weigh healthy and infected samples and grind using autoclaved mortar and pestle in extraction buffer at a ratio of 1:10 (w/v) at room temperature. Centrifuge at 12,000 rpm for 2 min and separate supernatant.
4. Dispense 100 µl extract from test as well as healthy samples to each well of the microtitre plate. Cover the plate and incubate at 4°C overnight.
5. Decant and wash the plate thrice as described above.

6. Dilute specific antibody enzyme conjugates (as recommended by manufacturer) in PBS-TPO buffer and dispense 100 µl to each well and incubate at 37°C for 4 h.
7. Decant and wash the plate thrice as described above.
8. Dispense 100 µl freshly prepared substrate (p-nitrophenyl phosphate-PNPP, Sigma, USA) solution in substrate buffer (5 mg PNPP tablet in 10 ml substrate buffer) to each well. Incubate at 37°C for 1 h.
9. Measure the intensity of color in each well at 405 nm by using ELISA reader after 1 h and 2 h. Stop the reaction by adding 50 µl of 3 M NaOH (Stop solution) to the wells.
10. Compare the absorbance values of the test samples with healthy control. Consider samples showing absorbance (OD_{405}) values more than two times of healthy control as positive.

Note: DAS-ELISA protocol is different for each commercial kit. Kindly follow manufacturer's protocol as mentioned on the kit. Prepare buffers (especially extraction buffer) as per manufacturer's instructions.

Reference

Clark, M.F., Adams, A.N., 1977. Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. Journal of General Virology; 34(3):475-483.

Clark, M.F., Bar-Joseph, M., 1984. Enzyme immunosorbent assays in plant virology. In: Methods in Virology. K. Maramorosch and H. Koprowski (Eds.). Academic Press, ISBN 0124702082, New York, USA; 51-85.

Immunocapture - Polymerase Chain Reaction (IC-PCR) based detection of plant viruses
(A case study of Banana streak virus detection)

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PCR is a very sensitive method for detection of plant viruses; however the presence of inhibitory plant compounds or low titres of virus can cause hindrance to PCR assay. An initial immuno-capture step by virus specific antibodies can overcome this problem. Immuno-capture polymerase chain reaction (IC-PCR), is a highly specific and sensitive method for detection of viruses, in which actual viral particles are trapped with virus specific antibodies (Sharman *et al*., 2000). It is a useful technique especially for detection of badnaviruses infecting banana. There are currently four recognized banana-infecting badnavirus (BIB) species: Banana streak GF virus (BSGFV), Banana streak MY virus *(*BSMYV), *Banana streak OL virus* (BSOLV), and *Banana streak VN virus* (BSVNV). BSVs exist in two forms, either the infectious episomal form with a circular double stranded DNA genome, or the endogenous form in which the viral genome is integrated into the host genome.

The most common badnavirus infecting banana in India is BSMYV. Indexing of BSMYV poses a challenge as the integrated virus sequences in the host genome might lead to false positives in PCR. ELISA usually gives erratic results due to low virus titre in infected hosts. Hence, IC-PCR can be performed for specific detection of episomal BSMYV using BSMYV antibody to capture virus prior to amplification by PCR (Sharma *et al*., 2014).

Requirements

As described under chapter 1.

For immuno-capture step, coating buffer & PBS-T is required. The composition is same as described in chapter 7. Virus specific antibody can be procured commercially.

Protocol

It is usually performed in a single polypropylene microfuge tube, to which BSV-antibodies are being coated. The tubes are washed with PBS-T buffer. The washing steps are employed to eliminate traces of plant material (which may harbor integrated BSV) from PCR reaction. The use of BSV-specific antibody is crucial as the traces of plant material are furthermore eliminated from the PCR reaction.

Antibody coating steps A diluted BSMYV antibody (*BSV-Ab; 1:500) in 1X carbonate coating buffer (containing 2% PVP) was coated in sterile polypropylene microfuge tubes with 50 µl BSMYV-A and kept for incubation at 4ºC for overnight.

Note: *Antibody dilution will depend on the source. Antibody used here is generated inhouse at Advanced Centre for Plant Virology, IARI. Commercially procured Ab will have separate working dilution.

