DIAGNOSTIC METHODS FOR PLANT VIRUSES

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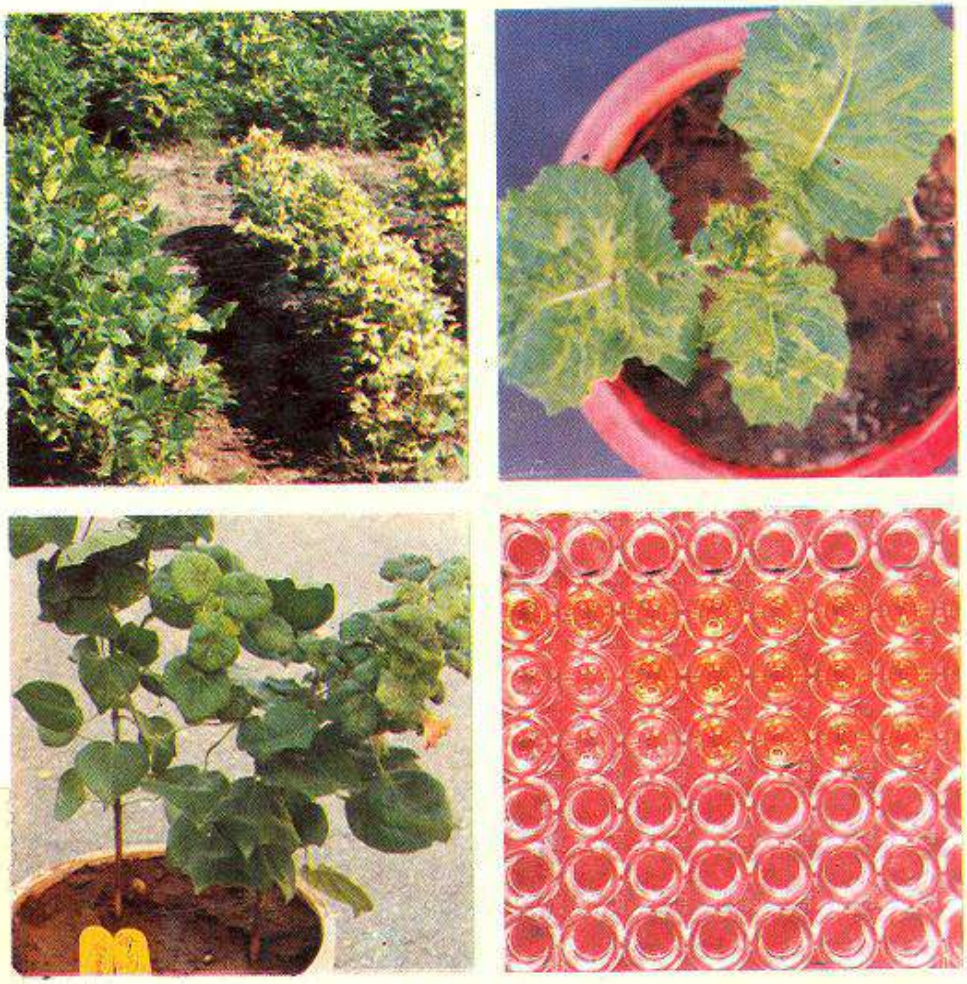

PAKISTAN AGRICULTURAL RESEARCH COUNCIL
P.O. BOX 1031, ISLAMABAD, PAKISTAN

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Mahmood Bodhi

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FOREWORD

Plant viruses are of great significance because they cause serious plant diseases, and result in economic losses. Numerous cases of economic loss caused by virus diseases have been reported world wide. Cotton production in Punjab province of Pakistan for example decreased drastically due to cotton leaf curl virus with a loss of 3 million bales during 1992-93. Similarly in Sind province an economic loss of Rs. 915 million was reported due to banana bunchy top virus during 1992.

Proper identification, characterization and documentation of the disease causing agents is a prerequisite in order to plan suitable control measures for averting losses. The books on diagnosis of plant viruses written by foreign authors are available in the country. However, they do not meet our requirements, as they are meant to cater the specific needs of the developed countries. Therefore, the need for locally-written books on plant virology/pathology has been felt since a long time. "***Diagnostic Methods for Plant Viruses***" is the first ever book in Pakistan written by highly qualified and leading Plant Virologists of the country. The knowledge and art of identifying plant viruses will help in devising appropriate disease management strategies. The diagnostic techniques described in the book will not only help in identification of plant virus diseases but also in understanding the behaviour and characteristics of different viruses. The book should be a good guide for plant pathologists, virologists, molecular biologists, researchers, teachers and graduate students in the fields of plant pathology and virology. My sincere appreciation goes to the authors for their selfless hard work and devotion in accomplishing the formidable task for the advancement of plant virology in this country.

Dr. Muhammad Akbar
Chairman
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Islamabad
June 15, 1998

Preface

Modern agriculture is technology based and causes perturbations in the naturally equilibrated ecological situations. Intensive monoculture of high yielding crop varieties over extensive areas coupled with improved tillage, water management, and extensive use of fertilizers have created micro-and macro-environments extremely favourable for viral diseases. This large scale cultivation of genetically uniform crops is highly unnatural conditions and inevitably invites its own destruction by parasites. This has already resulted in the destruction of various crops by many plant viruses. Failure of cotton crop by cotton leaf curl virus, banana crop by banana bunchy top virus, and chili crop by a virus complex are a few examples of such destruction in Pakistan.

The first requirement for the control of such diseases is the identification of the causative virus or viruses. Over recent years such identification of viral pathogens has become easier due to two new developments (i) a comprehensive and internationally agreed classification system for viruses and (ii) a variety of technical improvements have been made in the methods and techniques that can be used for virus detection and identification. During the past, in Pakistan the subject of Plant Virology has been given less importance mainly due to shortage of trained manpower and well equipped virology laboratories. Difficulties were faced to diagnose the viral pathogens in the absence of proper books or laboratory manuals on diagnostic techniques.

Scientific knowledge on plant viruses is rapidly increasing, but practical information on virus diagnosis is often lacking. There are a couple of books available on diagnosis of plant viruses by foreign authors, but they are either too expensive or not available for non-specialists and for most students especially in developing countries like Pakistan. This book reflects the most recent work and conveys the principles of current techniques in Plant Virology. The need for a suitable book describing the methodology for characterization of plant viruses was felt for a long time. The present volume of this book contains twelve chapters, arranged in a sequence which may provide a route to virus characterization. Wherever, possible the techniques are

described first in outline to provide a basic understanding, the material required are listed, and then step-by-step the procedure.

In Chapter 1, some basic information regarding structure and properties of plant viruses are given. Chapter 2 deals with the most recent plant virus classification. Properties of each virus family and genus have been described. Role and importance of virus disease symptoms in virus diagnosis are discussed in Chapter 3. Mechanical and vector transmission are described in Chapters 4 and 5 respectively. Chapter 6 covers the virus transmission by fungal and nematode vectors. Virus isolation, purification and production of antisera are discussed in Chapter 7. Serological procedures have been developed and diversified rapidly in recent years, and have become one of the most important tools in virus diagnosis. These techniques with practical details are given in Chapter 8. Recently, enzyme-linked immunosorbent assay (ELISA) has gained much importance in virus detection and identification due to its simplicity, sensitivity and reliable quantitative data for better interpretation of the results. Variants of ELISA have been discussed in Chapter 9. The use of electron microscopy to characterize virus particles, with particular reference to negative staining and decoration procedures is described in Chapter 10. The very recent advances into virus detection by use of monoclonal antibodies are discussed in Chapter 11. Theory and practical aspects of nucleic acid hybridization, polymerase chain reaction (PCR) and characterization of virus proteins are described in the last Chapter 12. The appendices given at the end of this book contain complete list of definitive and tentative members of each genus, alphabetical list of viruses with their acronym, list of antisera available with American Type Culture Collection (ATCC) and description of preparation of buffers at various pH levels.

Finally, the objective, of writing this book is to provide a basis for detection, identification and characterization of plant viruses. Such information should help to better understanding of viral disease management and to develop control strategies. This book should be of value to undergraduate and graduate students, teachers and researchers of Plant Pathology and Plant Virology. It should serve as a bench book to the understanding of plant viruses.

The text of this manuscript will certainly not be perfect in presentation and precision of the methodology described. We would therefore greatly appreciate the readers's lenience in judgment and their kindness in informing us of whatever errors they may encounter and of any comment that may help us to improve the next edition of this book.

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Chapter 1
STRUCTURE AND PROPERTIES OF PLANT VIRUSES

1.1 Introduction: Literally the term "*virus*" is used to denote a distinct group of sub-microscopic disease causing agents. Previously "*virus*" was defined as an obligate particulate infectious parasite of sub-microscopic size, with dimensions of less than 200 nm, but after generation of wealth of information about the chemical and physical characteristics of these infectious agents, the term "*virus*" has been defined in a more comprehensive way. According to Matthews (1992) *"a virus is set of one or more nucleic acid template molecules, normally encased in a protective coat or coats of proteins or lipoproteins, that is able to organize its own replication only within suitable host cells. Within such cells, virus replication is (i) dependent on the host's protein-synthesizing machinery (ii) organized from pools of the required materials rather than binary fission (iii) located at sites that are not separated from the host cell contents by lipoprotein bilayer membrane, and (iv) continually giving rise to variants through various kinds of changes in the viral nucleic acid"*. All viruses are parasitic in nature and cause a variety of diseases to all forms of living organisms and have four characteristics in common (i) they are infectious (ii) they are invisible under the light microscope (ii) they multiply only in living host tissue and (iv) they are nucleoprotein.

Viruses consist of nucleic acid (RNA or DNA) enclosed within a protective coat of protein or lipoprotein called "*capsid*". The nucleic acid is the infectious component and carries the genetic information and is responsible for replication of the virus. The nucleic acid is either ribose nucleic acid (RNA) or deoxyribose nucleic acid (DNA) in each virus. In most plant viruses, only one kind of protein is found. However, some of the larger viruses may have different proteins, each probably has different function. Complete virus particle with its nucleic acid, capsid, or envelop if any, is called *"virion"*. Capsid, the protein coat, is also called *"shell"*, is intact with nucleic acid. Nucleocapsid is the structure composed of the nucleic acid and the protein closely associated with it.

1.2 Shape and size of plant viruses: Plant viruses differ greatly from all other pathogens not only in size and shape, but also in simplicity of their chemical composition, physical structure, pathogenesis, multiplication, dissemination, translocation within the host and symptoms induction. Plant viruses have different shapes and sizes (Figure 1.1). They may be rod shaped, isometric (spherical) or bacilliform (bullet-shaped). Bacilliform viruses measure from 36-380 nm in length and 18-95 nm in width. Rigid rod shaped viruses range from 100-300 nm in length and 13-23 nm in width, whereas, the flexuous rods range from 470-2000 nm in length and 10-13 nm in width. Spherical viruses range from 17-70 nm in diameter. Viruses are classified in families, genera and species on the basis of shape and size of particles, type of nucleic acid and mode of transmission.

1.3 Composition and structure: Majority of plant viruses consist of ribose nucleic acid (RNA), but at least more than 25 viruses have been shown to contain deoxyribose nucleic acid (DNA). All have protein coat called *capsid*. However, a few also have lipoprotein membrane.

1.3.1 Nucleic acid: The nucleic acids (RNA or DNA) are chain-like molecules consisting of hundreds or more often, thousands of units called "*nucleotides*". Each nucleotide consists of a nitrogenous base (purine or pyrimidine) attached to a phosphoric acid molecule through a sugar (ribose in RNA and deoxyribose in DNA). RNA contains four different basis: adenine, guanine, cytosine and uracil. In case of DNA, uracil is replaced by thymine, the other three basis being the same. Approximately 400 plant viruses contain single stranded RNA (ssRNA), and about 60 or more contain double stranded RNA (dsRNA). Twelve plant viruses contain double stranded DNA (dsDNA), and about 15 contain single stranded DNA.

1.3.2 Capsid protein: The viral protein forming a protective coat around the nucleic acid is called *"capsid"*. Viral proteins, like all other proteins, consist of amino acids which are organized into protein sub-units. The amino acid contents are the same for the

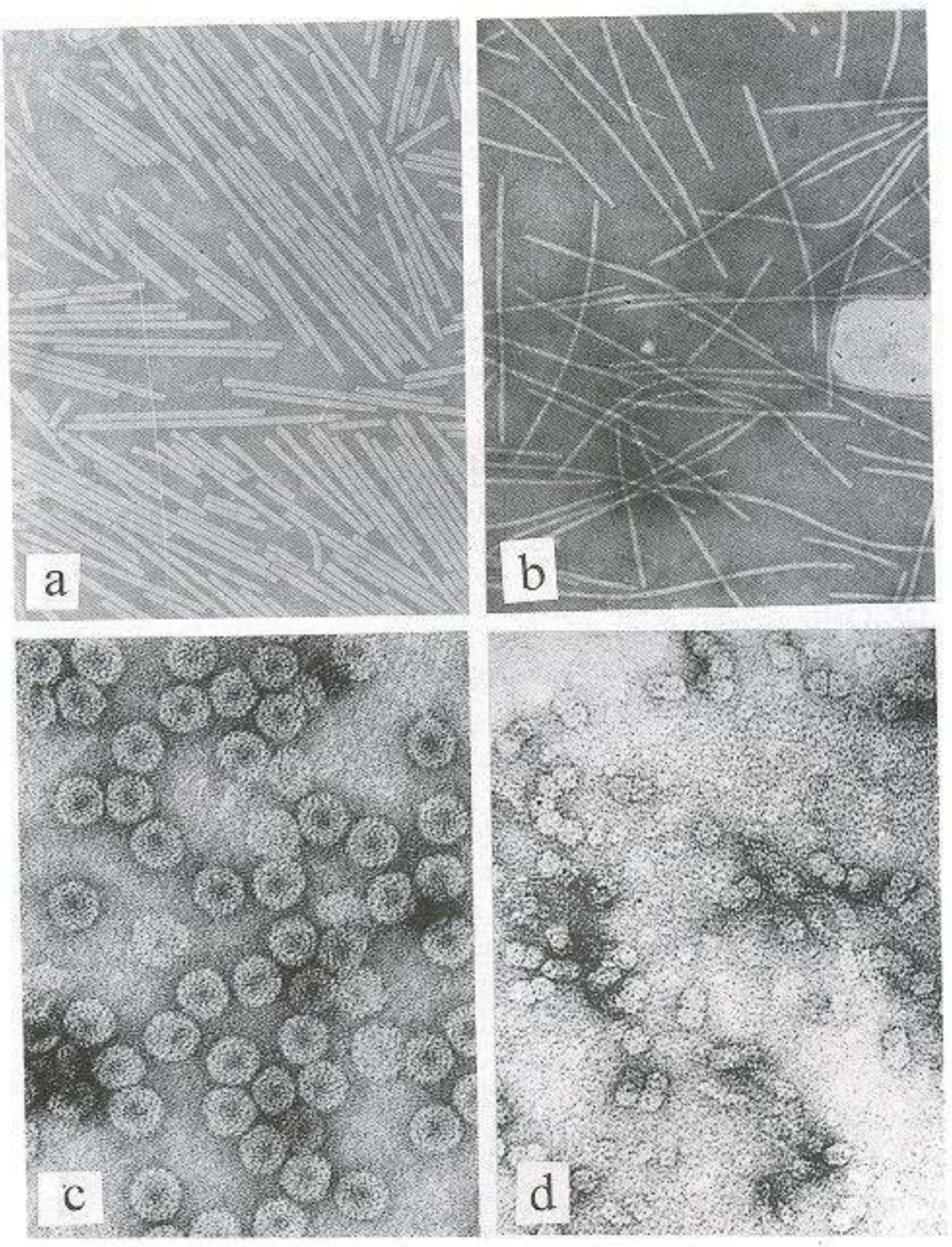

Figure 1.1: Various shape and size of virus particles (a) elongated rod shaped (b) elongated flexuous (c) isometric particles (d) geminivirus particles.

identical viruses, but may vary for different viruses, different strains of the same virus, and even for different proteins of the same virus particle. The number of amino acids per protein subunits vary among different viruses. For example, the protein subunit of tobacco mosaic virus (TMV) has 158 amino acids and that of turnip yellow mosaic virus (TYMV) has 189 amino acids. In case of rod shaped viruses the nucleic acid is packed tightly between the helix of protein subunits, whereas, in polyhedral (spherical) viruses the protein subunits are tightly packed in arrangements that produce 20 or multiple of 20 facets and form a shell, in which the nucleic acid is folded and organized.

1.4 Function of capsid: Protein shell protects the nucleic acid against nucleases and other degrading factors. It also plays some definite role in virus vector specificity during transmission.

1.5 Other components: In addition to nucleic acid and proteins, some plant viruses also contain polyamines, (e.g. turnip yellow mosaic virus), lipids (e.g. tomato spotted wilt virus) and divalent metal ions (e.g Ca^{+2} and Zn^{+2}). Water is a necessary component of all viruses.

1.6. Virus genome: The nucleic acid of a virus is referred as virus *"genome"*, which is either single stranded (ss) or double stranded (ds) RNA or DNA. The RNA genome may be of positive sense (polarity), when it directly functions as messenger RNA (mRNA) and encodes the relevant proteins, or of negative sense (polarity), when it cannot act as mRNA and fails to act as template for protein synthesis. The RNA genome of positive polarity may be either monopartite, when a single RNA strand contains all the genetic information or multipartite when the genetic information is divided into two or more than two RNA segments all of which must be present for the infectivity of a multipartite virus. The summary of genome types is as follows:

1.6.1 RNA Genome

1.6.1.1 Single stranded RNA (ssRNA)

a. Positive sense RNA

i. Monopartite RNA (e.g. tobacco mosaic virus)

ii. Multipartite RNA (e.g. barley stripe mosaic)

b. Negative sense RNA (e.g Rhabdoviruses)

1.6.1.2. Double stranded RNA (dsRNA) (e.g Reoviruses).

1.6.2 DNA Genome

1.6.2.1 Single stranded DNA (ssDNA; e.g Geminiviruses)

1.6.2.2 Double stranded DNA (ds DNA; e.g Caulimoviruses)

1.7. Function of viral genome: The RNA or DNA genetic material of plant viruses, controls infectivity and codes for specific structural and non-structural proteins. Enzymes and other proteins responsible for virus replication, virus movement and vector transmission are also encoded by viral genome.

1.8. Economic importance of plant viruses: Viruses attack almost all economically important crop plants. In most cases the viruses cause considerable reduction in yields and reduces quality of infected crop. Various workers have tried to assess crop losses caused by viruses in various crops. A few examples of losses caused by plant viral diseases are shown in Table 1.1.

Almost, all viruses cause economic losses under favourable conditions. However, in a few cases virus infection of a mild strain may protect the crop from subsequent infection by a severe strain, hence beneficial to the growers. Latent infection, in which the virus does not induce symptoms may cause little or no loss of yield in infected plants. Mild strains of a virus may be used artificially to protect against virulent strains of the same or related viruses. This phenomenon is called *"cross protection"*. This technique has been used successfully to control tomato mosaic virus in commercial tomato crop in Europe and papaya mosaic virus in Brazil and Taiwan.

Table1.1: Yield reduction and losses caused by various plant viruses in different crop species.

Crop	Virus	%Yield reduction	Country	Reference
Asparagus	MYMV	49	Pakistan	Aftab et. al., 1993
Bean	BYMV	33	USA	Hampton, R.O. 1975
Bean	BCMV	64	USA	Hampton, R.O. 1975
Banana	BBTV	30	Pakistan	Soomro, M.H. 1993
Cotton	CLCuV	59	Pakistan	Khan et. al., 1995
Lettuce	LMV	56	USA	Zink & Kimple, 1960
Mash	ULCV	81	Pakistan	Bashir, et. al. 1992
Tobacco	TEV	3-18	USA	Gooding, G.V. 1970
Wheat	BYDV	29	Australia	Smith & Sward, 1982

BYMV: Bean yellow mosaic virus
BBTV: Banana bunchy top virus
BYDV: Barley yellow dwarf virus
ULCV: Urdbean leaf crinkle virus
TEV: Tobacco etch virus
BCMV: Bean common mosaic virus
LMV: Lettuce mosaic virus
CLCuV: Cotton leaf curl virus
MYMV: Mungbean yellow mosaic virus

Chapter 2

CLASSIFICATION OF PLANT VIRUSES

2.1. Introduction: Classification is the arrangement of biological entities into taxonomic categories (taxa) on the basis of similarities and/or relationships, whereas nomenclature is the arrangement of names to taxa according to international rules. Similar to other organisms, plant viruses are also grouped and classified according to consistent and accurately determined characteristics and relationships. Naming of new viruses is mostly based on the host plant in which it was first found, and the disease symptoms it caused. For example, the virus inducing mosaic symptoms in tobacco is called tobacco mosaic virus (TMV). Another virus causing ring spot symptom in tomato is called tomato ring spot virus. With the discovery of more viruses and with the advancement of plant virology, suggestions were proposed by different virologists for virus classification. The requirement for criteria and accurate classification system based on well defined characters, became important during 1950s and 60s as more and more information on individual plant virus were accumulated. The requirement was met by the appointment at the International Congress of Microbiology held in Moscow in 1966 of a committee to investigate virus taxonomy. This committee later was designated as the International Committee on Taxonomy of Viruses (ICTV), and its objectives are; (a) to develop an internationally accepted taxonomy for viruses, (b) to establish internationally agreed upon names of taxonomic genera of viruses and (c) to communicate the latest results on classification and nomenclature of viruses to virologists by holding meetings and publishing results. As a result of the work of this committee, and its plant virus sub-committee, a system for plant virus classification was introduced based on such characteristics as virus particle morphology, type of nucleic acid, genomic structure and type of vector.

2.2. Properties of viruses used in taxonomy: The properties used in plant virus taxonomy are summarized below:

2.2.1 Particle morphology

Virion size
Virion shape
Presence or absence and nature of peplomers
Presence or absence of an envelop
Capsid symmetry and structure

2.2.2 Physiochemical and physical properties

Virion molecular mass (Mr.)
Virion buoyant density (in CsCl, sucrose etc.)
Virion sedimentation coefficient
pH stability
Thermal stability
Cation stability (Mg^{++}, Mn^{++})
Solvent stability
Irradiation stability

2.2.3 Nucleic acid structure

Type of nucleic acid (DNA or RNA)
Size of genome in kb/kbp
Strandedness (single or double)
Linear or circular
Sense (positive or negative)
Number or size of segments
Nucleotide sequence
Genome organization

2.3 The new classification and nomenclature: According to the new classification system, the plant viruses are now grouped in orders, families and genera i.e. formal taxa with Linnean hierarchy and phylogenic implications.

2.3.1 Orders: Orders are clusters of viruses denoted by names with suffix -virales, which embrace families and genera with common characteristics that distinguish them from other comparable taxa. Mononegavirales is the only order currently established, which contains two families with plant virus representatives i.e. *Rhabdoviridae* and *Bunyaviridae*.

2.3.2 Families: Families are clusters of viruses denoted by names ending with the suffix -viridae. Families are distinct from other comparable taxa and comprise genera with common characteristics. Thirteen families with plant virus representatives have been identified so far.

2.3.3 Genera: Genera are clusters of viruses denoted by names with the suffix -virus. They are distinct from similar taxa and comprise a single or several viral species that share certain properties. Currently, there are 22 "floating" genera awaiting assignment to families.

2.3.4 Species: Species are individual viruses possessing characteristics distinct from those of similar viruses so as to allow their unequivocal identification. Properly characterized species are called "definitive", whereas those incompletely known, or having unusual characters, are called "tentative".

The new classification of plant viruses (Table 2.1) differs from the former classification, as in the 5th Report of ICTV (Francki *et. al.* 1991), because (i) the noval taxonomic arrangement in families and genera (ii) the assignment of international names to all taxa (iii) the splitting of the potyvirus group (now family Potyviridae) into three genera, i.e. *Potyvirus*, transmitted by aphids, *Bymovirus*, transmitted by fungi, and *Rymovirus*, transmitted by mites (iv) the transfer of cryptoviruses in *Partitiviridae*, a family of fungal viruses (v) the addition of three new genera i.e. *Idaeovirus*, type species rasberry bushy dwarf virus; *Trichovirus*, type species apple chlorotic leaf spot virus and *Umbravirus*, type species carrot mottle virus. Another minor change is the substitution of maize chlorotic dwarf virus with rice tungro spherical virus as the type species of newly named genus *Waikavirus*.

Table 2.1: Classification of plant viruses in families, genera and species.

Family	Genus	Type species	Acronym
1. The DNA Viruses			
1.1 The ssDNA viruses			
Geminiviridae	Sub-group-I-Geminivirus	Maize streak virus	MSV
	Sub-group-II-Geminivirus	Beet curly top virus	BCTV
	Sub-group-III-Geminivirus	Bean golden mosaic virus	BGMV
1.2 The DNA and Reverse Transcriptase Viruses (dsDNA)			
?	Badnavirus	Commelina yellow mottle virus	ComYMV
?	Caulimovirus	Cauliflower mosaic virus	CaMV
2. The RNA Viruses			
2.1 The dsRNA Viruses			
Partitiviridae	Alphacryptovirus	White clover cryptic virus-1	WCCV-1
	Betacryptovirus	White clover cryptic virus-2	WCCV-2
Reoviridae	Fijivirus	Fiji disease virus	FDV
	Oryzavirus	Rice ragged stunt virus	RRSV
	Phytoreovirus	Wound tumor virus	WTV
2.2 The Negative Sense ssRNA Viruses			
Rhabdoviridae	Cytorhabdovirus	Lettuce necrotic yellow virus	LNYV
	Nucleorhabdovirus	Potato yellow dwarf virus	PYDV
Bunyaviridae	Tospovirus	Tomato spotted wilt virus	TSWV
?	Tenuivirus	Rice stripe virus	RSV
2.3 The Positive Sense ssRNA Viruses			
Sequiviridae	Sequivirus	Parsnip yellow fleck virus	PYFV
	Waikavirus	Rice tungro spherical virus	RTSV
Comoviridae	Comovirus	Cowpea mosaic virus	CPMV
	Fabavirus	Broadbean wilt virus	BBWV
	Nepovirus	Tobacco ringspot virus	TRSV

Table 2.1: Continued

Family	Genus	Type species	Acronym
Potyviridae	Bymovirus Potyvirus Rymovirus	Barley yellow mosaic Potato virus Y Ryegrass mosaic virus	BaYMV PYV RGMV
?	Enamovirus	Pea enation mosaic virus	PEMV
?	Luteovirus	Barley yellow dwarf virus	BYDV
?	Sobemovirus	Southern bean mosaic virus	SBMV
?	Umbravirus	Carrot mottle virus	CMoV
Tombusviridae	Carmovirus Tombusvirus	Carnation mottle virus Tomato bushy stunt virus	CarMV TBSV
?	Dianthovirus	Carnation ringspot virus	CRSV
?	Furovirus	Soil-borne wheat mosaic virus	SBWMV
?	Hordeivirus	Barley stripe mosaic virus	BSMV
?	Machlomovirus	Maize chlorotic mottle virus	MCMV
?	Necrovirus	Tobacco necrosis virus	TNV
?	Tobamovirus	Tobacco mosaic virus	TMV
?	Tobravirus	Tobacco rattle virus	TRV
Bromoviridae	Alfamovirus Bromovirus Cucumovirus Ilarvirus	Alfalfa mosaic virus Brome mosaic virus Cucumber mosac virus Tobacco streak virus	AMV BMV CMV TSV
?	Closterovirus	Beet yellow virus	BYV
?	Capillovirus	Apple stem grooving virus	ASGV
?	Idaeovirus	Rasberry bushy dwarf virus	RBDV
?	Marafivirus	Maize rayado fino virus	MRFV
?	Potexvirus	Potato virus X	PVX
?	Trichovirus	Apple chlorotic leaf spot virus	ACLSV
?	Tymovirus	Turnip yellow mosaic virus	TYMV

2.4 Description of plant virus families and genera: In this section the characteristics of plant virus families and genera are discussed.

2.4.1 The DNA Viruses

2.4.1.1 The ssDNA Viruses

2.4.1.1.1 Family: Geminiviridae

Taxonomic structure of the family

Family: Geminiviridae

Genus: Sub-group-1-Geminivirus

Genus: Subgroup-II-Geminivirus

Genus: Sub group-III-Geminivirus

Virions are geminate, 18 nm wide and 30 nm long. Virions contain a single structural protein and a single molecule of circular ssDNA, monopartite or bipartite. Transmitted in nature by insect vector belonging to the Aleurodidae or Cicadellade. Transmitted in a persistent manner. Non-vector transmission for some members is by mechanical inoculation, but with difficulty, not by seed, not by pollen.

2.4.1.1.1.1 Genus: Subgroup-I-Geminivirus

Type species: Maize streak virus (MSV)

The genome of Subgroup-I-Geminivirus consists of a single component. Close serological relationships exist between viruses originating from the same continent. Viruses originating from different continents are either unrelated or distantly related. Host range is narrow. Members of Subgroup-I-Geminivirus are limited to members of the Gramineae. In nature the viruses are transmitted persistently by leafhoppers (Homoptera: Cicadellidae). Sub-group-I Geminiviruses not transmitted by mechanical inoculation. The species of this genus are listed in Appendix-1.

2.4.1.1.1.2 Genus: Subgroup-II-Geminivirus

Type species: Beet curly top virus (BCTV)

The genome of Subgroup-II-Geminivirus consists of a single component. Close serological relationships exist between the members.

Type species beet curly top virus (BCTV) has a very wide host range. over 300 species in 44 plant families. The viruses are transmitted in nature by leaf hoppers in the persistent (circulative, non-propagative) manner. The species are listed in Appendix-1.

2.4.1.1.1.3 Genus: Subgroup-III-Geminivirus

Type species: Bean golden mosaic virus (BGMV)

The genome of the majority of Subgroup-III-Geminivirus consist of two components (A and B ssDNA). Serologically closely related. Individual Subgroup-III-Geminivirus generally have narrow host range among dicotyledonous plants. The viruses are transmitted in nature by whitefly (*Bemisia tabaci*). The species of the genus are listed in Appendix-1.

2.4.1.2 The DNA and Reverse Transcriptase Viruses (dsDNA)

2.4.1.2.1 Genus: Badnavirus

Type species: Commelina yellow mosaic virus (CoYMV)

Virions are bacilliform, non-enveloped. Virions are uniformly 30 nm in width. Modal length of the particle is 130 nm, but particles ranging in length from 60-900 nm are commonly observed. Virions contain a single molecule of circular dsDNA. Virions are only moderately immunogenic. Host range is restricted. The majority of the *Badnoviruses* occur is clonally propagated plant hosts and are therefore, spread by vegetative propagation of infected plant materials. The majority are transmitted in nature by mealybugs (Hompotera: Pseudococcidae), and several are also seed-and/or pollen transmitted. Rice tungro Badnovirus is transmitted by Cicadellidae leaf hopper vectors. The viruses are transmitted in semi-persistent manner. The viruses occur world wide. The most frequent symptom type is interveinal chlorotic mottling.

2.4.1.2.2 Genus: Caulimovirus

Type species: Cauliflower mosaic virus (CaMV)

Virions are isometric, about 50 nm in diameter, non-enveloped. Virions contain single molecule of dsDNA. The viruses are immunogenic. There are serological relationships among some members. The natural host range of most members is narrow. Disease symptoms are usually mosaic and mottle. The viruses are transmissible experimentally by mechanical inoculation. In nature, they are transmitted by aphids in a semi-persistent manner. the species are listed in Appendix-I.

2.4.2 The RNA Viruses

2.4.2.1 The dsRNA Viruses

2.4.2.1.1 Family: Partitiviridae

Taxonomic structure of the family

Family: Rartitiviridae

Genus: Alphacryptovirus

Genus: Betacryptovirus

Virions are isometeric, non-enveloped, 30-40 nm in diameter. Symmetry of particles has not been determined. Virions contain two unrelated linear dsRNA segments (1.4 - 3.0 kbp in size). The two segments of the individual viruses are usually of similar size. Virions are efficient immunogens. Memebers are serologically related, may be strains of single virus.

The viruses are associated with latent infections of their hosts. There are no know natural vectors. The plant cryptoviruses are transmitted by ovule and by pollen to the seed embryo. There is no graft transmission and apparently no-cell transport, except at cell division. Seed transmission is the only known mode for the transmission of cryptoviruses. The following two plant virus genera are included in this family.

2.4.2.1.1.1. Genus: Alphacryptovirus

Type species: White clover cryptic virus 1 (WCCV-1)

Virions are isometric, 30 nm in diameter. The virions typically contain two dsRNA segments. It is not known whether the dsRNA segments are packed together or separately. Viruses are serologically related. The species of the genus are listed in Appendix-I.

2.4.2.1.1.2 Genus: Betacryptovirus

Type species: White clover cryptic virus-2 (WCCV-2)

Virions are isometric, 38 nm in diameter. Particles show prominent subunits, but their precise geometrical arrangement is not clear. The particles are rounded and are not penetrated by stain. Viral nucleic acid comprises two dsRNA segments. Some viruses in the genus are serologicallay related, none are related to viruses in the genus *Alphacryptovirus*. The species of the genus are listed in Appendix-1.

2.4.2.1.1 Family: Reoviridae

Taxonomic structure of the family

Family: Reoviridae
Genus: Fijirivus
Genus: Oryzavirus
Genus: Phytoreovirus

Virions are icosahederal in structure, but many appear, spherical in shape. They are 60-80 nm is diameter. Virions contain 10,11,12 segments of linear dsRNA, depending on the genus. No antigenic relationship has been found between viruses in different genera. This family has nine genera and three of them infect plants. The others are pathogenic to arthropods and vertebrates.

2.4.2.1. 2 Genus: Fijivirus

Type species: Fiji disease virus (FDV)

Fijiviruses have a fragile structure and contain 10 dsRNA segments. They are transmitted by plant hoppers and infect phloem cells of susceptible plants. Virions are double shelled, spherical, 65-70 nm in diameter. Three groups of fijiviruses have been recognized based on antigens associated with core particles. All *Fijiviruses* induce hypertrophy of the phloem leading to vein swelling and sometimes gall formation. The species of the genus are listed in Appendix-1.

2.4.2.1.2 Genus: Oryzavirus

Type species: Rice raged stunt virus (RSSV)

Orzyaviruses appear to lack an outer capsid and possess a genome consisting of 10 dsRNA species. They are transmitted by viruliferous plant hoppers to plants in the family Gramineae. Particle diameter is in the range of 57-65 nm. Cross reaction among the viruses has been reported. Viruses infect plant in the family Gramineae, causing diseases of rice. The species of the genus are listed in Appendix-1.

2.4.2.1.3 Genus: Phytoreovirus

Type species: Wound tumor virus (WTV)

Virions are 65-70 nm in diameter. Phytoreoviruses have 12 segments of dsRNA. No antigenic relationship exists among the viruses. Plant hosts are either dicotyledons or the family Gramineae. Transmission is by leafhoppers. No seed transmission occurs. Experimentally not mechanically transmissible from plant to plant. The species of the genus are listed in Appendix-1.

2.4.3 The negative sense ssRNA viruses

2.4.3.1 Family: Rhabdoviridae

Taxonomic structure of the family

Family: Rhabdoviridae

Genus: Cytorhabdovirus

Genus: Nucleorhabdovirus

Virions are 100-430 nm long and 45-100 nm in diameter. Viruses infecting plants are bacilliform. The viruses which infect vertebrates are bullet shaped. A wide variety of plants are susceptible to plant Rhabdoviruses, although each virus has restricted host range. Most of the plant Rhabdoviruses are transmitted by leaf hoppers, plant hoppers and aphids.

2.4.3.1.1 Genus: Cytorhabdovirus

Type species: Lettuce necrotic yellow virus (LNYV)

Cytorhabdoviruses replicate in the cytoplasm of the infected cells. Information on the genome structure is limited. The species of the genus are listed in Appendix-1.

2.4.3.1.2 Genus: Nucleorhabdovirus

Type species: Potato yellow dwarf virus (PYDV)

Nuclorhabdoviruses multiply in the nucleus of the plants forming large granular inclusions that are thought to be sites of virus replication. The viruses have not be assigned to serogroups or other taxonomic groups. The species of the genus are listed in Appendix-1.

2.4.3.2 Family: Bunyaviridae

Taxonomic structure of the family

Family: Bunyaviridae

Genus: Tospovirus

Virions are spherical, 80-120 nm in diameter. Virions contain 3 molecules of negative or ambisence ssRNA. Virions are enveloped, and composed of 2-7% carbohydrates by weight. All viruses have four structural proteins, two external glycoproteins (G1,G2), nucleocapsid protein (N) and a large transcriptase protein (L).

2.4.3.2.1 Genus: Tospovirus

Type species: Tomato spotted wilt virus (TSWV)

Virus particles are 85 nm in diameter, enveloped. Nucleic acid is ssRNA, linear and tripartite. Virus preparations are poorly immunogenic. TSWV has very wide host range, and is common in temperate and sub-tropical regions causing diseases of economic importance in tomato, tobacco and potato. In nature transmitted by thrips. Non-vector transmission is by mechanical means and not by seed or pollen. TSWV is the only member of this genus.

Al least 9 species of thrips have been reported to transmit *Tospoviruses*. Transmission involves the sap of infected plants. More than 360 plant species belonging to 50 families are known to be susceptible to infection with *Tospoviruses*.

2.4.3.3 Genus: Tenuivirus

Type species: Rice stripe virus (RSV)

Virions have a thin filamentous shape, they consists of nucleocapsids, 3-10 nm in diameter with lengths proportional to the size of their RNA. The filamentous particles may appear to be spiral,

branched or circular. No envelope has been observed. Virus preparations are separated into 4 or 5 components by sucrose density gradient centrifugation. Virions contain ssRNA which is segmented. Rice stripe virus is related serologically to maize stripe virus and distantly related to rice grass stunt virus. *Tenuiviruses* are restricted to the host family *Gramineae*. Viruses are transmitted by plant hoppers in a persistent manner, and in some cases transovarial transmission by viruliferous females to progeny. Mechanical transmission is difficult. The species of the genus are listed in Appendix-1.

2.4.4 The positive sense ssRNA viruses

2.4.4.1 Family: Sequiviridae

Taxonomic structure of the family

Family: Sequiviridae

Genus: Sequivirus

Genus: Waikavirus

Particles are isometric, about 30 nm in diameter. The main virion component contains one molecule of infective, positive sense ssRNA. Sequivirus RNA is not poly-adenylated but Waikavirus RNA is. Polyclonal sera contain antibodies to all virion proteins. Natural host ranges are restricted. Transmission is semi-persistent by aphids or for most Waikavirus species by leafhoppers. A helper protein is needed which may be self-encoded (*Waikavirus*) or encoded by a helper virus (*Sequivirus*).

2.4.4.1.1 Genus: Sequivirus

Type species: Parsnip yellow fleck virus (Parsnip serotype) (PYFV)

The RNA is about 10 kb. PYFV RNA is not polyadenylated and lacks small ORF near the 3' -end. There are about 400 amino acids upstream of the structural proteins in the large polyprotein. Transmission of PYFV depends on the presence of a helper protein encoded by anthriscus yellow waikavirus. The species of the genus are listed in Appendix-1.

2.4.4.1.2 Genus: Waikavirus

Type species: Rice tungro spherical virus (RTSV)

The RNA is longer than 11 kb and has a poly (A) tail. RTSV RNA contains a small ORF near the 3'-end and has 600 amino acids upstream of the structural proteins in the large polyprotein. Transmission depends on a self-encoded helper protein. The helper protein of some members can assist transmission of other unrelated viruses. The species of the genus are listed in Appendix-1.

2.4.4.2 Family: Comoviridae

Taxonomic structure of the family

Family: **Comoviridae**
Genus: **Comovirus**
Genus: **Fabavirus**
Genus: **Nepovirus**

Virions are non-enveloped, 28-30 nm in diameter and are icosahederal. The genome consists of two species of linear positive sense ssRNA. The virions are good immunogens. Species belonging to the same genus are serologically interrelated, often distantly. Host range is narrow to wide. Symptoms vary widely within each genus. Member viruses of the family Comoviridae all have biological vectors. *Comoviruses* are transmitted by beetles, *Fabaviruses* by aphids and *Nepoviruses* by nematodes. Seed transmission is very common among *Nepoviruses*, but is rare or does not occur with *Como-* and *Fabaviruses*.

2.4.4.2.1 Genus: Comovirus

Type species: Cowpea mosaic virus (CPMV)

Virions are isometric, 24-30 nm in diameter, non-enveloped. Genome is linear, bipartite, ssRNA. Comoviruses have narrow host range, 11 of the 15 species being restricted to a few species of the family Leguminosae. Mosaic and mottle symptoms are characteristic. Transmission in nature is by beetles in a persistent manner. Beetles retain their ability to transmit virus for days or weeks. Non-vector transmission is by mechanical inoculation, by seed, or by pollen to seed. The species of the genus are listed in Appendix-1.

2.4.4.2.2 Genus: Fabavirus

Type species: Brood bean wilt virus (BBWV)

Virions are isometric, 26 nm in diameter, non-enveloped. Genome is ssRNA, linear, bipartite. Fabaviruses have wide host range among dicotyledenous and some families of monocotyledons. Symptoms are ringspot, mottle, mosaic, distortion, wilting and apical necrosis. In nature *Fabaviruses* are transmitted non-persistently by aphids. Non-vector transmission is by mechanical inoculation, not by seed, not by pollen. Preparations are imunogenic. The species of the genus are listed in Appendix-1.

2.4.4.2.3. Genus: Nepovirus

Type species: Tobacco ring spot virus (TRSV)

Virions are isometric, 30 nm in diameter, non-enveloped. Genome is ssRNA, linear, bipartite. Preparations are strongly immunogenic or moderately immunogenic. Nepoviruses are widely distributed in temperate regions. Natural host range vary from wide to restricted to a single plant species, depending on the virus. Ringspot symptoms are characteristic, but mottling and spotting are common. Viruses are transmitted by nematodes (*Xiphinema* or *Longidorus*) in a semi-persistent manner. Seed transmission is very common. The species of the genus are listed in Appendix-1.

2.4.4.3 Family: Potyviridae

Taxonomic structure of the family

Family: **Potyviridae**
Genus: **Bymovirus**
Genus: **Potyvirus**
Genus: **Rymovirus**

Virions are flexuous filaments with no envelop and are 11-15 nm is diameter. Particle length differs among members of the three genera. Members of the genus *Potyvirus* and *Rymovirus* are monopartite with particles modal length of 650-900 nm. Members of the genus *Bymovirus* are bipartite with particles of two modal lengths of 250-300 nm and 500 nm. Nucleic acid is ssRNA positive sense. Viral proteins are moderately immunogenic. There are serological relationships between members. All members of the family Potyviridae

form cytoplasmic cylindrical inclusion (CI) bodies during infection. Host range is narrow to wide. Easily transmitted by mechanical inoculation. Seed transmission is common. Members of the genus *Potyvirus* are vectored by aphids in a non-persistent manner. *Rymoviruses* are transmitted by mites and *Bymovirures* are transmitted by a fungal vector.

2.4.4.3.1 Genus: Bymovirus

Type species: Barely yellow mosaic virus (BYMV)

Virions are flexuous filaments of two modal lengths, 250-300 and 500-600 nm, both are 13 nm in width. Virions contain two molecules of linear positive sense, ssRNA. The viral proteins are moderately immunogenic. Serological relationships exist among members except barley mild mosaic virus (BaMMV). There are characteristic pinwheel-like inclusions and membranous network structures formed in the cytoplasm of infected plant cells. No nuclear inclusions are found. The host range of member viruses is narrow, restricted to the host family *Gramineae*. The viruses are transmitted by plasmodiophoraceous fungus *Polymyxa graminis*, and experimentally by mechanical inoculation. Species of the genus are listed in Appendix-1.

2.4.4.3.2 Genus: Potyvirus

Type species: Potato virus Y (PVY)

Virions are flexuous filaments, 680-900 nm long and 11-13 nm wide. Virions contain a single molecule of linear, positive sense ssRNA. Virions are moderately immunogenic. There are serological relationships among many members. Many individual viruses have a narrow host range. The viruses are transmitted by aphids in a non-persistent manner, and are transmissible experimentally by mechanical inoculation. Species of the genus are listed in Appendix-1.