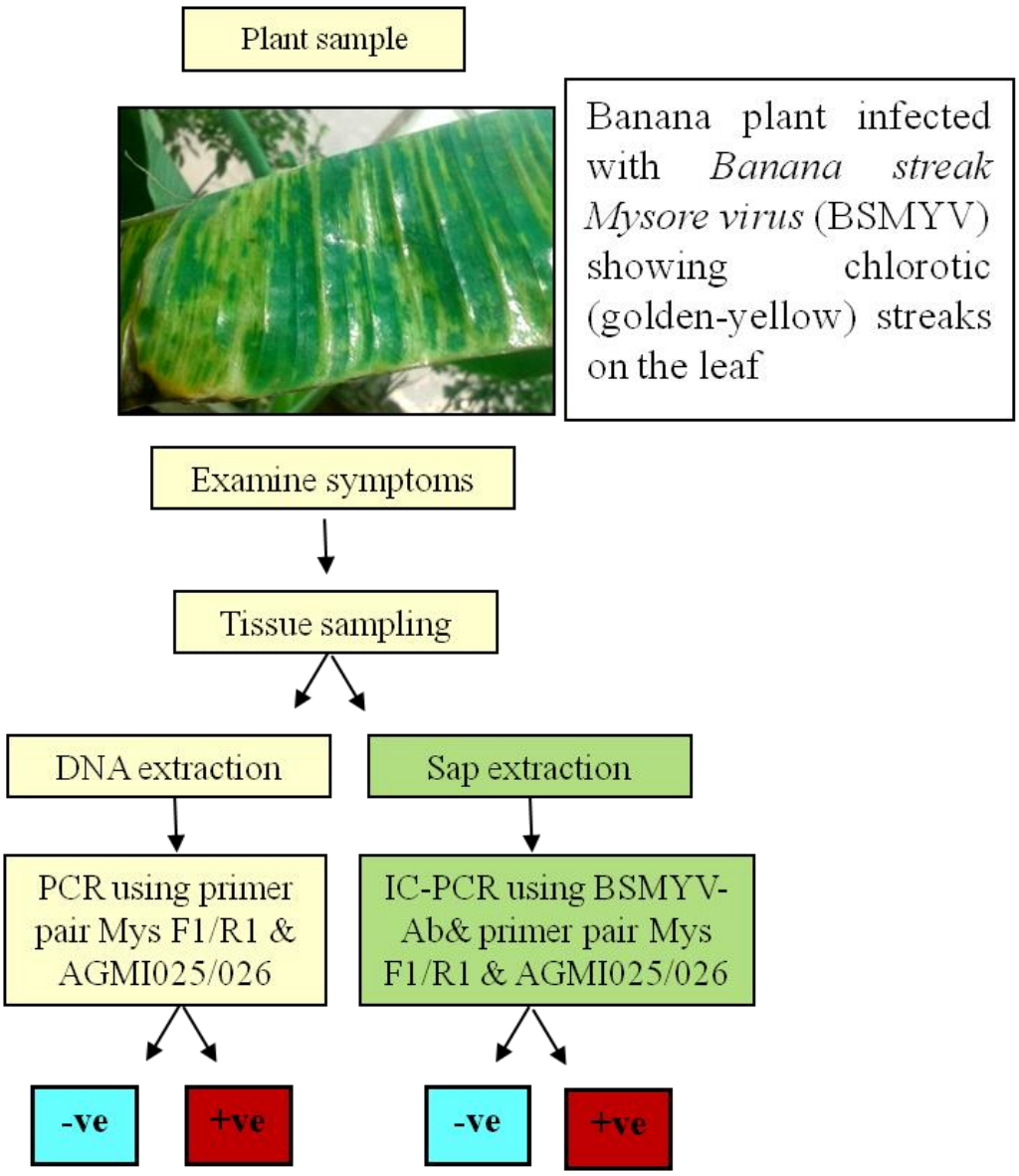

Fig. 1. Diagnostic Flow-chart for the BSMYV

Antigen capture steps

1. Pipette out BSMYV-Ab and dispense 1X PBS-T (100 µl) wash buffer. Keep at room temperature for 3 mins.

2. Pipette out wash buffer and repeat the step twice.
3. Wash tubes with 100 µl sterile distilled water for 3 mins and drain out residual liquid on tissue paper.
4. Weigh 500 mg test/healthy tissue and grind using autoclaved mortar and pestle in 2 ml grinding buffer (containing 2% (w/v) PVP, 0.2% sodium sulphite and 0.2% bovine serum albumin in 1X PBS-T) at a ratio of 1:4 (w/v) at room temperature.
5. Centrifuge the extract at 12,000 rpm for 2 min and dispense 50 µl supernatant in BSV-Ab coated tubes.
6. Incubate at 37ºC for 3 h.
7. Pipette out the diluted plant extract.
8. Wash the tubes three times as described above followed by a final wash with sterile distilled water.
9. Prepare PCR reaction mixture and carry out PCR directly in the tubes.

Immunocapture - PCR reaction

Assemble the PCR reaction components on wet ice and prepare amplification mix by dispensing into 200 µl microfuge tube in the order listed below [See box].