2.4.4.3.3 Genus: Rymovirus

Type species: Ryegrass mosaic virus (RMV)

Virions are flexuous filaments 690-720 x 11-15 nm in size. Virions contain a single molecule of linear, positive sense, ssRNA.

Most Rymoviruses are moderately immunogenic. No serological relationships among member viruses have been reported. Most members have limited host range within the family *Gramineae*, but some have relatively narrow host ranges. Natural transmission is by eriophyid mites and mechanical transmission have been reported for most members. Species of this genus are listed in Appendix-1.

2.4.4.4 Genus: Enamovirus

Type species: Pea enation mosaic virus (PEMV)

Virions are polyhederal and of two distinct sizes, approximately 25 nm and 28 nm. Virus preparations contain two species of linear, positive sense, ssRNA. The virus is moderately antigenic. The virus infects many legumes, and is transmitted by aphids in a persistent, non-propagative manner. Non-vector transmission is by mechanical inoculation. PEMV is the only species of this genus.

2.4.4.5 Genus: Luteovirus

Type species: Barley yellow dwarf virus (BYDV)

Virions are 25 to 30 nm in diameter. Virions contain a single molecule of infectious linear, positive sense ssRNA. The viruses are strongly immunogenic. Most luteoviruses are restricted to one plant family. Luteoviruses are transmitted in a circulative non-propagative manner by specific aphid species. Luteoviruses occur world wide. Some have restricted distribution. Luteoviruses are tissue specific and particles are detectable in phloem. Species of the genus are listed in Appendix-1.

2.4.4.6 Genus: Sobemovirus

Type species: Southern bean mosaic virus (SBMV)

Virions are about 30 nm in diameter. Virions contain a single molecule of positive sense ssRNA. Viral proteins serve as efficient immunogens. There are serological relationships between strains and some members of the genus. Host range is narrow. Disease symptoms are mainly mosaics and mottles. Seed transmission occurs in several host plants. In nature transmission occurs by beetles. species of the genus are listed in Appendix-1.

2.4.4.7 Genus: Umbravirus

Type species: Carrot mottle virus (CMoV)

The particles are approximately 52 nm in diameter with envelop and occurs in vacuoles of CMoV-infected cells and in partially purified preparations from such cells. It is not known whether these are (i) virus particles of a kind unusual among plant viruses but resembling those of some viruses infecting insects or vertebrates. Infected leaf tissue contains abundant dsRNA. Two species are common to all members: one is dsRNA1 and the other is dsRNA2. Host range is narrow. Umbraviruses are transmissible by mechanical inoculation, but in nature, each is dependent on a specific helper virus. Seed transmission has not been reported. Species of the genus are listed in Appendix-1.

2.4.4.8 Family: Tombusviridae

Taxonomic structure of the family

Family: Tombusviridae

Genus: Carmovirus

Genus: Tombusvirus

Virions are icosahederal. Virions contain a single molecule of positive sense, linear ssRNA. Virions are good immunogens. The natural host range of individual virus species is relatively narrow and restricted to dicotyledons. Diseases are characterized by mottling, crinkling and deformation of leaves. All species are readily transmitted by mechanical inoculation and through propagative plant material. Some are transmitted through seeds. Transmission by the chytrid fungus *Olpidium radicale* and beetles have also been reported.

2.4.4.8.1 Genus: Carmovirus

Type species: Carnation mottle virus (CarMV)

Virions are isometric, 30 nm in diameter, non-enveloped. Genome is ssRNA, linear, monopartite. Preparations are strongly immunogenic. Viral species are not serologically related. Most species are found in temperate regions. Several viruses are soil-borne, but only two cucumber leaf spot virus (CLSV) and melon necrotic spot virus (MNSV) are transmitted by *Olpidium radicale*. Others are transmitted by beetles (e.g. cowpea mottle virus-CPMoV, bean mild mosaic virus-BMMV). Non-vector transmission is by mechanical inoculation, by

grafting, not by seed, not by pollen. Host range is wide. Species of the genus are listed in Appendix-1.

2.4.4.8.2 Genus: Tombusvirus

Type species: Tomato bushy stunt virus (TBSV)

Virions are rod shaped with 300 nm long and 18 nm wide. Nucleic acid is ssRNA and monopartite. Virus preparations are strongly immunogenic. The viruses have relatively wide host range. All species are soil-borne, but only cucumber necrosis virus (CNV) has a recognized fungal vector (*Olpidium radicale*). Non-vector transmission is by mechanical inoculation, by grafting and through seed. Species of the genus are listed in Appendix-1.

2.4.4.9 Genus: Dianthovirus

Type species: Carnation ringspot virus (CRSV)

Virions are isometric, 32-35 nm in diameter. Virions contain two molecules of infectious, linear, positive sense ssRNA. The viruses are moderately to highly immunogenic. Serologically related strains have been identified. Host range is broad. The viruses are readily transmitted by mechanical inoculation. The viruses are not known to be seed transmitted. The viruses are not transmitted by insects, nematodes, or soil inhabiting fungi belonging to the Chytridiales. However, viruses are readily transmitted through the soil without the aid of a biological vector. There is no seed or pollen transmission. The species of the genus are listed in Appendix-1.

2.4.4.10 Genus: Furovirus

Type species: Soil-borne wheat mosaic virus (SbWMV)

Virions are rod-shaped, about 18-21 nm in width, with predominant length of 92-160 nm and 250-300 nm. Virions contain two molecules of linear, positive sense ssRNA. Most species are fairly good immunogens. The type species is serologically distantly related to potato mop top, broad bean necrosis, oat golden striple and sorghum chlorotic spot viruses. Host range is very narrow. The virions are transmitted by Plasmodiophorid fungi (*Polymyxa graminis*, *P. betae*,

or *Spongospora subterranean*). Peanut clump virus is also seed-borne. Non-vector transmission is by mechanical inoculation, not by pollen. Species of the genus are listed in Appendix-I.

2.4.4.11 Genus: Hordeivirus

Type species: Barley stripe mosaic virus (BSMV)

Virions are non-enveloped, elongated and rigid, about 110-115 nm long, and 20 nm wide. Virions contain three molecules of positive sense ssRNA. The viruses are good immunogens. Member species are very distantly related serologically. The natural host range of three species (ALBV, BSMV, PSLV) are grasses (family Gramineae). BSMV is efficiently transmitted through seeds. Species of the genus are listed in Appendix-I.

2.4.4.12 Genus: Machlomovirus

Type species: Maize chlorotic mottle virus (MCMV)

Virions are isometric, approximately 30 nm in diameter, non enveloped. Virions contain a single molecule of infectious, linear, positive sense ssRNA. The virus is moderately to highly immunogenic. The virus is restricted to members of the host family Gramineae. The virus is readily transmitted by mechanical inoculation. Seed transmission is also reported. Some isolates are transmitted by beetles. Species of the genus are listed Appendix-I.

2.4.4.13 Genus: Necrovirus

Type species: Tobacco necroses virus (TNV)

Virions are icosahederal, 28 nm in diameter. Virions contain one molecule of infectious, linear, positive sense ssRNA. Members are moderately immunogenic. Necroviruses have wide host range. Infections are restricted to roots in natural infections. Experimental inoculations usually cause necrotic lesions on inoculated leaves, rarely resulting in systemic infection. Viruses are readily transmitted by mechanical inoculation. Member viruses are transmitted naturally by the chytrid fungus *Olpidium brassicae*. Non-vector transmission is by

mechanical inoculation, not by seed, not by pollen. species of the genus are listed in Appendix-1.

2.4.4.14 Genus: Tobamovirus

Type species: Tobacco mosaic virus (TMV)

Virions are elongated, rigid cylinder, about 18 nm wide and 300 nm long. Virions contain a single molecule of positive sense, linear ssRNA. Preparations are strongly immunogenic. Most species have moderate to wide host range. They are transmitted in nature without the help of vectors by contact plants and sometimes by seed. Species of the genus are listed in Appendix-1.

2.4.4.15 Genus: Tobravirus

Type species: Tobacco rattle virus (TRV)

Virions are tubular with no envelop. There are two predominant lengths, (L) 180-215 nm and (S) ranging from 46 to 115 nm. The genome consists of two molecules of positive sense ssRNA. Virions are moderately immunogenic. There is little or no serological relationship between members of the genus. The host range is wide. The natural vectors are nematodes in the orders *Trichodorus* and *Paratrichodorus* (Trichodoridae). Non-vector transmission is by mechanical inoculation, by grafting, by seed and not by pollen. Species of the genus are listed in Appendix-1.

2.4.4.16 Family: Bromoviridae

Taxonomic structure of the family

Family: Bromoviridae
Genus: Alfamovirus
Genus: Bromovirus
Genus: Cucumovirus
Genus: Ilarvirus

Virions of members of the genera *Bromovirus*, *Cucumovirus* and *Ilarvirus* are 26-35 nm in diameter and spherical. Virions contain three genomic and one sub-genomic ssRNA molecules. RNA 1 and RNA2 are contained in separate particles, while RNA3 and RNA4 (subgenomic) are contained in one particle. Virions of members of the

genus *Alfamovirus* are mostly bacilliform. There are four particle sizes, three containing single copies of each of RNA1(B), 2(M) and 3(Tb), and the fourth containing two copies of RNA4 (Ta).

Virions are typically moderate to poor immunogens. There are no serological relationships between genera. All the virions are readily transmissible by mechanical inoculation; otherwise, *Cucumovirus* and *Alfamovirus* are non-persistently transmitted by a wide variety of aphids, and some *Hordeiviruses* are seed-transmitted in some host species.

2.4.4.16.1 Genus: Alfamovirus

Type species: Alfalfa mosaic virus (AMV)

Virions are bacilliform, non-enveloped, 13 nm in length (Ta), 35 nm in length (Tb), 43 nm in length (M), and 56 nm in length (B). Viruses are non-persistently transmitted by aphids and have a very wide host range (10 or more families), often cause yellowing under field conditions. Non-vector transmission is by mechanical inoculation, by seed or by pollen to seed. Purified virus preparations are poorly immunogenic. Species of the genus are listed in Appendix-I.

2.4.4.16.2 Genus: Bromovirus

Type Species: Brome mosaic virus (BMV)

Virions are polyhoderal, non-enveloped, 26 nm in diameter. Genome is ssRNA, linear, tripartite and all the same size. All members are serologically related. All species are beetle-transmitted in a persistent manner. Non-vector transmission is by mechanical inoculation, by grafting, rarely by seed, not by pollen. Bromoviruses have narrow host range in the families *Graminеae* and *Leguminosae*. Viruses are serologically related. Species of the genus are listed in Appendix-I.

2.4.4.16.3 Genus: Cucumovirus

Type Species: Cucumber mosaic virus (CMV)

Virions are polyhedral, not enveloped, 26 nm in diameter. Genome is ssRNA, linear, tripartite and all the same size. Preparations are immunogenic or strongly immunogenic. All *Cucumovirus* are serologically related. All are aphid-transmissible in a non-persistent

manner. Non-vector transmission is by mechanical inoculation, grafting, by seed, or by pollen to seed. CMV has a very wide host range, others are more limited. species of the genus are listed in Appendix-1.

2.4.4.16.4 Genus: Ilarvirus

Type species: Tobacco streak virus (TSV)

Virions are isometric or bacilliform, non-enveloped, and are about 30 nm in diameter. The virus infect mainly woody plants. Viruses are serologically related. Some *Ilarviruses* are transmitted via pollen. Transmitted in nature without the help of a vector, by unknown means, or by insect vector belonging to the Thysanoptera, transmitted in a non-persistent manner. Non-vector transmission is by mechanical inoculation, by grafting, usually by seed and by pollen to seed. Experimental host range of individual viruses are wide. Preparations are immunogenic or poorly immunogenic. Ilarviruses have tripartite genome. Species of the genus are listed in Appendix-1.

2.4.4.17 Genus: Capillovirus

Type species: Apple stem grooving virus (ASGV)

Virions are flexuous filaments, 600-750 nm long, 12 nm wide. Virions contain linear, monopartite, positive sense ssRNA,. Virions are moderately antigenic. Serologically related. No vectors are known. Host range is narrow. Non-vector transmission is by mechanical inoculation, root grafting, by seed, not by pollen. Species of the genus are listed in Appendix-1.

2.4.4.18 Genus: Carlavirus

Type species: Carnation latent virus (CLV)

Virions are slightly flexuous filaments, 610-700 nm long and 12-15 nm wide. Virion contain single molecule of linear ssRNA. The virions are good immunogens. Some members of the group are serologically interrelated, but others are apparently distinct. Viruses have narrow to wide host range. Member viruses are transmitted by aphids is a non-persistent manner, two possible member viruses are transmitted by whiteflies. Some are seed-borne is leguminous species.

All viruses are mechanically transmissible. Species of the genus are listed in Appendix-1.

2.4.4.19 Genus: Closterovirus

Type species: Beet yellows virus (BYV)

Virions are very flexuous filaments, non-enveloped, 1000-2200 nm long and about 10-12 nm wide. Virions contain a single molecule of linear, positive sense, ssRNA. Virion proteins are moderately antigenic. Most of the species are serologically unrelated to one another. Host range is restricted. Disease symptoms are of the yellowing type i.e. rolling, yellowing or reddening of the leaves. Transmission is mainly through propagative material. Transmission through seeds is very rare. Natural vectors are aphids, which transmit in a semi-persistent manner, whiteflies and Pseudococcid mealybugs. Species of the genus are listed in Appendix-1.

2.4.4.20 Genus: Idaeovirus

Type Species: Rasberry bushy dwarf virus (RBDV)

Virions are isometric, 33 nm is diameter and are non-enveloped. Virion preparations contain three species of linear, positive sense, ssRNA. The genome is bipartite. Particles are moderate immunogens. In nature the host range is confined to *Rubus* species. Pollen transmission is the only known method of transmission. RBDV is the only member of this genus.

2.4.4.21 Genus: Marafavirus

Type species: Maize rayado fino virus (MRFV)

Virion are icosahedral, 28-32 nm in diameter, and do not have an envelop. Virion contain one molecule of linear positive sense ssRNA. Virions are moderately immunogenic. No serological relationship exists between maize rayado fino virus and oat dwarf virus. Bermuda grass etched-line virus is serologically related to both maize rayado fino virus and oat dwarf virus. The viruses generally have narrow host ranges restricted to the family *Gramineae*. One member, oat blue dwarf virus, has a wide host range including dicotyledonous

plants. The viruses are transmitted by leafhoppers, mechanical transmission is difficult. Species in the genus are listed in Appendix-1.

2.4.4.22 Genus: Potexvirus

Type species: Potato virus X (PVX)

Virions are flexuous helical rods, 470-580 nm long and 11-13 nm wide. The genome is a single linear molecule of positive sense ssRNA. Virions are highly immunogenic. Some members are antigenically related. The virions cause mosaic or ringspot symptoms in a wide range of mono-and dicotyledonous plants. The host range is limited. The viruses are readily transmitted by mechanical inoculation, no vectors are known. The viruses are transmitted in nature by mechanical contacts and have world-wide distribution. species of the genus are listed in Appendix-1.

2.4.4.23 Genus: Trichovirus

Type species: Apple chlorotic leaf spot virus (ASCLV)

Virions are very flexuous, 640 nm long and 12 nm wide. Virions contain a single molecule of linear, positive sense, ssRNA. The virions serve as moderate to poor antigens. Species are not serologically related. Host range is narrow. Transmission is by mechanical inoculation, by grafting, propagative material and through seeds. Some are transmitted by mealybugs and aphids. Species of the genus are listed in Appendix-1.

2.4.4.24 Genus: Tymovirus

Type species: Turnip yellow mosaic virus (TYMV)

Virions are icosahederal, non-enveloped, and have a diameter of about 30 nm. Particles are of two types. B particles contain one molecule of infectious linear positive sense ssRNA. Virions are moderately to highly antigenic. Serological relationships between different species range from very close to distant, to not detectable. Tymoviruses are possibly restricted to dicotyledonous hosts. The viruses are transmitted by beetles in a persistent manner. Non-vector

transmission is by mechanical inoculation, grafting, rarely by seed, not by pollen. Mostly cause bright yellow mosaic symptoms or mottling. Species of the genus are listed in Appendix-1.

Chapter 3

SYMPTOMS IN VIRUS DIAGNOSIS

3.1 Introduction: Viruses are economically important only when they cause some significant abnormality in plant growth. An abnormal appearance is usually the first indication that a plant is virus infected. Symptoms are the observable effects that a virus has on growth, development and metabolism of an infected plant. In nature, severe disease symptoms normally occur only when a virus has infected the plant systemically.

In the early days of plant virology, symptoms were of major importance, for they were the main means by which a virus disease was diagnosed. Viruses are still named after the type of symptoms they produce in the diseased plants, but many other techniques have now become available to assist in virus diagnosis. The most common and sometimes the only kind of symptoms produced by virus infection is reduced rate of plant growth, resulting in various degrees of dwarfing or stunting. The most obvious symptoms of virus infected plants are usually the abnormal appearance of leaves, but some viruses may cause striking symptoms in the stem, fruits, and roots with or without symptom development on the leaves.

Most symptoms are still important to plant virologists for diagnosing virus diseases under field conditions. In the field the disease symptoms give the first clue of virus identification, and in the laboratory, the symptoms produced in a range of test plants may be of useful and diagnostic value. For the growers, it is important to know about symptoms produced in response to infection with a virus disease. It is the nature and severity of the disease symptoms that determines the economic importance of a particular virus, in terms of yield and reduced quality.

Several types of symptoms are produced in virus infected plants because of many reasons, the large number of viruses and strains, varying reactions of different plants to different or even to the same virus, age of the plant at the time of infection, organ and tissue infected, and environmental conditions particularly temperature and light. When considering virus symptoms, it must be remembered that a

virus not only causes just one type of symptom, but most often infection results in more than one type of symptoms and frequently there may be a series of symptoms as the disease persists within the plant. For example, stunted growth and dwarfing, may be associated with necrotic symptoms and in extreme cases, the necrosis may spread to the whole plant to cause plant death. The occurrence of more than one type of symptoms in a diseased host plant is called "*syndrome*". Under field condition, most often the same host plant is infected by more than one virus, and the effect may be synergistic.

Virus infection does not necessarily cause disease or produce visible symptoms at all times in all parts of an infected plant. There are some situations in which the virus infected plants do not show observable symptoms due to (i) infection with a very mild strain of a virus (ii) tolerant host plant (iii) absence of virus in newly formed leaves and (iv) latent infection under certain environmental conditions.

Symptom expression is not usually a precise indication of virus identity and its interpretation should be treated with caution. In some cases the type of symptoms may be indicative of a distinct virus or virus group (e.g. pea enation mosaic virus), but symptoms on plants in the field may be unreliable because (i) several viruses may cause similar symptoms in the same plant (ii) a single virus may cause highly variable symptoms depending on virus strain (iii) a mixture of viruses or virus strains (iv) different crop varieties may be affected differently by the same virus, and (v) different soil and weather conditions may alter disease expression. Environmental factors, especially light and temperature, can often confuse and influence the symptom expression. A brief description of the symptoms of the diseased plants may help in virus diagnosis, but it should be kept in mind that other confirmatory tests must be followed to reach a final conclusion.

In this chapter some major external and internal symptoms caused by virus infection in host plants are discussed.

3.2 External symptoms: Most viruses spread through their host, producing "*systemic infection*". In certain cases the primary symptoms may differ from those produced in later stages of infection. Sometimes the symptoms result in rapid death of the invaded cells, thus preventing further spread of infection, and such response is referred to as

"hypersensitive reaction". Mostly in hypersensitive response the virus infection induces localized necrotic or chlorotic lesions, and the systemic spread of virus is stopped. The most obvious symptoms are external and restricted changes in foliage colour or growth abnormalities. Internal symptoms are also produced in the leaf cells. Such symptoms can only be detected either by light microscope or under the electron microscope.

The external symptoms caused by viruses may be due to *"primary infection"* which is limited to the inoculated cells of the host plant or due to secondary or *"systemic infection"* when the virus moves from the sites of primary inoculation into the whole plant parts.

3.2.1 Primary leaf infection: The initial symptoms that develop at the site of virus entry in the inoculated leaves are known as *"local symptoms"*. These symptoms develop in the form of distinct areas of diseased cells commonly know as *"local lesions"* (Figure 3.1). Local lesions vary in size and may be chlorotic due to loss of chlorophyll or necrotic if the cells die. Local lesions most frequently occur on virus indicator hosts, when mechanical inoculation is carried out under greenhouse conditions. Local lesions on indicator plant species are very useful for diagnosis of plant viruses.

3.2.2 Secondary systemic infection: In case of secondary infection, systemic symptoms develop in a sequential manner in the infected plants. The virus moves in the vascular system and from vascular system to adjacent cells, in roots, fruits and other organs. Systemic symptoms that appear in various forms are as follows:

3.2.2.1 Dwarfing and stunting: These are the most common symptoms caused by plant viruses. The plant size is reduced and stunting is confined to specific plant parts. For example, chickpea stunt, peanut stunt, soybean stunt and apical stunting of pea caused by red clover vein mosaic virus are few examples of dwarfing and stunting (Figure 3.2a).

Figure 3.1: Necrotic lesions induced in tobacco leaf by CMV.

3.2.2.2 Mosaic: Mosaic pattern of leaf symptoms are the most common symptoms caused by plant viruses. In leaves the cells of some infected areas become discoloured due to loss of chlorophyll and the other cells in other area remain green. The mosaic pattern varies considerably. If the boundaries between dark and light areas of the leaf are not well defined, the symptoms are called "*mottle*". Mosaic symptoms also occur on the stems and fruits. For example mosaic symptoms of cucumber due to cucumber mosaic virus (CMV) (Figure 3.2b)

3.2.2.3 Chlorosis: In certain cases the leaves of the virus infected plants become chlorotic due to decreased chlorophyll production and breakdown of chloroplasts. Most chlorotic symptoms are linked with internal histological disorders, such as abnormal changes in the plastid cells and intracellular vacuoles. Chlorosis is mainly caused by viruses called "*yellowing viruses*" e.g. mungbean yellow mosaic virus (Figure 3.3a) and barley yellow dwarf virus (Figure 3.3b). In some instances the chlorosis is confined to veins called "*vein yellowing*" or "*vein clearing*" in which the cells adjacent to the vein become translucent.

3.2.2.4 Necrosis: Sometimes the systemic infection results in the death of cells which is called "*necrosis*". Systemic necrosis may take the form of small or large lesions, for example, turnip yellow mosaic virus (TYMV), on the external and internal leaves of cabbage. Blackeye cowpea mosaic virus (BlCMV) also causes vein necrosis in some cowpea cultivars. Necrosis, resulting in collapse of superficial tissue is called "*etch*" (as in tobacco with tobacco etch virus).

3.2.2.5 Ring spots: The symptoms of ring-spotting is a characteristic symptom of some viruses. Ring spots are characterized by the appearance of chlorotic or necrotic rings on the leaves and some times on fruits and stems (Figure 3.4a). In these infections, the diseased area is restricted to a ring or broken ring of infected cells. In many ring spot diseases the symptoms, but not the virus, tend to disappear after onset and reappear under certain environmental conditions.

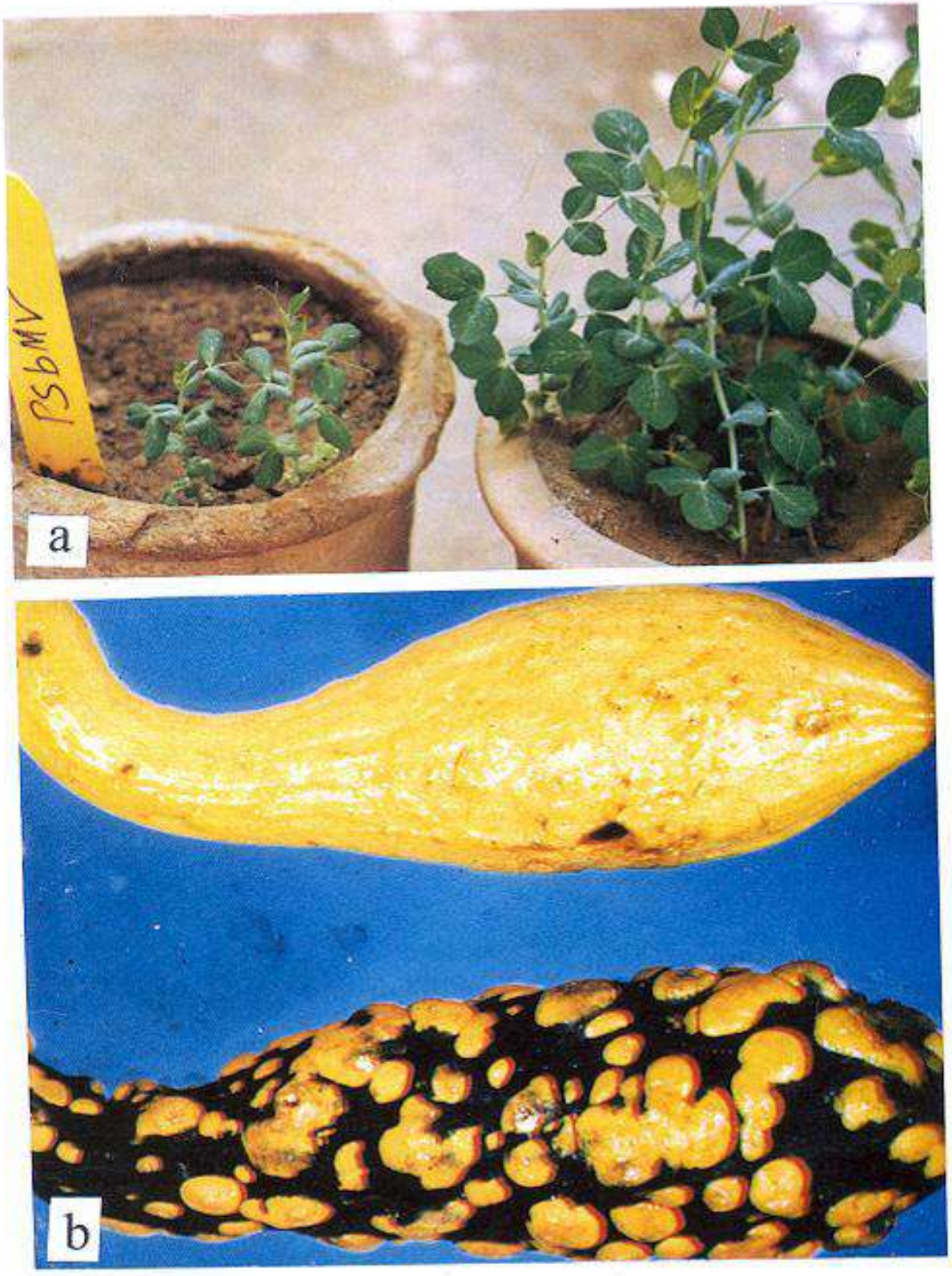

Figure 3.2: (a) Dwarfing and stunting of pea plant due to PSbMV (b) mosaic symptoms on cucumber fruit due to CMV.

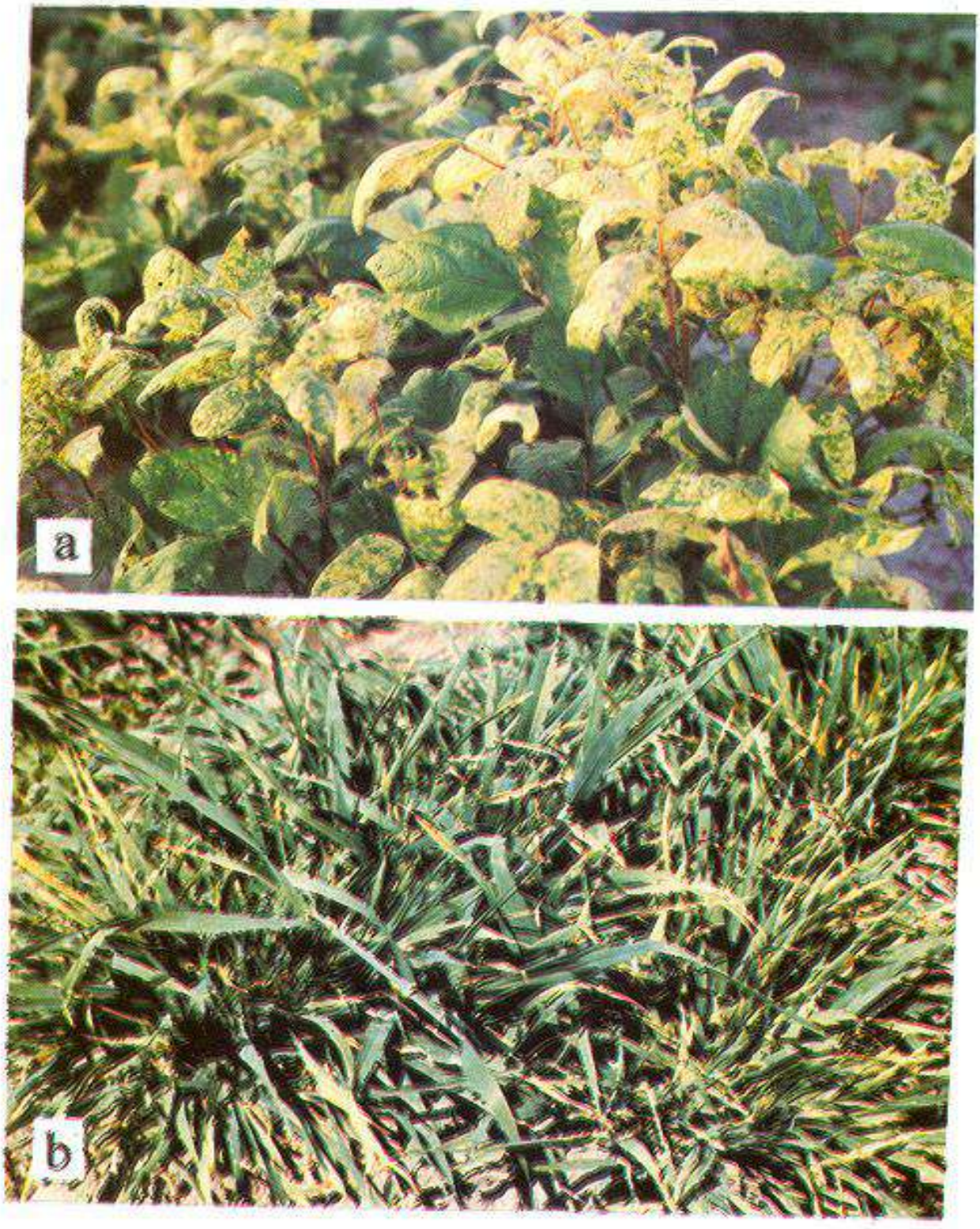

Figure 3.3: (a) Yellowing of mungbean plants infected with MYMV (b) yellowing of barley plants infected with BYDV.

3.2.2.6 Leaf and stem distortion. Some viruses cause leaf and stem distortion. For example bean common mosaic virus (BCMV) in *Phaseolus vulgaris* and strawberry latent ring spot virus (SLRSV) in celery cause such type of symptoms. Such abnormal growth is due to hormonal imbalance within the leaves and is similar to herbicide (2,4-D) injury (Figure 3.4b).

3.2.2.7 Enations or tumors: Some virus infections are characterized by leaf like or tumor-like outgrowths on the leaves and roots. The leaf like outgrowths are referred to as "*enation*" and these appear like "*warts*" on the upper or lower surface of the infected leaf. Such type of symptoms are common in pea plants caused by pea enation mosaic virus or in cotton due to cotton leaf curl virus infection (Figure 3.5 b)

3.2.2.8 Flower discolouration: Some viruses produce very striking changes in flower colour called "*flower breaking*". Such colour "*break*" symptoms are common in flowers of plants infected with tulip mosaic virus, gladiolus infected with bean yellow mosaic virus or cucumber mosaic virus (CMV). The "*break*" symptoms may appear in the form of streaking, flecking or variegation of petal tissues with a colour different from normal flowers.

3.2.2.9 Fruit and seed symptoms: Fruits may show colour changes when the parent plant is virus-infected. Fruits from infected plants are reduced in size, misshapen or changed in texture. Mottling can be found on tomato fruit due to tomato spotted wilt virus (TSWV). Bean common mosaic virus (BCMV) may cause brown necrotic areas and internal necrosis of pods of legumes. Seedless pods are produced by soybean infected with soybean mosaic virus (SBMV). A large number of other less common virus symptoms have been described, and include leaf roll (potato leaf roll virus), leaf curl (cotton leaf curl virus) (Figure 3.5a), leaf crinkling (Figure 3.6a), yellows (beet yellows), streak (tobacco streak), pox (plum pox), and stem pitting (apple stem pitting virus).

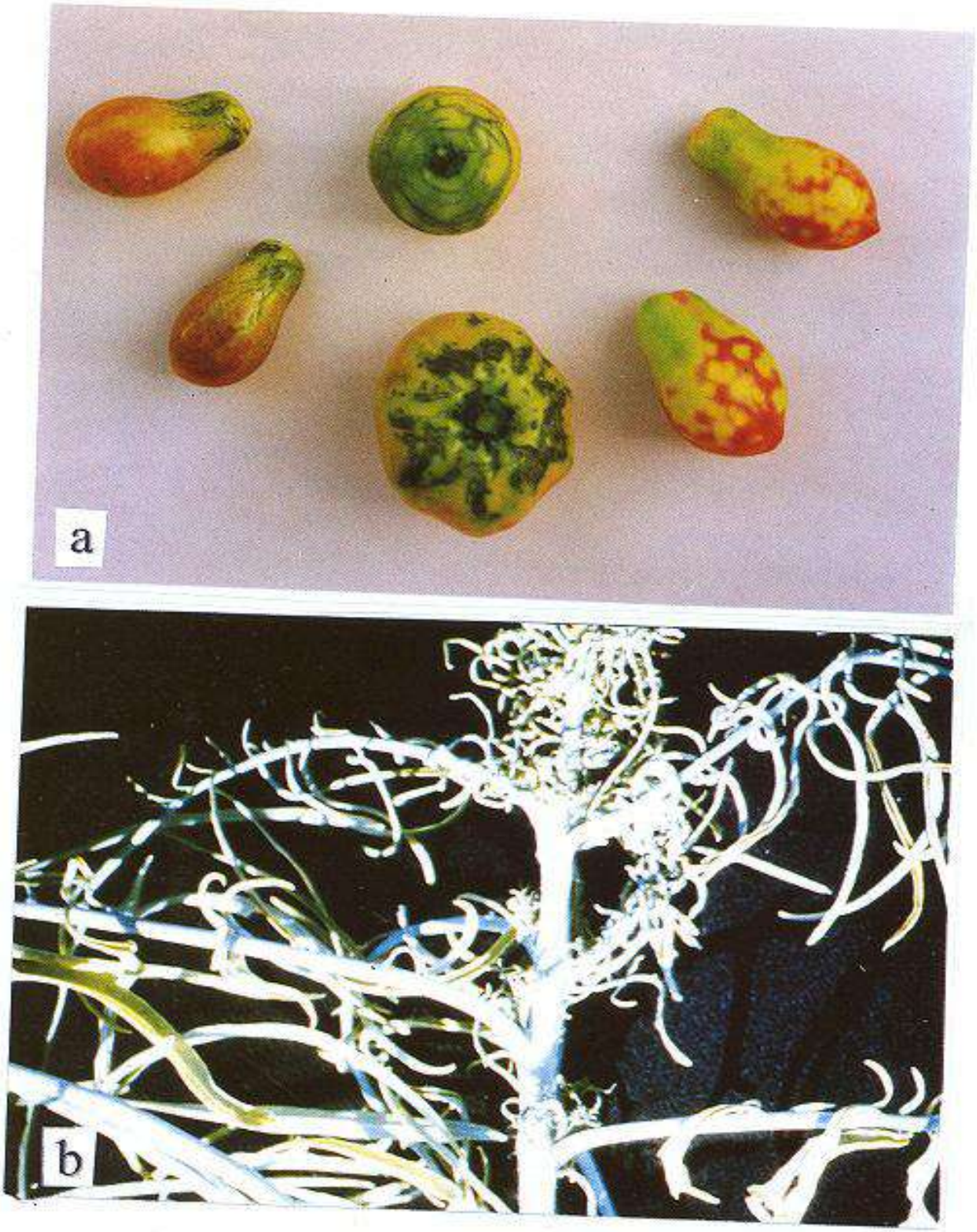

Figure 3.4: (a) Ringspot symptoms induced on tomato fruits by TSWV (b) leaf and stem distortion of tomato plants by CMV.

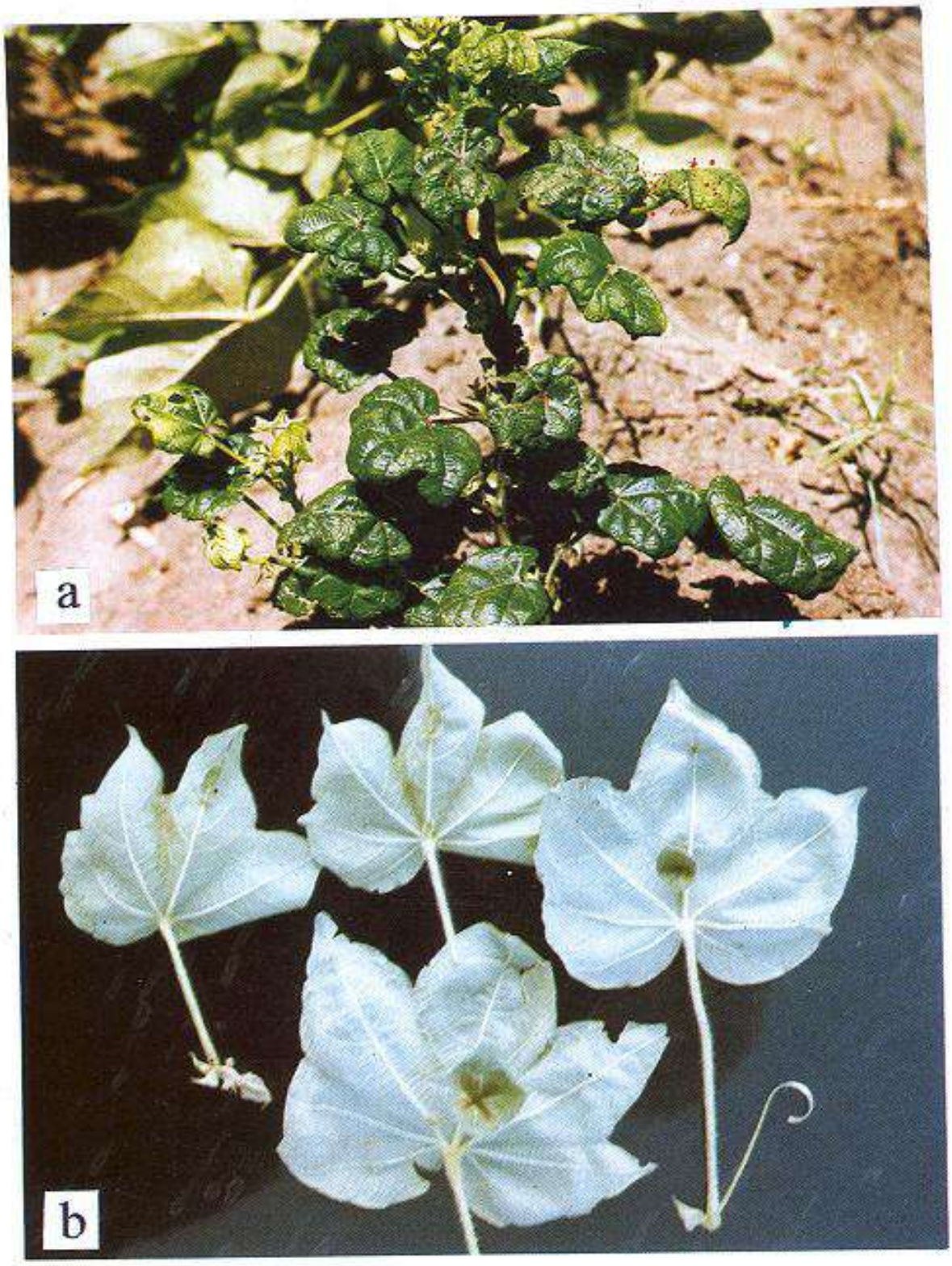

Figure 3.5: (a) Downward leaf curling induced by CLCuV (b) enation on lower leaf surface of cotton induced by CLCuV.

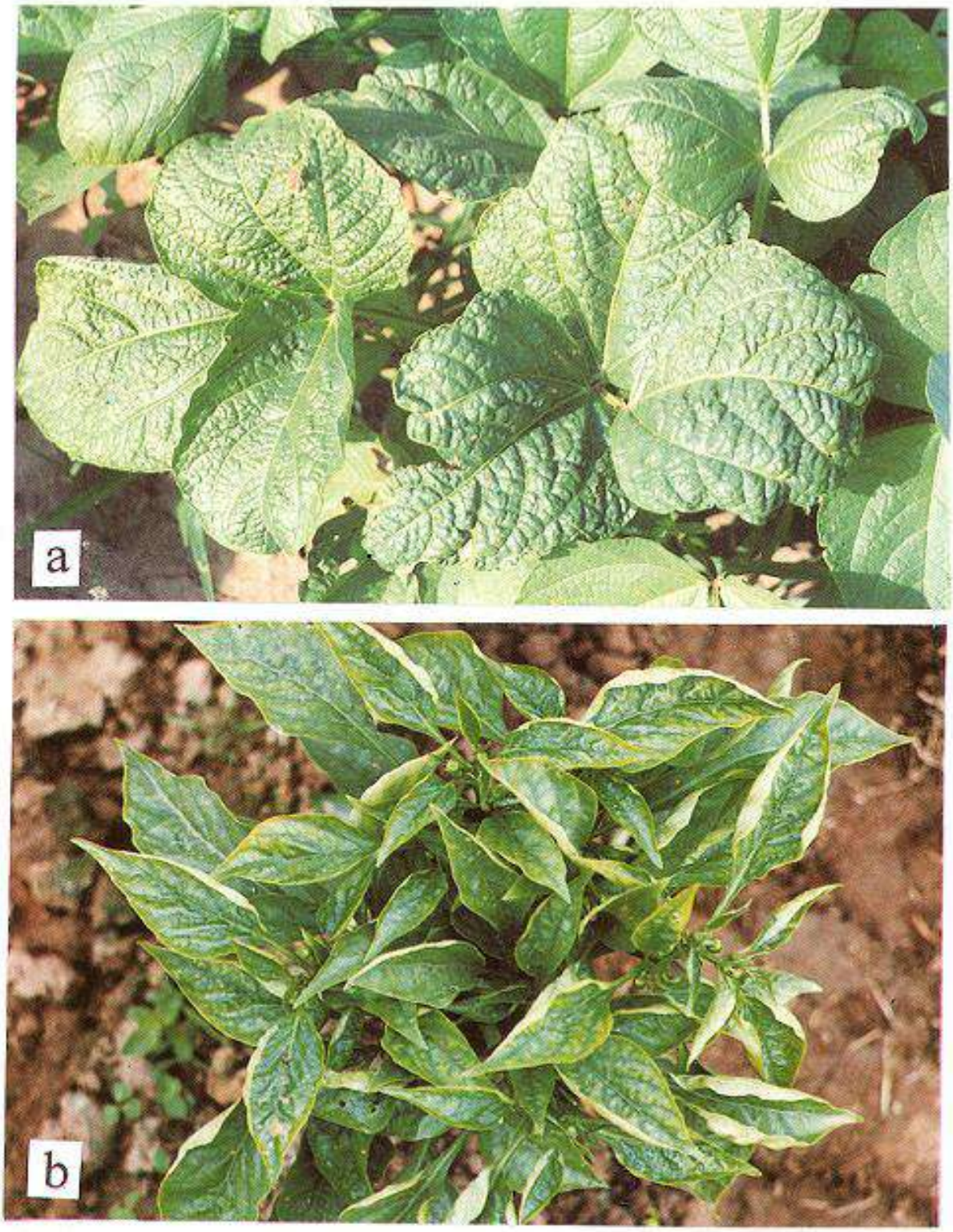

Figure 3.6: (a) Leaf crinkling induced by urdbean leaf crinkle virus in urdbean (b) upward leaf curling induced in chilies by chili leaf curl virus.