Recipe of reaction mixture

Reagents	Volume (µl)
10X Enzyme Buffer	2.0
$MgCl_2$ (25 mM)	1.0
dNTP Mixture (10 mM)	1.0
Forward primer BSV Mys F1 (10 µM/µl)	1.0
Reverse primer BSV Mys R1 (10 µM/µl)	1.0
Forward primer AGMI025 (10 µM/µl)	1.0
Reverse primer AGMI026 (10 µM/µl)	1.0
Taq DNA Polymerase (2 U/µl)	0.5
Sterile distilled water	11.5
Total volume	20.0

Note: Primers AGMI025 and AGMI026 are specific to *Musa* genomic DNA and can be used as internal control to detect amplification from *Musa* genome. Details of primer sequences are given in Appendix.

Mix the PCR reaction mixture well and place it in a thermal cycler with the following reaction conditions [See Box].

PCR Temperature profile

Steps	Temperature	Time	Cycle
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 sec	
Annealing	58°C	30 sec	30
Primer extension	72°C	1 min	
Final primer extension	72°C	10 min	1

Note: Annealing temperature is specific to a particular combination of primer used for amplification and can be optimized further.

Electrophoresis analysis of amplicons

Analyze the IC-PCR products in 1% agarose gel in Tris-acetate EDTA (TAE) buffer containing ethidium bromide (0.5 µg/ml) as described below (Fig. 1 and 2).

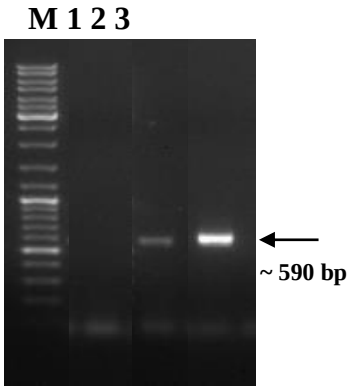

Fig. 1. Electrophoresis analysis of IC-PCR using specific primers for BSMYV detection. Lane 1: Healthy banana leaf sample (no amplification seen); Lane 2: Test sample (~590 bp amplified fragment BSMYV DNA); Lane 3: Positive control (~590 bp amplified BSMYV DNA); M = 100 bp DNA ladder

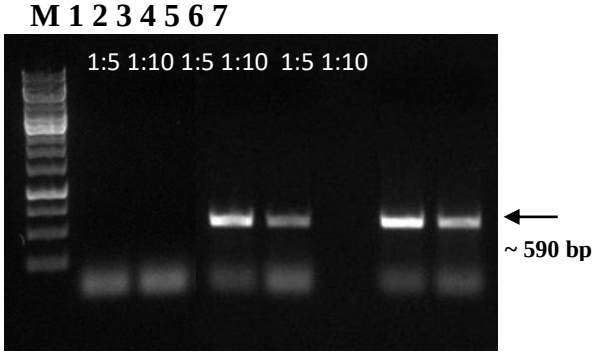

Fig. 2. Electrophoresis analysis of IC-PCR using sap dilutions (1:5, 1:10). Lane 1 & 2: Healthy banana leaf sample (no amplification seen); Lane 3 & 4: Test sample (~590 bp amplified fragment indicating BSMYV infection); Lane 6 & 7: Positive control (~590 bp amplified BSMYV DNA), M = 1 kb DNA ladder

References

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Sharman M, Thomas JE, Dietzgen RG. 2000. Development of a multipleximmunocapture PCR with colourimetric detection for viruses of banana. Journalof Virological Methods; 89:75-88.

Appendix-I

Details of primer sequences for virus / phytoplasma detection

Name	Primer sequence 5'---3'	Annealing Temp (°C)	Specific detection	Expected amplicon (bp)	Reference
CMV Forward CMV Reverse	5' GGATCCATGGACAAATCT 3' 5' ACTTTCGTGGGGCCTCCA 3	58	CMV	~657 bp	Shetti *et al*. (2012)
BSV Mys F1	5'-TAAAAGCACAGCTCAGAACAAACC-3'	58	BSMYV	~589	Geering *et al.* (2000)
BSV Mys R1	5'-CTCCGTGATTTCTTCGTGGTC-3'				
AGMI 025	5'-TTAAAGGTGGGTTAGCATTAGG-3'	58	STMS	~248	Lagoda *et al*. (1998)
AGMI 026	5'-TTTGATGTCACAATGGTGTTCC-3'				
BBTV F	5'-ATGGCGCGATATGTGGTATGC-3'	58	BBTV	~ 439	Selvarajan *et al*. (2011)
BBTV R	5'-TCACGTTTTATTCATCTCTGCTTG-3'				
P1-F	5'-AAG AGT TTG ATC CTG GCT CAG GATT-3'	55	Phytoplasma	~1800	Deng and Hiruki (1991)
P7-R	5'-CGT CCT TCA TCG GCT CTT-3'				
R16F2n-F	5'-GAA ACG ACT GCT AAG ACT GG-3'	56	Phytoplasma	~1200	Gundersena and Lee (1996)
R16R2-R	5'-TGA CGG GCG GTG TGT ACA AAC CCCG-3'				
BBTV F2/ BBTV R2	5'-ACAAGAATCGAAGGTCCCTTCGAGTTTGGTGC-3' 5'-CTCTGTATAATGTATCCTTACTTCTATCGAAGG-3'	40	BBTV through RPA assay	163 bp	Kapoor *et al.* (2017)