3.3 Internal symptoms: Internal symptoms of virus diseases are those detected by light and electron microscopy. In the virus infected plants some histological changes occur. In addition to abnormal cell structure, various virus induced structures are present in the infected cells. Such structures are called *"inclusion bodies"*. Presence of inclusion bodies within virus infected cells is one of the most characteristic internal symptoms, because inclusions occur only in virus infected cells and not observed in healthy cells or in associations with other type of diseases. Inclusion bodies incited by several, but not all plant viruses are the most common in epidermal cells of leaves, stems, roots and flowers. These inclusions are microscopic bodies differing from other cell structures.

In this section, the major cytological and histological changes that occur in the diseased cell are described briefly together with the most characteristic types of inclusion bodies.

3.4 Cytological and histological changes: Plant viruses affect the size of the plant organs and tissue by increasing cell numbers, a condition known as *"hyperplasia"*. Excessive growth due to enlargement of individual cell is termed as *"hypertrophy"*.

Among the most important cytological effects of virus infection are changes in cell nuclei. Pea enation mosaic virus (PEMV) has been observed to cause the breakdown of cell nuclei. The nuclei of bean plant cells infected with bean golden mosaic virus (BGMV) have been reported to increase in size. Changes in chloroplasts have also been observed. The chloroplasts become colourless with the loss of chlorophyll, misshapen or grouped into abnormal clumps within the cell.

Some histological effects of virus infection that occur internally in diseased plants are associated with externally visible symptoms. These symptoms may appear in form of reduction or increase in cell numbers or internal cell necrosis. An example of reduced cell formation is seen with apple grooving virus.

3.5 Inclusion bodies induced by plant viruses: Inclusion bodies are defined as *"intracellular structures produced de novo as a result of virus infection"*. These structures may contain virus particles, virus related material or ordinary cell constituents in a normal or degenerate conditions, either singly or more often in various proportions. The inclusion bodies vary in morphology and composition. They are located either in the cytoplasm or in the nucleus. The study of inclusion bodies in some cases may help in virus identification.

3.6 Types of inclusion bodies: Inclusion bodies may occur in the nucleus, but are most common in the cytoplasm. Before discussing the methods of studying inclusion bodies for virus identification, it seems appropriate to briefly discuss types of inclusion bodies induced by plant viruses. For convenience inclusion bodies are discussed under two groups:

3.6.1 Nuclear inclusions: These may occur in the nucleoplasm (the viscous fluid within the body of the nucleus) or between the membranes of nuclear envelope (the perinuclear space). The inclusions in the nucleoplasm may be crystalline in structure, amorphous or fibrous e.g. inclusion bodies induced by bean yellow mosaic virus (BYMV) and tobacco mosaic virus (TMV). Intracellular membrane inclusions have been reported in cowpea mosaic virus (CPMV).

Nucleolus-related inclusions may be amorphous or crystalline in structure. Amorphous inclusions induced by been common mosaic virus (BCMV) can be seen in infected cells of *Gomphorena globosa* and *Chenopodium quinoa*. Bean yellow mosaic virus (BYMV) induces crystalline inclusions.

3.6.2 Cytoplasmic inclusions: When viruses multiply, they may accumulate in large number within the cell to form inclusion bodies composed of entirely virus particles. These particles are arranged side by side, end to end or aggregate at random. Such inclusion bodies are either fibrous, paracrystalline or crystalline. Cytoplasmic inclusions may occur in the following forms:

i. Amorphous inclusions
ii. Fibrous, banded and paracrystalline inclusions
iii. Crystalline inclusions
iv. Pinwheel and laminated inclusions

Amorphous structures in TMV-infected tobacco cells have been observed and named as X-bodies. Amorphous inclusions are not only associated with *Tobamoviruses*, but also with *Potyviruses* such as bean yellow mosaic virus (BYMV).

Fibrous cytoplasmic inclusions are pure or nearly pure virus aggregates which accumulate in the cytoplasm in various ways. Such inclusions are induced by elongated viruses such as *Potexviruses*, *Carlaviruses* and *Potyviruses*. Paracrystalline bodies are produced by most *Rhabdoviruses*, *Comoviruses* and *Nepoviruses*. Crystalline inclusions are produced both by elongated and isometric viruses.

Pin wheel and laminated inclusion bodies are characteristic of the genus *Potyviruses*. These are complex of three dimensional proteinacious structures, which in section appear as a group of curved membranous arms diverging from central core, and hence called *"pin wheel"*.

3.7 Inclusion bodies in virus diagnosis: While some viruses in their host cells form distinct cytological structures, which can be used for rapid identification of the virus, their use is rather limited in case of many viruses, where inclusion bodies are not very characteristic. Many viruses do not form inclusion bodies. Type and intracellular locations of inclusion bodies are among the several criteria to be used for classifying viruses in families or genera. Characters of inclusion bodies can be used for identification of certain virus genera. There are cases, where inclusions in one genus appear similar to those in other genus, therefore, we can not rely upon such bodies at the genus level. Many inclusions however, are distinctive when they occur consistently. Within a genus, they may be used for diagnosis of infection at species level. At present inclusions are listed as main characteristics of plant *Reoviruses*, *Caulimovirus*, *Closteroviruses* and *Potyviruses*. The diversity of potyvirus induced cytological alteration is so great that several of them can serve as markers in diagnosis work. In certain cases strains of potyviruses may be differentiated by the presence of

inclusions, although they are very similar and not distinguishable by serology. Cytological investigations of inclusions can be used to indicate relationships among many viruses, and in some cases for diagnosis. Examples of some viruses which produce inclusion bodies that are quite distinctive for rapid identification are (i) bean yellow mosaic virus (ii) cabbage black ring virus (iii) cactus mosaic virus (iv) cauliflower mosaic virus (v) lily symptomless virus (vi) petunia ring spot virus (vii) red clover vein mosaic virus (viii) tobacco mosaic virus and (ix) watermelon mosaic virus

Characteristics of inclusion bodies combined with other diagnostic tests would be helpful for rapid identifications of plant viruses.

3.8 Methods for studying plant virus inclusion bodies: Detection of inclusion bodies with the help of light microscope is a useful and rapid method for identification of many plant viruses. The method is simple and quick, but the difficulty is to recognize and differentiate the type of inclusion bodies. This can be overcome by practice and experience.

3.8.1 The stains used: Recognition of inclusion bodies under the light microscope after proper staining provides a reliable method for identification of plant viruses at genus level. The unstained inclusions are hyaline and it is difficult to distinguish such structures from the surrounding cytoplasmic organelles without staining. Staining of tissue enhances the process of detection. The stains can also be used to differentiate inclusions bodies.

Two stains have been developed to study inclusion bodies induced by plant viruses in the epidermal tissue. These stains are O-G, a combination of calcomine orange 2RS and Luxal (E.I. due Pont de Nemours & Co. Inc., Wilmington, DE 19898), brilliant green BL dyes, and Azure A. Azure A stain and the dyes for O-G stain are available from Aldrich Chemical Company, P.O.Box 355, Milwaukee, WI 53201.

Stock solutions of calcomine orange 2RS and Luxol brilliant green BL are prepared by suspending each dye in 2-methoxyethanol (Ig/100 ml), mixed thoroughly and filtered through coarse filter paper.

Both stocks remain stable indefinitely at room temperature. A standard O-G stain solution is prepared by mixing the green dye, the orange dye, and water in a ratio of 8:1:1. The ratio varies according to the type of tissue being studied. This is done by keeping the orange dye and water content at 1:1 and increase or decrease the green dye content. The stock solution of Azure A in 2-methoxyethanol (0.1 g/100 ml) also remain stable at room temperature. The staining solution is prepared just before use by mixing the stock solution with 0.2 M dibasic sodium phosphate (Na_2PO_4) in a ratio of 9 : 1. The stain should be prepared fresh in small quantity for each batch of tissue and should not be reused.

3.8.2 Selection of tissue: Generally epidermal tissue or mesophyll tissue are used to study inclusion bodies. Epidermal tissue is easy to obtain, easy to remove and contain numerous inclusions.

3.8.3. Staining methods

1. Remove the epidermal strips by inserting the tip of a sharp pointed tweezers (0.8 Dumont NO.5) under the epidermis of a vein (preferably at vein junction) on the lower surface of a leaf. In some plant species, a strip from the upper epidermis is easier to obtain. In order to avoid folding of the epidermal strip, it is brought in contact with the surface of the stain solution in watch glass before being separated from rest of the tissue. The removed tissue should float in the staining solution and not touch the glass surface.
2. After keeping the tissue for 5-10 minutes, the stain is removed by pasteur pipette. Excess stain is eliminated by several changes of 95% ethanol, 5-10 seconds per change for a total of about 50 seconds.
3. After removing the excess stain, the epidermal strips are lifted out with a wooden applicator stick and mounted on a drop of Euparal (Carolina Biological Supply, Burlington, NC 27215) on a glass slide; mounting media containing xylene should be avoided since this solvent adversely affects the stains. Place the coverslip over the tissue and observe under the light microscope for the presence of inclusions. The magnification of 1000 X or higher are needed to resolve small inclusions and to distinguish many features that are useful for diagnosis.

The slides prepared in this way may last for several months or years when properly stained and mounted. Refrigeration helps even for longer storage. When O-G combination is used, the stained plastids confuse with small inclusions. These plastids can be dissolved by floating the epidermal strip on 5% solution of Triton X-100 for 5 minutes before staining. The Triton X-100 solution is removed and the O-G stain is added, but water is omitted from staining solution, because residual Triton X-100 replaces it. The staining time after Triton X-100 treatment is about one-half that for untreated tissue. After staining, the tissue is dehydrated and mounted as previously described.

The Triton X-100 treatment is normally not used with the Azure A stain, which does not stain plastids and thus does not interfere with detection of inclusions.

3.9 A simple technique for studying inclusion bodies: Recently a very simple technique for studying plant virus inclusion bodies has been introduced. A stain known as " Toluidine Blue " is used in this technique. The procedure is as follows:

1. Wash off dirt from leaves with running water and dry with blotting paper.
2. Peel off the epidermal layer with fine point tweezer. Either side of the leaf can be used but the abaxil side is preferable.
3. Float the epidermal strip on 2.5 % aqueous Triton X-100 solution for 2-3 min to remove plastids.
4. Transfer the strips to distilled water to wash off the Triton X-100 for 1 min.
5. Mount the strips directly in 0.05% Toluidine blue in 0.05 M potassium phosphate buffer (pH 7.0) on a clean glass slide.
6. Cover the strips with a cover slip, leave for 1-2 min, examine under 40 X objective of light microscope.

Nucleus will stain blue, while nucleolus stain purple. Inclusion bodies of potyviruses will appear as granulated or fibrills bodies adjacent to the nucleus and are light blue in colour. It is important to stain both a known healthy and virus-infected tissue and examine them for comparison.

Chapter 4

MECHANICAL TRANSMISSION

4.1 Introduction: Symptoms caused by a virus infection are helpful in identification, but symptoms alone are insufficient for virus diagnosis, because symptoms may result from the presence of more than one virus or alternatively, several different viruses may individually cause similar symptoms in the same plant. After observing virus-like symptoms on the infected plants the first step is to isolate the virus. This is achieved by mechanical transmission to the original host. Majority of the viruses causing diseases in plants are mechanically transmitted. If mechanical transmissions is not possible the transmission is achieved by using different biological vectors.

4.2 Virus isolation: Transmission of viruses in the laboratory is generally necessary to isolate viruses from diseased plants collected from the field in order to identify the virus and separate it from mixed infections. This is generally accomplished by sap transmission.

4.2.1 Sap transmission: Sap transmission, also called "*mechanical inoculation*" is the application of plant extract containing virus (i.e. inoculum) on the leaf surface of healthy host plants. In order for the virus particles to penetrate the cuticle and cell wall of a healthy leaf epidermal cells, the surfaces must be artificially wounded. Once introduced into host cell, a virus may spread systemically to other parts of the host. Most plant viruses cause systemic infection resulting in characteristic disease symptoms in the inoculated plant.

In some plant virus combinations, virus movement is restricted and symptoms appear as local lesions (small chlorotic or necrotic spots). Some viruses cause local lesions as well as systemic symptoms on the same host. In some cases there are no systemic symptoms because the virus, although it has entered the plant, does not multiply and invade other parts of the plant. This type of reaction is referred to as *"hypersensitivity"*. Absence of symptoms may also be due to tolerant or resistant reaction of the plant to virus infection. In this case the virus multiplies, but the host is not producing a visible reaction.

When the plants totally prevent the multiplication of the virus and the virus is not detected by any assay such reaction is called *"immune"*.

Not all plant viruses are sap transmissible. The viruses which multiply in epidermal or mesophyll cells and reach to a high concentration in plants are usually mechanically transmissible. The sap transmissible viruses usually produce mosaic, mottle, or ring spot symptoms.

4.2.2. Selection of indicator host: Some viruses develop characteristic diagnostic symptoms in certain hosts known as indicator hosts. They can be used to distinguish between viruses, usually by their immunity to one and susceptibility to the other, and also by the type of symptoms produced. The most commonly used indicator plants for diagnosing plant viruses are listed in Table 4.1.

Table 4.1: Plant species used to identify plant virus genera

Genus	Host plants	Non-host plants
Bromovirus	*Chenopodium amaranticolor* *Chenopodium hybridium* *Nicotiana clevelandii* *Pisum sativum*	*Brassica oleracea* *Nicotiana tabacum* cv. Samsun
Carlavirus	*Chenopodium quinoa* *Datura metel* *Nicotinana clevelandii* *Pisum sativum*	*Cucumus sativus* *Datura stramonium* *Phaseolus vulgaris* cv. Red Kidney
Caulimovirus	*Brassica campestris* *Glycine max* *Phaseolus vulgaris* cv. Kintoki	*Vigna sinensis* cv. Blackeye *Chenopodium quinoa* *Datura stramonium* *Gomphorena globosa*
Closterovirus	*Chenopodium amaranticolor* *Chenopodium quinoa* *Nicotiana clevelandii*	*Cucumus sativus* *Datura stramonium* *Nicotinana glutinosa* *Vigna unguiculata*

Cont.d....

Table 4.1: (Continued)

Genus	Host plants	Non-host plants
Comovirus	*Brassica campestris*	*Datura stramonium*
	Chenopodium amaranticolor	*Nicotiana glutinosa*
	Chenopodium quinoa	*Nicotiana tabacum*
	Cucurbita pepo	
	Vicia faba	
	Vigna unguiculata	
Cucumovirus	*Chenopodium quinoa*	*Nicotiana tabacum*
	Cucumus sativus	*Beta vulgaris*
	Nicotiana benthamiana	*Pisum sativum*
	Nicotiana glutinosa	*Vicia faba*
Furovirus	*Chenopodium quinoa*	*Datura stramonium*
	Dianthus barbatus	*Glycine max*
	Gomphorena globosa	*Petunia hybrida*
	Nicotiana clevelandii	*Pisum sativum*
Geminivirus	*Datura stramonium*	*Chenopodium amaranticolor*
	Nicotiana benthamiana	*Chenopodium quinoa*
	Nicotiana clevelandii	*Gomphorena globosa*
	Phaseolus vulgaris	
	Vigna radiata	
Nepovirus	*Chenopodium quinoa*	*Capsicum annum*
	Cucumis sativus	*Cucurbita maxima*
	Gomphorena globosa	*Datura metel*
	Nicotiana clevelandii	*Vicia faba*
	Phaseolus vulgaris	*Zinia elegans*
Potexvirus	*Chenopodium amarnticolor*	*Cucurbeta pepo*
	Cucumus sativus	*Glycine max*
	Datura stramonium	*Petunia hybrida*
	Nicotiana benthamiana	*Zinia elegans*
	Nicotiana clevelandii	
	Nicotiana glutinosa	
	Nicotiana tabacum	
Potyvirus	*Agropyron repens*	*Cucuuis sativus*
	Avena sativus	*Medicago sativus*
	Beta vulgaris	*Phaseolus vulgaris*
	Chenopodium amaranticolor	*Vigna unguiculata*
	Chenopodium quinoa	*cv. Blackeye*

Cont.d...

Table 4.1: (Continued)

Genus	Host plants	Non-host plants
Potyvirus	*Cucurbita pepo*	
	Datura stramonium	
	Gomphorena globosa	
	Nicotiana clevelandii	
	Nicotiana glutinosa	
Sobemovirus	*Chenopodium amaranticolor*	*Cucumis melo*
	Glycine max	*Cucumis sativus* cv. Delicates
	Nicotiana clevelandii	*Datura stramonium*
	Oryza sativa	*Phaseolus vulgaris*
	Zea mays	*Vicia faba*
Tobamovirus	*Capsicum spp*	*Petunia hybrida*
	Cucumus sativus	*Pisum sativum*
	Nicotiana clevelandii	*Vigna unguiculata*
	N. glutinosa cv. Samsum	cv. blackeye
	Phaseolus vulgaris cv. The Prince	
Tobravirus	*Chenopodium amaranticolor*	*Cucurbita pepo* cv. Capitatum
	Nicotiana clevelandii	*Medicago sativus*
	Phaseolus vulgaris	
Tospovirus	*Nicotiana clevelandii*	*Tetragonia expensa*
	Nicotiana glutinosa	
	Nicotiana tabacum	
Tymovirus	*Chenopodium amaranticolor*	*Capsicum annum*
	Cucumus sativus	*N. tabacum* cv. Xanthi
	Datura stramonium	*Phaseolus vulgaris* cv. Pinto
	Nicotiana clevelandii	*Zinia elegans*
	Phaseolus vulgaris cv. Great Northern	

(Matthews, 1993)

Initially a small number of seeds of the indicator host plants are obtained and propagated under insect proof greenhouse conditions. Sterilized soil (steam sterilized, at 100° C for 30 min) to kill the microbial pathogens, soil inhabiting viruses and virus vectors is used for filling the pots. If possible, healthy plants should be separated from

inoculated plants. Insect vectors inside the greenhouse should be killed regularly with appropriate insecticide sprays or fumigants.

4.2.3 Preparation of inoculum: Inoculum is prepared by extracting sap from the diseased tissue, and this is sometimes sieved through cheese cloth. This sap is then used for rub inoculation on the indicator hosts. The following three points must be kept in mind when choosing virus infected tissue for inoculum preparation:

1. The virus content often, but not always, correlates with severity of the symptoms
2. The highest virus content is often found in young tissue.
3. Some viruses can only be transmitted at certain time of the year under special conditions.

4.2.4 Selection of test plants

1. It is essential that plants selected for inoculation, are healthy looking plants, raised in well fertilized soil in pots under shade, having succulent leaves.
2. For inoculation of cowpea (*Vigna unguiculata*) and beans *Phaseolus* sp., plants with fully expanded primary leaves should be used. *Chenopodium* plants should have expanded mature leaves.

4.2.5 Selection of infected tissue

1. Try to select young infected tissue with primary symptoms. Leaves from younger plants are preferable to those from older plants because of high virus concentration.
2. As much as possible, use infected tissue from greenhouse grown plants with precautions to exclude accidental contamination.
3. Periodically test the tissue, using serological tests, to be sure that the plant has not become contaminated with other viruses.

4.2.6 Preparation of inoculation buffer: Most commonly 0.05 M phosphate buffer of pH 7.0 is used for grinding virus infected tissue to prepare inoculum. Prepare this buffer in one liter distilled water as follows:

Potassium phosphate (monobasic) KH_2PO_4 :	2.4 g
Potassium phosphate (dibasic) K_2HPO_4 :	5.4 g
Sodium sulfite:	1-2 g

4.2.7 Inoculum additives

4.2.7.1 Abrasives: Abrasives facilitates wounding on the leaf surface of plants and entry of virus particles. The most commonly used are carborundum (silicon carbide, 400 - 600 mesh) and celite (diatomaceous earth). The abrasive is either dusted over the leaf surface before inoculation or suspended in the inoculum (0.5 - 1% w/v).

4.2.7.2 Stabilizing additives: Many plants contain inhibitors that may inactivate the virus, decrease or inhibit its infectivity, or interfere with its transmission. The following compounds, when added to the inoculum, are known to have stabilizing effect on viruses in plant extracts containing such inhibitors. They also protect unstable viruses.

a. Ethylene diamine tetraacetic acid trisodium salt (EDTA): 0.05 - 0.1 M
b. Thioglycolic acid : 0.01 - 0.1 M
c. 2-Mercaptoethanol (MEC) : 0.015 - 0.15 M
d. Ascorbic acid (Vitamin C): 0.02 - 0.17 M
e. Sodium sulfite (Na_2SO_3): 0.02 - 0.05 M
f. Bovine serum albumin: 0.01 %.

Material

1. Young systemically virus-infected leaf tissue.
2. Test plants to be inoculated.
3. Phosphate buffer 0.05 M with pH 7.0.
4. Carborundum powder (600 mesh).
5. Mortar and pestle.
6. Piece of cheese cloth, scissors.
7. Disposable gloves.

Precautions

1. Soak all glassware, mortars and pestles to be used in 8 % trisodium phosphate prior to cleaning with any laboratory detergent. Wash them with tap water and rinse several times in distilled water.
2. Dry them thoroughly either in a hot air oven or at room temperature.
3. All the mortar and pestles should be sterilized in an autoclave.
4. Do not touch any virus infected plant prior to inoculation .
5. Inoculate the plants either early in the morning or late in the evening.
6. Keep plants in the dark room prior to inoculation.
7. Host plants must be at right stage of development.

4.2.8 Procedure for sap inoculation

1. Grind infected leaf tissue in a mortar with pestle, preferably in chilled 0.05 M phosphate buffer (normally use a 1:9 dilution i.e. 1 g tissue in 9 ml buffer), till a fine homogenate is obtained. Filter the extract through cheese cloth. It is better to use the inoculum immediately after preparation and do not store for any length of time.
2. Dust the leaves of the test plants with carborudum powder (600 mesh) using aspirator.
3. Hold the leaves in the left palm and apply plant sap inoculum (already prepared) gently with folded cheese cloth or with fore-finger of right hand (Figure 4.1) covered with glove, or with a cotton swab.
4. Mechanically inoculate at least 4 plants of each plant species, label each plant with date, time and name of virus inoculated.
5. Inoculate the same set of plants with buffer without inoculum to serve as negative control.
6. Rinse the inoculated plants with water immediately after inoculation.
7. Wash hands thoroughly with soap or trisodium phosphate or change new gloves before proceeding to inoculate next set of plants.