Annexure-II

Reagents/Buffers

- **0.5 M EDTA**
  Add 186.1 g of EDTA salt (Mol. wt. 372 g) to 800 ml distilled water and stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with NaOH (20.0 g of NaOH pellets). Make up final volume to 1 L distilled water and sterilize by autoclaving.

- **1M Tris**
  Add 121.13 g of Tris base salt (Mol. Wt. 121.17 g) to 700 ml distilled water and stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with diluted HCl. Make up final volume to 1 L distilled water and sterilize by autoclaving.

- **5NaCl**
  Add 292.5 g of NaCl salt (Mol. Wt. 58.5 g) to 700 ml distilled water and stir vigorously on a magnetic stirrer. Make up final volume to 1 L distilled water upon complete dissolving the salt and sterilize by autoclaving.

- **10 % CTAB**
  Add 100 g of CTAB salt (Mol. Wt. 364.5 g) to 700 ml distilled water and stir vigorously on a magnetic stirrer. Make up final volume to 1 L distilled water upon complete dissolving the salt and sterilize by autoclaving.

- **Ethidium bromide**
  Dissolve 1 g ethidium bromide in 100 ml H_2O and transfer to a dark bottle and store at 4°C.

- **Running Buffer (50X Tris Acetate EDTA, pH 8.0)**

Tris base	242.0 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml

Adjust pH 8.0 with 1 N NaOH. Make up volume to 1 litre. Dilute 50 X TAE buffer to1X before use.

Note: Other buffers such as Tris-Borate EDTA (TBE buffer) can also be used to run agarose gel but is not recommended for preparative gels for recovery of nucleic acids.

Annexure-III

(iii) Reagents/Buffers

- **PBS Stock buffer (Phosphate Buffer Saline, pH 7.4)**

NaCl	8.0 g
$Na_2HPO_4.2H_2O$	1.44 g or
$Na_2HPO_4.12H_2O$	2.90 g or
Na_2HPO_4	1.50 g
KH_2PO_4	0.20 g
KCl	0.20 g
Distilled water	to make 1 litre

Store PBS at 4°C.

- **Wash Buffer (PBS-Tween 20/ PBS-T)**

Add 500 µl Tween-20 to 1000 ml PBS.

- **Coating Buffer (Carbonate buffer, pH 9.6)**

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled waterto make	1 litre

Store coating buffer at 4°C.

- **Enzyme conjugate diluent buffer (PBS-TPO)**

Polyvinyl-pyrrolidone (PVP, MW 40,000)	20.0 g
Egg ovalbumin	2.0 g
PBS-T	1.0 litre

- **Antibody diluent buffer (PBS-TPO)**

Same as PBS-TPO.

- **Blocking solution**

Add 10.0 g bovine serum albumin (BSA/spray dried milk to 1 litre PBS-T).

- **Substrate buffer**

Diethanolamine	97 ml
Distilled water	800 ml

pH adjusted to 9.8 with 1N HCl (add about 67 ml) and volume made up to 1 litre with distilled water.

Store substrate buffer in a dark bottle at 4°C.

- **Substrate**
  Prepare 5 mg p-nitrophenyl phosphate-PNPP tablet in 10 ml substrate buffer. The solution should be prepared fresh prior to use.

- **Fixing / Stop solution**

3 M NaOH	120.0 g
Distilled water	to make 1 litre

Note: Diethanolamine is toxic and harmful to eyes. Take necessary care to avoid contact with skin. PNPP converts to p-nitrophenol after reacting with alkaline phosphatase. P-nitrophenol is corrosive and thus plates after adding substrate must be handled extremely carefully. 0.02 % of sodium azide may be added as a preservative to the buffers for long term storage.

NAHEP sponsored training programme on

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(October 15 - 24, 2019)

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