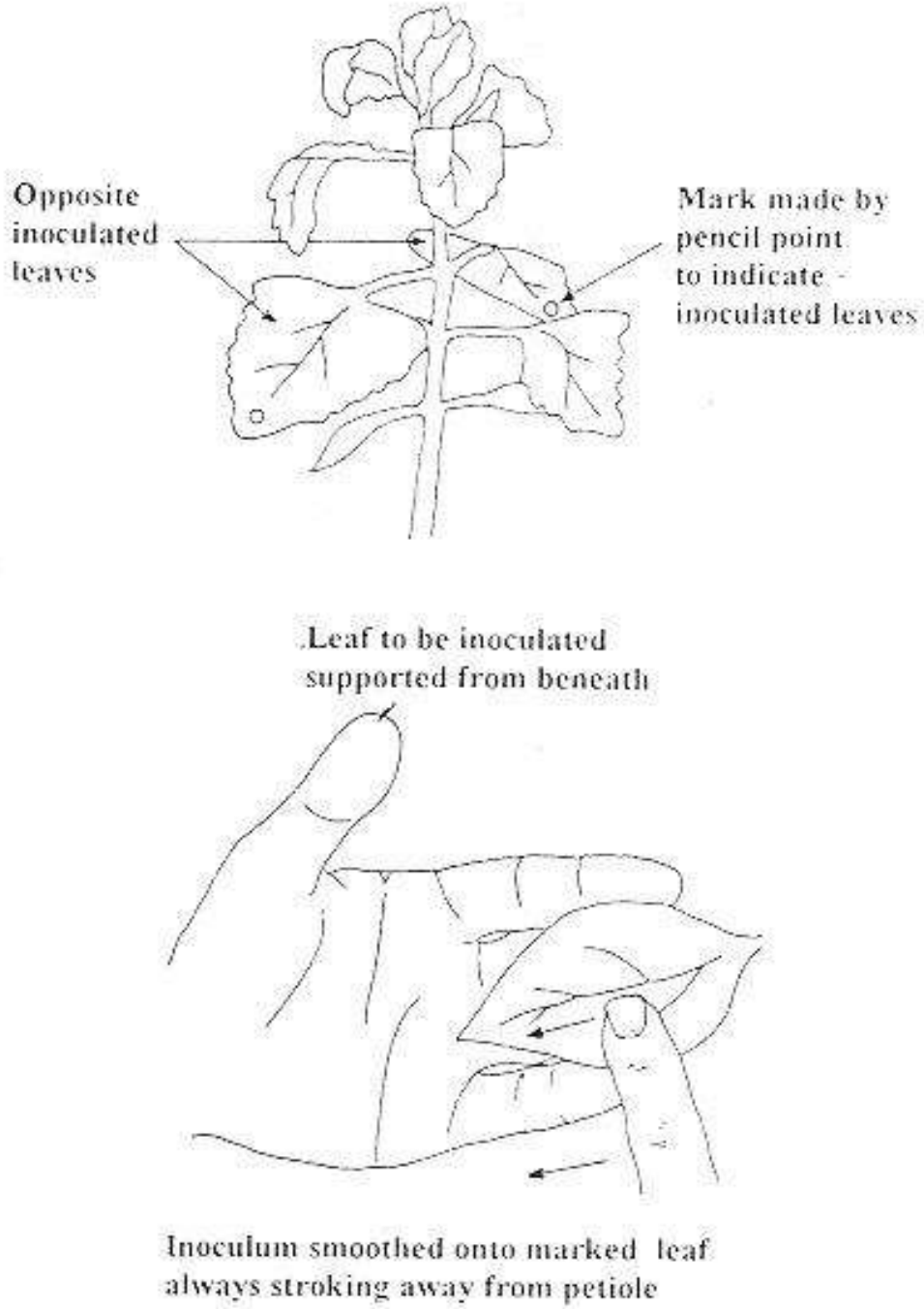

Figure 4.1: Mechanical sap inoculation on *Chenopodium* sp.

4.2.9 Recording of results

1. Observe inoculated plants daily and record the time of appearance of the first symptoms and types of symptoms produced.
2. Record the symptoms on the inoculated leaves and on subsequently produced leaves.
3. In case of local lesions, record number, their diameter and colour, if they are chlorotic or necrotic or both.
4. Observe systemic symptoms such as vein clearing, various types of mosaic, necrosis, vein banding, green islands, stunting of plants, malformation and distortion.
5. If no symptoms are observed on inoculated leaves or systemic, check the presence of latent infection by infectivity assay or by enzyme-linked immunosorbent assay (ELISA).
6. Take photographs of the plants showing symptoms and healthy plants of similar age for comparison.

Some of the common symbols used for recording symptoms are:
LL: local lesions , nLL: necrotic local lesions, cLL: chlorotic local lesion Vc: vein clearing, M: mosaic, Mo: mottle, N: terminal necrosis, Mal: malformation, E: etching, RS: ring spots etc.

4.2.10 Preservation of virus inoculum: In order to identify a new virus and for further studies, it is often useful to store inocula rather than maintaining cultures in the greenhouse for a long time. This helps in saving space in the greenhouse and minimizes the risk of contamination or change in virus isolate. The most widely used storage methods to preserve virus for future use are:

1. Keep viruses in suitable, actively growing storage hosts e.g. infected bulbs, seeds, and stems (potatoes).
2. Virus infected plant material wrapped in plastic can be preserved in a deep freezer at -20 ° C. Infectivity may be lost through repeated freezing and thawing.
3. Leaf material can also be rapidly dried and stored over anhydrous calcium chloride at 0-4°C.
4. Virus infected leaf tissue can be lyophilized and stored at -20 ° C, but this needs special equipment.

4.3 Infectivity Assay: Quantitative bioassay is done through mechanical sap transmission using suitable host plants. Usually, virus infectivity is measured by the number of local lesions induced in the inoculated leaves by the application of virus inoculum. The values of virus concentration obtained by infectivity assay are not absolute but are relative to total number of virus particles present in the inoculum. This is because all the virus particles present in the inoculum are not infectious and not all the cells inoculated become infected.

The relationship between inoculum dilution and local lesion numbers is not always linear and is frequently variable from one virus to another. For many viruses the infectivity/dilution curves are sigmoid in shape, although for some viruses they may be linear (Figure 4.2).

Infectivity assay is widely used by plant virologists for the comparison of different virus preparations. This method has the advantage of quantifying the relative amounts of infectious virus rather than the total amount of nucleoprotein, all of which is not infectious. With physical and chemical assay methods, the total viral nucleoprotein can be estimated, but infectivity assay only indicates that a particular sample is more infective than the other. This method is used to measure the relative virus concentration in crude sap.

4.3.1 Experimental design for infectivity assay: The number of local lesions produced in inoculated leaves varies among plants, and among leaves. There is a little difference even on the same opposite halves of the same leaf. Precautions must be taken to minimize such variation. As far as possible, in all infectivity assays the selected plants should be uniform and of the same age. Proper assay design must be followed to compare virus samples for local lesions study on the same plant. The selected leaves for each sample should be as similar as possible. With plants where leaves are arranged opposite to each other (such as *Chenopodium* sp.), two virus samples can be compared on opposite leaves at the same level on the stem. The assay may be replicated by inoculating other pairs of leaves on the same plant. The actual number of replications depend on the number of plants available and number of leaves on each plant, but the greater the number of replications, the better and more reliable results would be.

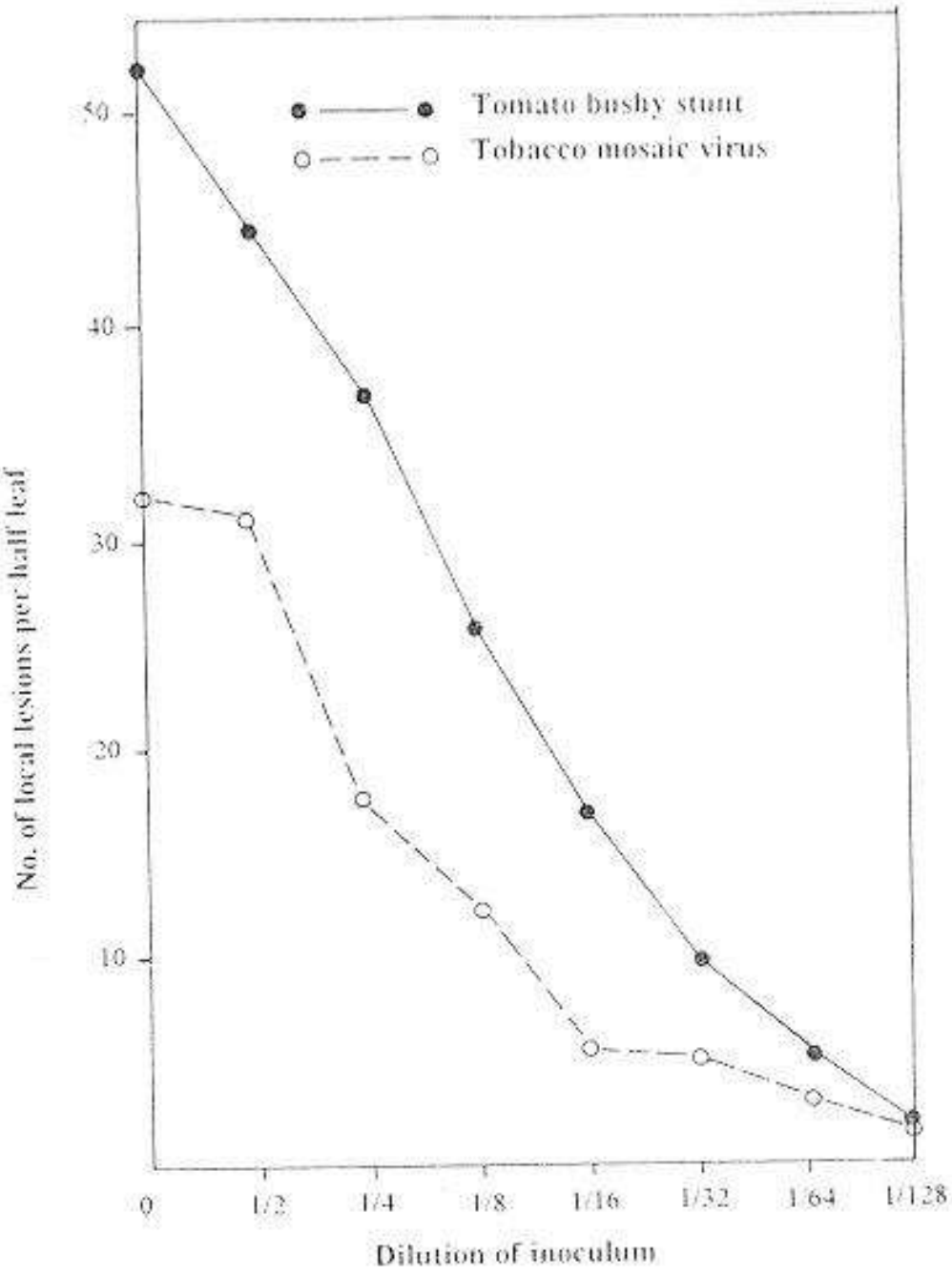

Figure 4.2: The relationship between dilution of virus inoculum and local lesion number for TBSV and TMV assayed on *Nicotiana glutinosa* (Walkey, 1985).

In order to reduce variation, each of the two samples can be inoculated to opposite halves of the same leaf, and this is only possible if the leaf is divided by a midrib into two equal halves. This procedure is more economical and more replications can be achieved.

If more than two samples are to be compared, then more complex designs (Figure 4.3) are to be used. One possibility is to compare each individual virus inoculum with every other sample on opposite half leaves on equal numbers of times. For example, if six comparisons are to be made the design will be as follows:

A	A	A	A	A	B	B	B	B
B	C	D	E	F	C	D	E	F
C	C	C	D	D				
D	E	F	E	F				

Such a design is possible with a host such as bean (*Phaseolus vulgaris*) or cowpea (*Vigna unguiculata*), in which only two opposite primary leaves are inoculated on each plant. Increased replications can be achieved by duplicating the complete design with a second batch of plants.

When no suitable local lesion host is available, the virus is assayed by mechanically inoculating a susceptible host and recording systemic symptoms. When such method is used, it is essential to leave a uniform group of assay plants, and each virus inoculum to be tested is usually diluted in a tenfold dilution series e.g. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} etc. Two or more test plants are used for each dilution of each inoculum tested.

In general, experimental design to conduct infectivity assay should be simple and all efforts should be made to minimize the effects of various variables by selecting test plants of uniform size and age.

4.4 Host range tests: The host range of an unknown virus and the symptoms it induces provides some clue for virus identity, but these tests should be treated carefully, because the type of symptoms produced on a particular hosts depend on a particular virus strain and several other factors. The host range of the virus, that is the host on which the virus induces symptoms and the type of symptoms, may help to differentiate a specific virus from several others. Transmission studies indicate whether the virus is sap transmissible and to what hosts

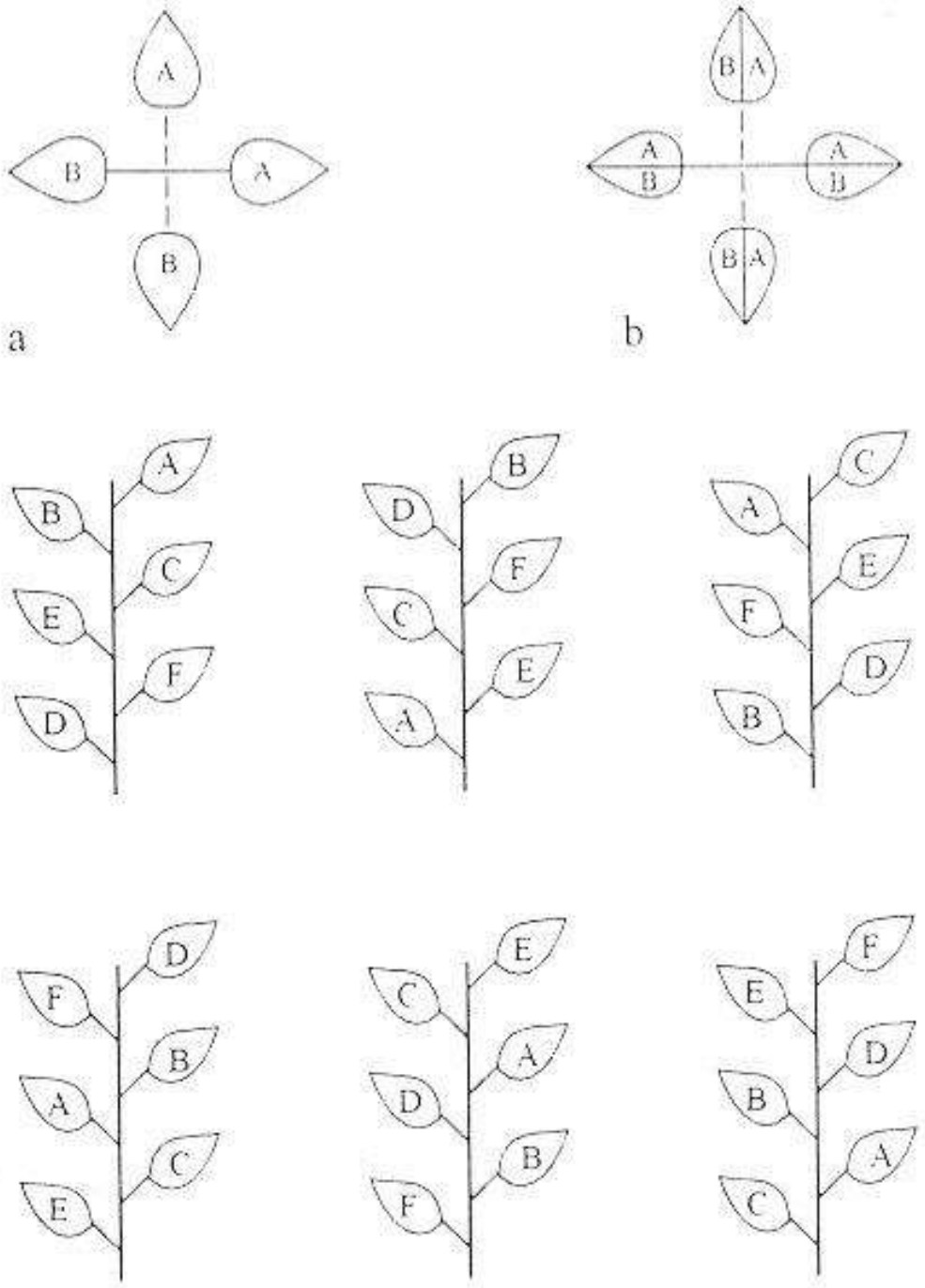

Figure 4.3: Various assay designs for comparing the relative virus concentration in different preparations (a) two virus samples inoculated to whole, opposite leaves at the same level on the stem (b) two samples compared on opposite half of the leaf (c) Latin square design for comparing six different samples on six leaves of six plants (Walkey, 1985).

and by which insects it can be vectored. Some viruses have very narrow host range, which help in their identification. The host range of celery mosaic virus for instance is restricted to species of the Umpbelliferae family. If a rod shaped virus with 750 nm long particles is isolated from celery and is restricted to the species of the Umbelliferae family, then one can tentatively diagnose it as celery mosaic virus.

It should be remembered that host range is not a reliable criteria for a virus identification, however, a preliminary host range studies may provide some useful information on the best host for propagation, assaying and maintaining cultures of newly isolated viruses. For host range studies a set of plants of various families is inoculated in a greenhouse mechanically or by using vector. Symptoms observation on the inoculated test plants are taken within 2-3 weeks.

4.5 Other transmission tests: In addition to sap inoculation, the plant viruses are also transmitted by other means, such as graft transmission and dodder transmission.

4.5.1 Graft Transmission: Almost all diseases caused by viruses are transmitted by grafting. Grafting involves allowing contacts between the meristematic tissue of a healthy plant with the meristematic tissue of a virus-infected plant. This union between meristematic tissue facilitates transport of plant metabolites and pathogens which produce systemic infection. There are three types of graft transmission.

4.5.1.1 Approach grafting: In this system the scion is not detached from the mother plant. A splice is made on the scion and the stock, and both surfaces are united by direct contact (Figure 4.4a).

4.5.1.2 Wedge grafting: A wedge is made in the root stock and scion is cut to be inserted the wedge. It is also called *"top cleft grafting"* (Figure 4.4b). It is widely used with both herbaceous and woody plants.

4.5.1.3 Bud grafting: A small piece of stem tissue known as scion is grafted onto the root stock after making a splice to the scion (Figure 4.4c).

Material

1. Test plants
2. Scalpel blade or sharp knife
3. Parafilm or polythene ribbons
4. Beakers
5. Sterile distilled water
6. Plastic bags to cover the plants

Procedure

1. Wash the diseased plants in sterile distilled water.
2. On potted healthy plants (stock) select branches of same size and approximately of same age of diseased plants (scion). By using a blade make a slanting cut of about 10 mm on the stock.
3. Give a wedge shaped cut on scion. The exposed area on the scion should match the cut area on the stock.
4. Insert the wedge shaped end of the scion into the cut on the stock. Immediately tie both by rolling a parafilm or a polythene ribbon over the joint. Cover the plant completely with a plastic bag to maintain humidity.
5. Keep the plants under shade and cover the plants with plastic sheet to create humid conditions which enhances the successful union between stock and scion.

Precautions

1. Handle the plants at 15-25^{o}C.
2. Maintain turgidity of the diseased plants until grafted.
3. Do not subject the grafted plants to moisture stress.

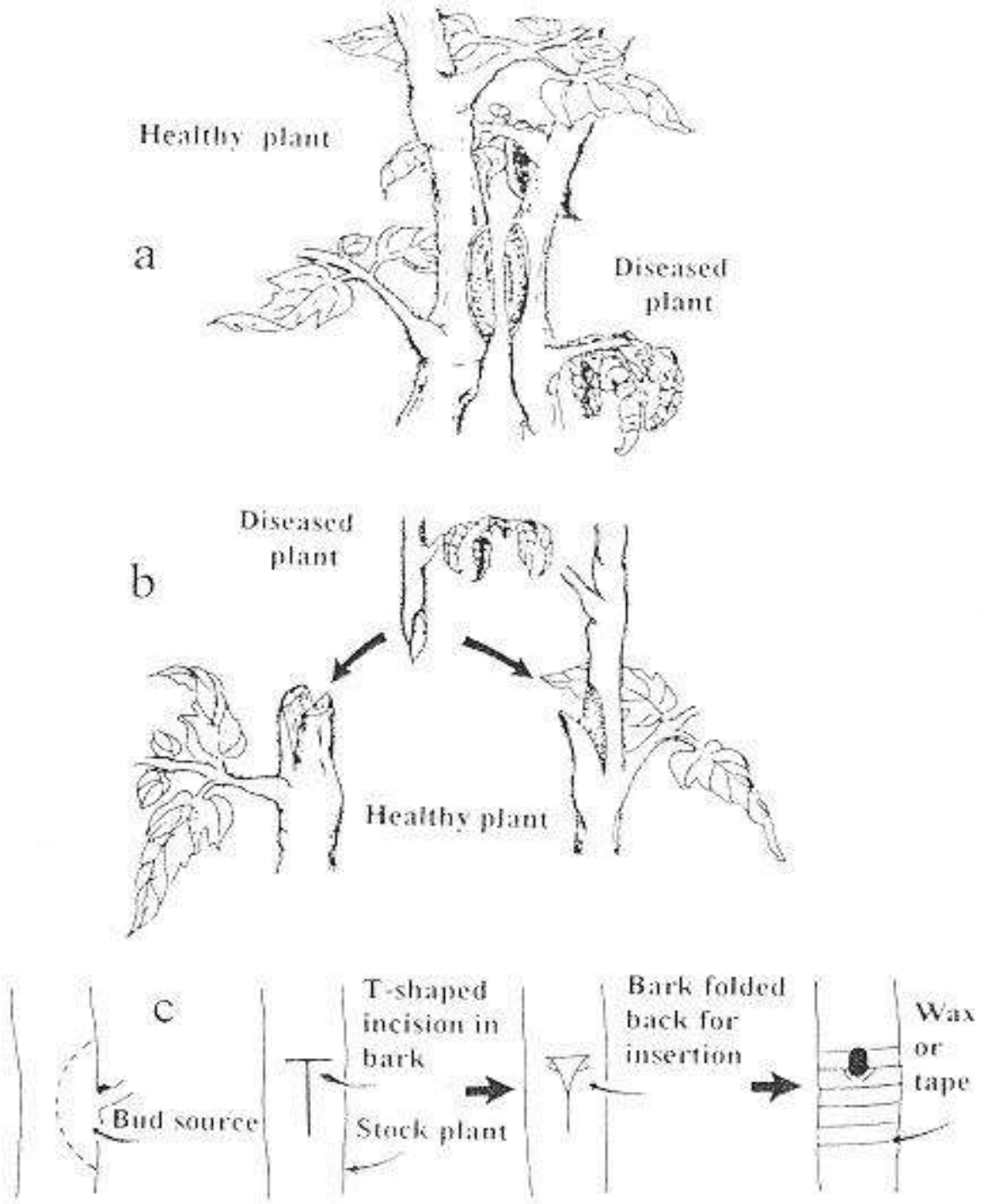

Figure 4.4: Three main types of grafting for virus transmission (a) approach grafting (b) wedge grafting (c) bud grafting.

4.6 Transmission by dodder: Dodder (*Cuscuta* sp) is a semiparasitic plant which attached itself to other plants and draws nutrients from host plants by means of sucking structures "*haustoria*". Several species of *Cuscuta* are known to transmit viruses. Dodder plants used for transmission studies must be grown from seed so that they will be virus-free.

Procedure

Place the virus-free dodder plant in close contact with the virus infected plant. The dodder will wrap itself around the stems and leaves of the virus infected plant and send out haustoria to form unions with the virus infected plant. After the dodder has become well established on the diseased plant, train its stems towards the healthy plants. If the virus is transmissible by dodder, virus symptoms will eventually appear on the healthy plant. Viruses such as cucumber mosaic and tobacco rattle replicate in the dodder and are more efficiently transmitted.

Chapter 5

INSECT TRANSMISSION

5.1 Introduction: The ability to be transmitted among plants is a fundamental property of plant viruses that is necessary for their survival and spread. Plant viruses are transmitted mechanically and through a wide range of agents like vegetative propagative organs, grafting, seed and pollen, fungi, mites, insects and nematodes. The later two are very effective transmitting agents. They feed on plants and move from plant to plant and thus transmit many viruses very efficiently and successfully. Majority of the viruses depend exclusively or largely on insects and nematodes for their transmission. Transmission characteristics are amongst the important features which are used for characterizing and identifying plant viruses. Mode of transmission of a virus by a specific vector may indicate the type of virus under investigation and suggests its probable identity. Insect transmission experiments are conducted to (i) identify the vector of a plant virus (ii) assay viruses which are not mechanically transmitted (iii) obtain information about the mode of transmission. In this chapter the insect transmission is discussed briefly and various techniques used for virus transmission by their insect vectors are described.

5.2 Insect transmission: Insects are the most efficient and important vectors of plant virus transmission. A large number of viruses are transmitted by a large number of insects under field conditions. Of 381 species of animals reported to transmit viruses, approximately 94% belong to Phylum "Arthropod", and approximately 99% are insects.

Some plant viruses could be transmitted mechanically by adults and Juvenile Orthoptera-Grasshoppers and Crickets, Lepidoptera butter flies and moths, and Diptera true flies, but these are not important virus vectors.

Most insect vectors (70%) of plants viruses belong to the order "Homoptera", and the aphids (family, Aphididae) are the most important vectors of this group. The leaf hoppers (family, Cicadellidae), plant hoppers (family, Delphacidae), and the tree hopper

(family, Membracidae) are also important vectors. The other important insect vectors are whiteflies (family, Aleurodidae), beetles (order, Coleoptera), and mealy bugs (family, Pseudococcidae). Generally, the viruses which are transmitted by vectors in one group are not transmitted by vectors from another group, but a few exception are there. The important groups of insect vectors are discussed below.

5.2.1 Aphid transmission: Aphids form the largest group of insect vectors. About 370 species of aphids have been reported transmitting about 300 plant viruses. The aphid, *Myzus persicae* alone is estimated to transmit about 100 viruses, while, each of the many other aphid species transmit more than 30 viruses. Some aphids, on the other hand, can only transmit one virus each.

5.2.2 Terminology: In experiments and studies of insect transmission of viruses, the following terms are often used:

Acquisition access period: This is the total time, when a vector is allowed upon a source of virus, whether it feeds or not.

Acquisition feeding period: This is the feeding period for which a virus free vector actually feeds on a virus infected plant to become viruliferous (infective).

Latent period or incubation period: Having acquired virus, there may be a waiting period or latent period before virus can be transmitted.

Inoculation access period: The period of time allowed for a viruliferous insect to be able to feed on virus free plants and transmit virus.

Inoculation feeding period: The actual period of feeding of a vector on a healthy plant is called the "inoculation feeding period".

Transmission time or transmission threshold period: The minimum period of time that a vector needs for the acquisition and subsequent

inoculation of a virus free plant is called "transmission time or transmission threshold period".

***Retention period of vector*:** The period for which an insect remains viruliferous.

5.2.3 Virus-vector relationship: Plant virus-vector relationships are classified according to the length of time the virus continues to be transmitted by the vector. Aphid transmission of plant viruses can be divided into three basic types. These terms are sometimes also applied to vectors other than aphids.

5.2.3.1 Non-persistent viruses: Non-persistent viruses are retained by the aphid vectors for a few minutes or at most a few hours. These viruses are considered economically more important, and are more numerous than those transmitted by aphids in the semi-persistent or persistent manners. These viruses are also mechanically transmitted. They are acquired and transmitted by the aphids within seconds to a few minutes. Aphids loose their infectivity upon molting. Preliminary fasting increases capacity of aphids to acquire and transmit virus. Viruses belonging to the genera *Potyvirus*, *Cucumovirus* and *Caulimovirus* are transmitted by non-persistent manner.

5.2.3.2 Semi-persistent viruses: These viruses are considered between non-persistent and persistent viruses, and possess some characteristics of both. Such viruses do not circulate within the vectors, but their vectors retain the ability to transmit them for as long as three to four days. There is no latent period, and transmission efficiency increases with increased acquisition and inoculation feeding times. Virus is not lost during molting. Beet yellows and parsnip yellow fleck viruses have the properties of semi-persistent viruses.

5.2.3.3 Persistent viruses: Persistent viruses are not easily mechanically transmitted and are acquired after relatively long feeding period. These viruses are not immediately transmitted and have latent period, after which the aphids remain infective for a long period, and in

some cases for the rest of its life. The vector does not loose virus after molting. These viruses need a long acquisition feeding time (0.5-2 hr) and a latent period of few hours. These viruses circulate in the vector and in some cases also replicate within the vector. Persistent viruses usually show more vector specificity and are restricted to phloem tissue of the plant.

5.2.4 Transmission tests by aphid vectors: Two techniques described below are for tests using aphid vectors.

5.2.4.1 Transmission of non-persistent viruses

Material

1. Vector aphid (must be viruliferous)
2. Petri dish
3. Cling film to close dish
4. Indicator plant species
5. Insecticide and sprayer
6. Small hair brush
7. Small piece of filter paper

Procedure

1. Collect viruliferous aphids from aphid culture maintained on host plants in a Petri dish. Close the dish with Cling film.
2. Store the aphids in a cool shaded place for about 1 hr to starve them.
3. Open the Cling film by cutting a top door.
4. Transfer aphids to infected plant material (detached leaves in a separate Petri dish) to feed for about 2 minutes. Watch to see if aphids feed.
5. Transfer at least five aphids to each separate indicator plant.
6. Cover the plants and allow a transmission feed of no more than 1 hr (this can be done for small plants using inverted plastic, the bottom of which have been replaced by aphid proof gauze).
7. Kill vector aphids by spraying with insecticides, and set test plants aside in the glasshouse to grow-on for symptom observation.

Note: For semi-persistent viruses increase acquisition feeding time to 4 hr, then transfer the aphids to the test plants for a transmission feed of about 1 hr.

5.2.4.2 Transmission of persistent viruses: Aphids are handled in the same way as for non-persistent virus transfer, except that no starvation period is required before acquisition and the transfer or inoculation feed must be longer (i.e. more than 24 hr).

Material

Same as in case of non-persistent virus transmission test.

Procedure

1. Transfer aphid to infected plant material in a Petri dish.
2. Leave in a suitable cool shaded situation to feed for at least 24 hr.
3. Carefully transfer the viruliferous aphids to indicator plants and leave for a further 24 hr, while covering the plants with aphids.
4. Kill all aphids as before and keep the plants in the greenhouse for symptom observation.

Note: In aphid transmission of some viruses there is a latent period following virus acquisition before transmission can take place. Where such a period is known to exist, the duration of the transmission feed should be increased accordingly.

In all vector transmission tests, appropriate control tests must be made in order to ensure the symptoms develop are not simply due to feeding of aviruliferous vectors or extraneous factors.

5.2.5 Rearing of aphids: In order to use aphid vectors regularly in transmission tests, cultures of relevant species must be maintained as a separate colonies. Aphids grow and multiply at temperature between 15 and 25° C. Such temperatures, and the provision of continuos light suppress the development of winged aphids, which are not easy to handle and not efficient vectors. Aphids prefer actively growing, healthy plants and care should be taken to ensure that proper light and temperature requirements are provided.

5.2.5.1 Aphid cages: The cages to rear aphid colonies should be such to accommodate the housing test plants during transmission and to large enough to keep the stock aphid cultures. A variety of cages can be used for such purposes. Traditionally, cages consisted of wooden or iron frame built over an enclosed tray. The top and sides are covered with aphid proof mesh (meshes not wider than 0.3 mm) or some (top and back) surfaces are of glass or perspex to allow better access to light (Figure-5.1). The front side should be lifted easily to water plants and to replace old plants by new ones.

5.2.5.2 Example of aphid transmission test: In this example transmission test for barley yellow dwarf virus (BYDV) has been explained.

Indicator plants

Plant oat seed (susceptible to BYDV) in pots (4 inches diameter) filled with compost, 5 mm deep. Plants will be ready for use when 20-50 mm tall, usually 50 days after emergence.

Vector: *Rhopalosiphum padi* and *Sitobion avenae*.

Acquisition feed

1. Take ten leaves from plants infected with BYDV.
2. Cut two 60 cms pieces from each leaf and place the separated pieces into each of two Petri dishes or damp filter paper arranging the leaves in a criss-cross manner to avoid close packing.
3. Carefully introduce about twenty aphids of *R. padi* into first Petri dish and a similar number of *S. avenae* into the second. Aphids should be handled very carefully.
4. Store the dishes in a cool, well lit place.
5. Allow the aphids to feed for two days.

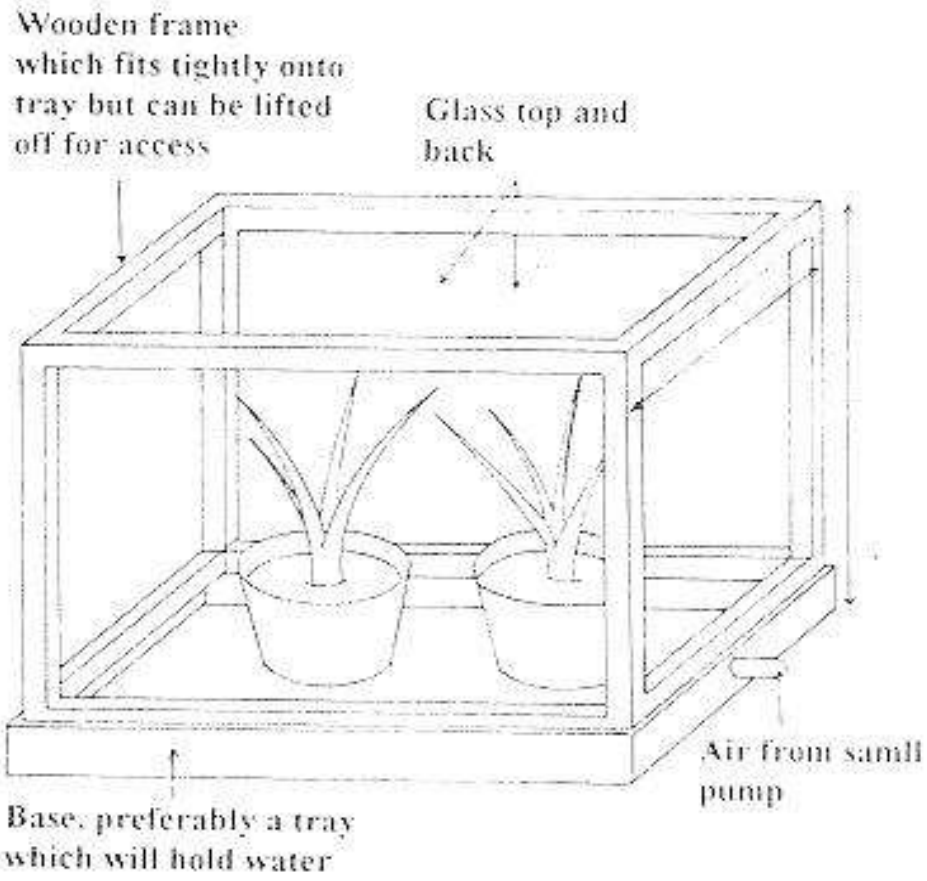

Figure 5.1: Culture cage for aphid stocks. The sides and front of the cage are covered with gauze (mesh less than 0.3 mm).

Transmission feed

6. Cover the non-inoculated seedlings with glass specimen tube to prevent aphid colonization (control).
7. Move at least ten aphids onto each pot of indicator plants. label with specimen no. and aphids species and date.
8. Store in a well lit, cool place. Cover with cage (plastic beaker).
9. Allow the aphids to feed for two days.
10. Kill the aphids with Metasystox or similar insecticide spray. After spraying, the pots should be kept in a confined space for one hour.
11. Examine to ensure that aphids are dead.

Observations of test

12. Keep pots in good light, but cool temperature. Water the plants regularly.
13. Examine the plants for symptoms after 14, 21, and 28 days for
    a. orange-brown discoloration.
    b. scorching of leaf edges.
    c. reduction in growth compared with control.
    d. chlorosis and water soaked patches when held to light.

5.2.6 Hopper transmission: The next important group of insects associated with plant virus transmission after aphids are leaf hoppers (Cicadellidae), plant hoppers (Delphacidae) and tree hoppers (Membracidae). More than 30 species of leaf hoppers have been reported to transmit at least 30 different viruses, and about 22 species of plant hoppers, and one tree hopper species are reported to be virus vectors.

These insects have sucking mouth parts similar to aphids, but they pierce plants more rapidly and cause more damage to plant tissues. Leaf hoppers transmit viruses in semi-persistent and persistent manners. Some viruses are also propagative in the vector and transovarially transmitted to progeny. These viruses are mostly concentrated in the phloem tissues of the host plants.

5.2.7 Whitefly transmission: Virus diseases of legumes, transmitted by whiteflies are economically important in the tropical

areas. In addition to legume viruses, cotton leaf curl, tomato leaf curl and tobacco leaf curl geminiviruses are the most economically destructive diseases. The whitefly vectors multiply to a great number on the underside of the leaves, and are very common in glasshouses. The viruses transmitted by them mostly cause mosaic and leaf distortion symptoms in infected plants. These viruses are not sap transmissible. *Bemisia tabaci* is the most important and wide spread vector of the most economically important whitefly-transmitted viruses. These viruses are phloem limited. Minimum acquisition access time is 6 minutes, but transmission efficiency increases with increase in acquisition feeding. The latent period lasts from 4 to 48 hours and transmission continues for up to 20 days, but viruses are not transovarially transmitted. Mung bean yellow mosaic, cotton leaf curl and tomato leaf curl viruses are few examples of viruses transmitted by whiteflies.

5.2.8 Beetle transmission: Beetles have chewing type of mouth parts and transmit about 45 different viruses. At least 74 beetle species have been reported as vectors of plant viruses. Usually the beetle-transmitted viruses have no other vectors. Members of four plant virus genera, *Bromovirus*, *Comovirus*, *Tymovirus* and *Sobemovirus* are transmitted by beetles. Acquisition feeding period is 24 hours or less; and increase in acquisition feeding time also increases virus transmission. No latent period and transovarial transmission has been reported for beetle vectors. Virus does not multiply in the vector.

5.2.9 Thrip transmission: Three plant viruses i.e. tomato spotted wilt virus (TSWV), peanut bud necrosis virus (PBNV), and pigeonpea sterility mosaic virus (PSMV) have been reported to be transmitted by thrips. TSWV is acquired by the larvae of *Thrips tabaci*, but not by adults, whereas only adults transmit the virus. The shortest acquisition time is 15 minutes. There is a latent period of 4-10 days. Adults are infective after 22-30 days, and retain virus for life. Virus is not transmitted to progeny.

5.2.10 Mealybug transmission: Nineteen species of mealybugs (Pseuodcoccidae) have been reported to transmit six viruses. Two most important viruses transmitted by mealy bugs are cacao swollen shoot virus and cacao mottle virus. Mealy bugs are not efficient vectors, because they are not very mobile and rely on crawling to move from one plant to other. Mealy bugs transmit viruses in a semi-persistent manner. Acquisition feeding time of 48 to 72 hours leads to efficient transmission, although minimal acquisition feeding period is 5-7 hours. Virus can be transmitted after 15 minutes of acquisition period. The insect can retain virus for 3-4 days. Nymphs are more effective vectors than adults.

5.3 Procedures for insect transmission studies

Materials: The materials needed for studying insect transmission includes, cages, insect handling tools, test plants, fumigation chamber, insecticides, incubators (controlled temperature)

5.3.1 Types of cages: Several types of cages can be used, such as wooden plant cages 35 x 35 x 50 cms in size (Figure 5.2a). The sides of the cage are covered either with fine wire netting or pollen netting (15 mesh/cm or saran screen). The top and front door of the cage must be covered with a glass plate. For whiteflies, use a cage with two wooden walls. Each wall should have a round access hole of approximately 18 cms diameter, just large enough for a head to pass through. Prevent the whiteflies from escaping during handling by attaching black blotch tubes to the holes on one end, held close by rubber bands on the other.

5.3.2 Plastic cylinder whole plant cage: The top of this type of plastic cylinder is 33 cm in diameter, covered with cheese cloth, and the bottom is pressed into the soil of the pot (Figure 5.2b). If a potted plant is not used, fresh leaves in a water-filled test tube can be placed in the plastic cylinder. Cellulose nitrate plastic or butyrate plastic should be used, as certain other kinds (cellulose acetate with diethyl phalate) are toxic to plants and insects.

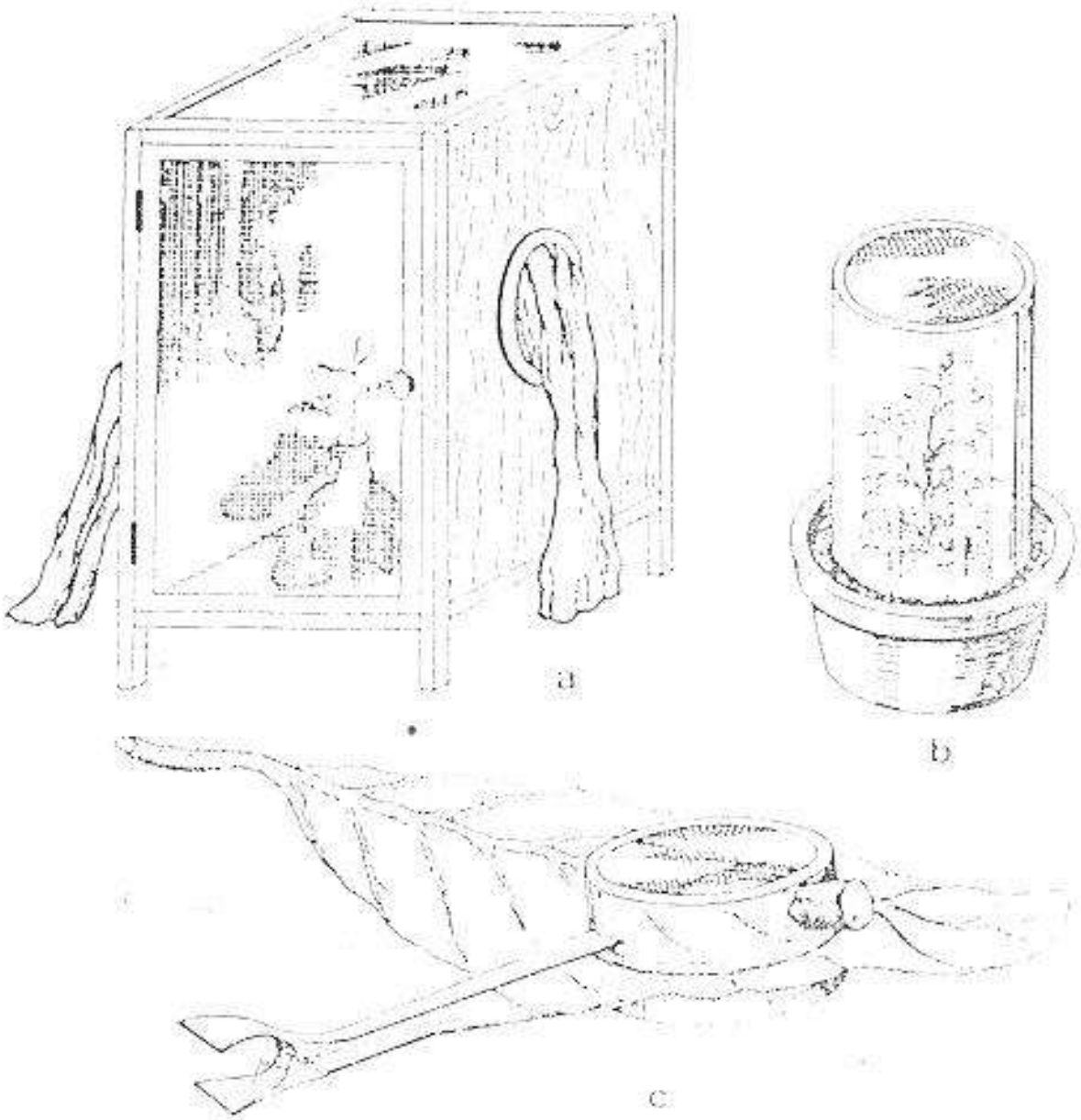

Figure 5.2: Types of cages used for insect virus transmission (a) wooden plant cage (b) plastic cylinder whole plant cage (c) plastic cylinder leaf cage.

5.3.3 Plastic cylinder leaf cage: Such cylinders are made by sectioning plastic tubing approximately 3 cm in diameter and 15 cm long, covered on one side with a screen from a nylon stocking (Figure 5.2c). These cages are used for small insects. The insects are transferred through a small hole in the wall of the tube which is then closed with a cork. The cages are attached to the leaves with the aid of hair clips. The hair clips are attached by heating them and pushing them through the wall of the plastic tube.

5.3.4 Plastic or glass containers: These containers are used to transport insects collected from the field. They should have a screen covering and be large enough to allow for ample space and ventilation.

5.3.5 Insect handling tools: Hair brush, aspirator (used for more active insects e.g. whiteflies and leafhoppers). The aspirator consists of a glass bottle closed with a two-hole rubber stopper. A small straight glass tube is inserted through one hole. The other end is connected to a piece of rubber tubing which serves as a mouthpiece of screen (Figure 5.3). A slightly longer glass tube which has been bent to the desired shape is inserted through the other hole. Insects are sucked into the bottle through the tube.

5.3.6 Test plants: Usually the same plant species from which the insects were collected from the field are used.

5.3.7 Collection of insects: Insects are collected by sweeping and brushing over low vegetation with a net or by beating the plant and collecting the fallen insects on dark sheet spread below. Colour traps, light traps, suction traps and sticky traps are also used to collect the insects.

5.3.8 Maintenance of insects: Generally the conditions which favour host plant growth also favour the development of vectors. Most vectors can be reared on their host plants or on detached leaves of the same plants. Some insects can be reared on artificial diet.

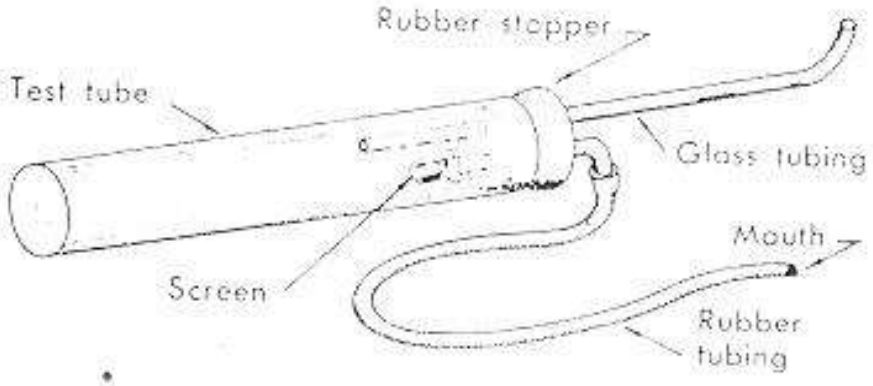

Figure 5.3: Aspirator used to catch active insects e.g. whitefly and leaf hoppers.

5.3.9 Maintenance of virus-free insect cultures: After collecting insects from the field they should be transferred to virus indicator plants to determine whether the insects are virus free. The insects should be maintained on host plants which are not susceptible to virus being studied. If the virus is not carried in the vector's egg (transovarially) the eggs can be used to start a virus-free culture. The eggs are put on blotting paper until they hatch. The nymphs are then transferred to healthy plants. Virus free colonies of aphids can be developed by feeding aphids collected from the field on healthy leaf under a binocular. The newly born nymphs which are usually virus free may be reared on healthy plants in the insectory.

5.3.10 Insect handling: Handle insects using fine paint brush, aspirator, or a single hair brush. Use pointed artist's brush for aphids. Moisten the tip to make the insect adhere to the brush, use aspirator for more active insects e.g. leaf hopper and whiteflies. Aspirator has a small rubber tube with a fine plastic tip to which insect attaches when it is sucked. Use a single hair fastened to a tooth pick or a thin wooden stick for mites and very small insects, such as thrips. Insects can be collected from the fields by sweeping or brushing over low vegetation with a net.

The same plant species from which the insects were collected in the field are generally used as test plant. In general, the conditions which favour the growth of host plants also favour the vector development. Insects should be maintained on host plants which are not susceptible to the virus being studied.

When insects collected in the field are used for transmission tests, they need to be transferred to virus indicator plants to determine if the insects are virus-free. If the susceptible plants do not show virus symptoms, then the insect culture can be used for transmission tests. Keep plants on which the insects are placed for acquisition or inoculation feeding, absolutely free from insecticides.

5.3.11 Inoculation procedure

1. Place virus free insects on a virus-infected test plant to feed and acquire the virus (acquisition feeding). Depending on the virus, it may take from a few seconds up to a few days for the insect to become

infective. The optimum acquisition period varies with the insect, the virus and the host plant. After the insects have acquired the virus they are usually immediately transferred to a virus-free test plant for transmission feeding (inoculation feeding). Some insects can transmit the virus immediately, but others need a latent period, which may vary from a few hours to seven weeks. The latent period can be determined by successive transfers of the insects to virus-free test plants at hourly/daily intervals following the acquisition feeding.

2. Some insects, such as aphids which may carry virus on their stylets, retain it for as little as 30 minutes or even less. Most leaf hoppers (and certain aphids which carry the virus in their guts) are able to transmit the virus throughout their life time.

3. The insects are usually killed after the inoculation feeding period with the aid of insecticides or by fumigants and the inoculated plants are kept under observation for development of typical virus symptoms. Keep the plants under observations for one to three months.

4. To check the possibility of the insect culture being contaminated with a virus, transfer some insects from the culture directly to test plants without feeding on a virus source. In addition, use non-inoculated plants in a greenhouse to detect accidental spread and ensure that the test plants were not infected before inoculation.

5.4 Virus transmission by thrips: Tomato spotted wilt virus (TSWV), peanut bud necrosis virus (PBNV) and peanut yellow spot virus (PYSV) are transmitted by thrips. Two species of thrips commonly occurring on groundnut are *Frankliniella schultzei* and *Scirotothrips dorsalis*, but *F. schultzei* is a major vector of TSWV, and *S. dorsalis* is a major vector of PYSV and poor vector of TSWV.

5.4.1 Collection of thrips: Both *F. schultzei* and *S. dorsalis* inhabit terminal shoots while *C. indicus* feeds on the lower leaves of groundnut plants. *F. schultzei* also occurs in flowers. For mass collection of thrips, a large number of leaves or flowers are removed early in the morning and placed in a plastic jar. The open end of the jar is inserted over the stem of the funnel. The adult thrips crawl along the walls of the funnel and gather into the glass vial. The glass vials are changed at 2 hr intervals, longer intervals at high temperatures may

result into death of the thrips. For collecting a small number of thrips, an aspirator can be used.

5.4.2 Examination: Before examining thrips under the microscope, they are immobilized by exposure to low temperature. This is done by keeping the aspirator containing thrips in a refrigerator at 4° C for 15 min. The thrips become sluggish and are dislodged onto a filter paper in a petridish, which is placed on ice. By using a light microscope at 70 X, individual thrips are picked with a fine camel hair brush (size 00 or 000) and transferred to vials containing AGA fluid (60% alcohol: 10 parts, glycerine: 1 part, and acetic acid: 1 part). The AGA solution keeps the specimens in a relaxed condition but if they are kept longer than one month in this fluid it results in discoloration and disintegration of the tissues. Therefore, thrips are transferred to 60 % alcohol for permanent storage.

5.4.3 Identification: Identify thrips by establishing homogeneous colonies with the help of an insect taxonomist. The thrips collected in AGA fluid, and stored in 60 % alcohol, can be sent to other places for identification.

5.4.4 Raising virus-free culture: Start culturing of thrips from field collected females. Try to keep each species separately. Five females and 2 to 3 males are picked individually by means of a moistened camel hair brush and are released gently into a glass vial, 3 cm in length and 1 cm in diameter, held in an inverted position. The thrips move upward and gathered in the upper portion of the inverted vial. Introduce a young groundnut leaflet and close the vial with bark cork. Keep the vials in the incubator adjusted to 12 hourly cycles of 26° C with a light period (4500 Lux), and 21° C with a dark period. After allowing a 24 hr oviposition access, the thrips are lodged from the leaflet onto the sides by trapping the individual vial held in an inverted position. After the thrips are moved to the upper portion of a vial, the cork is removed and the leaflet is transferred to a new vial for the incubation of eggs. A fresh leaflet is introduced into the original

vial for thrips to feed and oviposit. This process is continued for 10 days during which 90 % of the total fecundity has occurred.

Thrips from cultures are frequently examined to see if the colonies are homogeneous. They are also released on the test plants to ascertain that they are virus-free. The method of culturing thrips on detached leaflets is simple, economical and allows rearing in controlled environment throughout the year and insects of specific age can be obtained for transmission tests. This method is suited for rearing large as well as small numbers of thrips.

5.4.5 Inoculation procedure: A host that provides complete nutrition for larvae, and contain a high titer of virus is required to ensure normal feeding and virus acquisition. For example for peanut yellow spot virus (PYSV), groundnut is a suitable host. The leaves showing early virus symptoms are removed from plants. The leaves are floated on water in a petridish. The first instar nymphs are released on each leaflet with the help of camel hair brush. The nymphs are allowed an acquisition access period e.g 24 hr for TSWV. After the requisite acquisition access period, the nymphs are transferred to small vials containing healthy leaves. The exposed adult thrips are used in transmission tests. The thrips exposed to healthy leaves are used as controls. Individual test seedlings are inoculated by at least two adult thrips. After the inoculation access period, the plants are sprayed with 0.025 percent demeton-s-methyl (Metasystox-i-(R) Bayer) and are transferred to greenhouse for observations. To confirm virus transmission, the inoculated seedlings are tested by ELISA or infectivity assay.

Chapter 6
TRANSMISSION BY AGENTS OTHER THAN INSECTS

6.1 Introduction: In addition to insects plant viruses are also transmitted by nematodes, fungi and mites. Nematodes which belong to the class Nematode and order *Dorylamida* (family: Trichodoridae) are known as vectors of the genus *Tobraviruses*. Eight species of *Xiphinema* and about five species of *Longidorus* (family: Longidoridae) transmit about 20 *Nepoviruses*. There are at least 11 examples of viruses that are transmitted by soil inhabiting plasmodiophorid fungi. These viruses are transmitted by fungi belonging to two groups of obligate parasites i.e. *Olpidium* sp. (Chytridales), which transmit viruses with isometric particles such as tobacco necrosis (TNV), satellite and cucumber necrosis viruses, and *Polymyxa* and *Spongospora* sp. (Plasmodiophorales) which transmit a number of viruses including wheat mosaic and potato mop top viruses.

There are only three confirmed cases of plant virus transmission by mites. These are rye grass mosaic virus transmitted by *Abacarus hystis* and wheat spot mosaic and wheat streak mosaic viruses transmitted by *Acerina tulipe*. All these viruses are filamentous and readily sap transmissible.

6.2 Nematode transmission: Approximately 20 plant viruses are known to be transmitted by one or more species of soil inhabiting parasitic nematodes. Four genera of nematodes are reported to transmit plant viruses, and these are *Xiphenema*, *Longidorus*, *Trichodorus* and *Paratrichodorus*. Two distinct genera of plant viruses i.e. *Tobraviruses* and *Nepoviruses* are transmitted by nematodes. *Tobraviruses* are elongated viruses transmitted by *Trichodorus* and *Paratrichodorus*. The *Nepoviruses* are isometric and are transmitted by *Xiphinema* and *Longidorus*.

All nematode transmitted viruses are relatively stable *in vitro*, possess a wide host range of woody and herbaceous plants, and are also transmitted through seed and pollen. Tobacco rattle, tobacco ring spot and pea early browning viruses are a few examples of nematode transmitted viruses.

6.3 Fungal transmission: Some viruses persist in the soil for a longer time and have been found to be transmitted by fungi. The root infecting fungus *Olpidium* transmits at least four plant viruses. Another fungus *Polymyxa* transmits two viruses and a third fungus *Spongospora* transmits one virus. Some of these viruses are borne internally in the zoospores and others are carried externally on the resting spores and the zoospores. When these spores infect the healthy plants, the virus is transmitted. Examples of fungal transmitted viruses are peanut clump virus, tobacco necrosis, potato mop top and soil-borne wheat mosaic viruses.

6.4 Mite transmission: Mites (Acarina) are not insects and are related to spiders (Arachnids). Plant mites feed by sucking the contents from plant cells. The mites of the families Tetranychidae and Eriophyidae are virus vectors. Eriophid mites are more important vectors of plant viruses. Nymphal stage of *Aceria tulipae* can acquire wheat streak mosaic virus. Acquisition and transmission takes place within 15 minutes each, and virus is retained after molting. No evidence of transovarial transmission is reported.

6.5 Study of nematode transmitted viruses

6.5.1 Sampling: Under field conditions, the outbreaks of nematode-borne viruses are usually characterized by the occurrence of crop infection in patches. Soil sampling is done around the roots of the plants in infected and non-infected areas to determine the kinds of nematodes present. For sampling procedure it should be noted that the largest number of nematodes are present around young roots of host plants in the top 20-60 cm of soil. Soil samples should be taken at various depths (20, 40 and 60 cm etc.) in plastic bags and kept cool until use. The top few centimeters of field soil are usually scraped away and the soil beneath is taken with a trowel or spade at proper depth.

6.5.2 Isolation: Nematodes are isolated from soil samples by wet-sieving method. For this purpose 200 to 300 g of soil sample is suitable. The technique for nematode isolation has been demonstrated in Figure 6.1

The soil sample is soaked in 2 to 3 ml of water for 1-2 hr (Figure 6.1a) and then 400 to 800 ml of water is added and suspension is agitated vigorously to separate nematodes from soil particles and debris (Figure 6.1b). The mixture is allowed to settle for 15 to 20 seconds before decanting the supernatant into a second container through a 2-mm sieve to remove the larger soil particles, debris and small stones (Figure 6.1 c,d). The fluid is then sieved progressively through sieves of decreasing mesh size (mesh aperture 150, 75 and 53 um) (Figure 6.1 e, f, g). The 150-um mesh sieve traps mainly the larger nematodes such as *Longidorus* and *Xiphinema* sp., mainly the juvinils, while those remained by the other two sieves are the smaller nematodes, *Trichodorus* and *Paratricodorus*. The material on the sieves must not be allowed to dry and should be washed immediately into a beaker with water.

The further clarification is done on a Baermann funnel. The suspension is stirred, allowed to settle for 15 seconds and the supernatant is poured onto a 95-um mesh sieve. The sieves are placed on the top of the glass funnel filled with aerated water at about 20° C. Living nematodes actively move through the sieves into the water beneath and settle by gravity in the narrow neck of the filter funnel that is sealed with a clip (Figure 6.1k, l). Collect nematodes within 24 and 48 hr.

6.5.3 Handling and identification: Use a binocular microscope with magnification of 20 to 40 X for identification and handling nematodes. Manipulate the nematodes with a fine metal tool or eyelash. Identify nematodes with the help of a Nematologist or using various keys for *Xiphinema* sp., *Longidorus* sp. and *Trichodorus* sp. Preserve the specimens for further identification by gently heating in water at 65° C for 2 min to kill them and then fix them.

6.5.4 Maintaining culture: The first step after collecting nematodes from field sample is to get virus-free culture for further transmission studies, which is not an easy job. Most nematode-borne viruses are also seed-transmitted with high frequency in common weed species. The germination of such infected seeds following disturbance

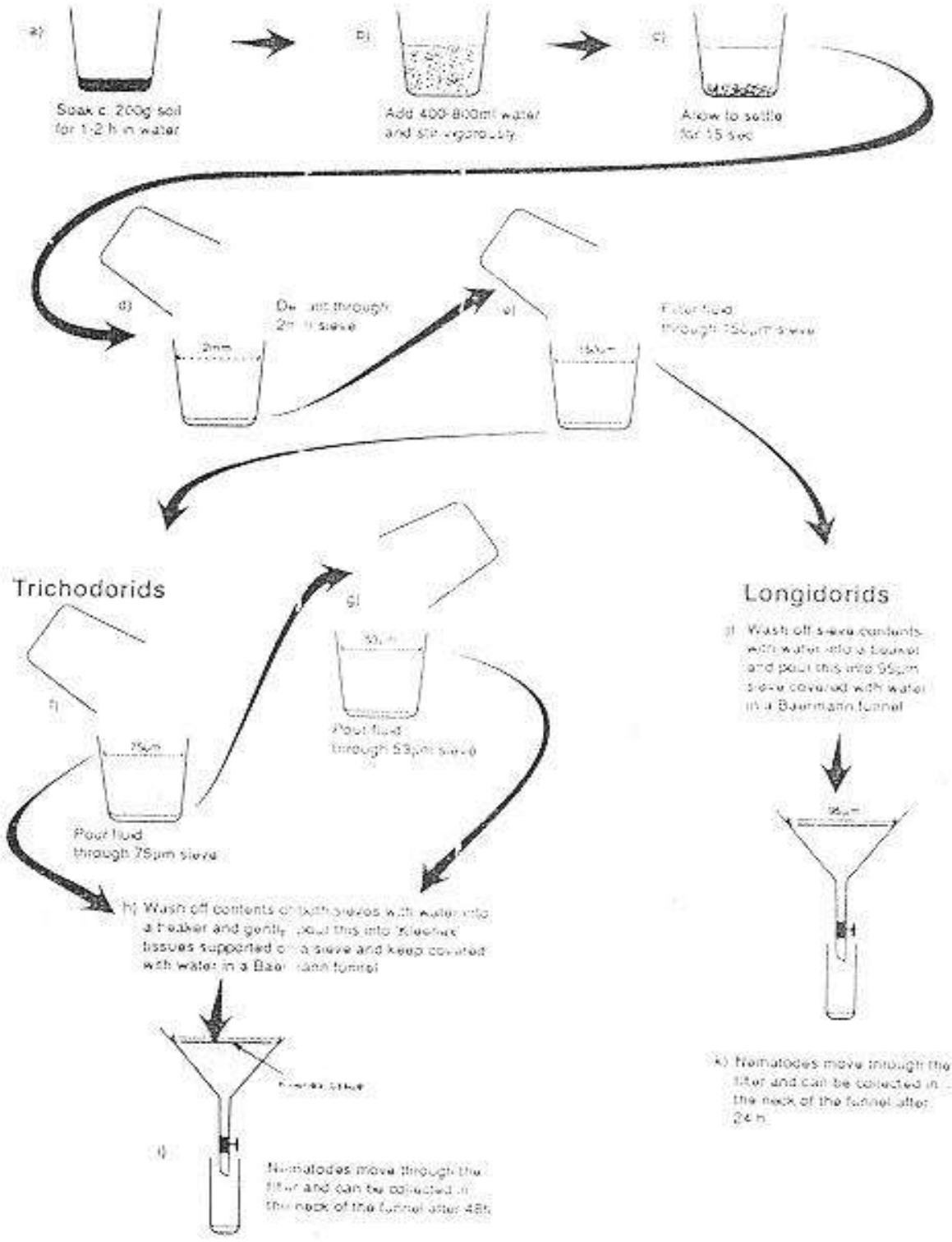

Figure 6.1: Procedure for isolation and inoculation with nematodes from soil samples (Matthews, 1993).

of the soil may provide an inoculum source for initially virus-free nematode.

Some nematodes are easy to culture in glasshouse and reach a large number very quickly e.g. *Xiphinema index*. However, most nematode vectors multiply very slowly. Temperature of about 20° C has usually been used for culturing nematodes in the greenhouse. High temperature and very wet conditions should be avoided.

6.5.5 Procedure for inoculation

1. First prepare micro pots (25 ml for *Longidorus* and 1-2 ml for *Tricodorus* without holes) containing sterile medium (sand for *Trichodorus*: 3:1 mix sand and palm oil for *Longidorus*).
2. Transplant small indicator plant seedlings in the pots partially filled with medium, and allow the plants to establish for 2-3 days before virus inoculation or addition of nematodes.
3. Once the nematodes are added, a further amount of the growing medium is also used.
4. Insert the micro pots in moist sand with proper conditions, i.e. 20°C temperature and high humidity.
5. The virus source and bait plants must be suitable for both virus and nematode multiplication. *Chenopodium quinoa*, *Nicotiana tabacum*, and *Petunia hybrida* can be used for this purpose.
6. Try to use adult nematodes and not the juveniles, as virus specifically attached to the feeding mouth parts is lost during molting.
7. Generally a single nematode is used for inoculation, however, when the transmission rate is low e.g. in case of *Longidorus* species, group of 10 or more are used. Keep as many replicates as possible, but at least 10 with control.
8. The length of acquisition and transmission access periods vary. Three to four weeks are recommended for *Longidorus* and 7-14 weeks for *Tricodorus*.
9. Extract the nematodes by immersing the micro pots in water, gently dislodge and shake to free them from soil particles.
10. Plant roots are washed in another water container and the water from this second wash is pooled with that from the first wash and

used for extracting nematodes. The method for extraction has already been described.

11. After washing, plant roots can be examined under the binocular microscope for root galls caused by nematode feedings.
12. For transmission test, it is better to test roots and tops of bait plants separately. If virus is present in plant tops, it indicates virus transmission by nematode. Virus presence should be confirmed by testing (e.g. ELISA).
13. The presence of virus in the extracted nematode can be confirmed by inoculating nematode extract to indicator test plants.
14. ELISA is not a recommended method for detecting virus in nematodes, as 20-50 nematodes are required to achieve sufficient sensitivity.

The outlines of the procedure for testing virus transmission by nematodes are given in Figure 6.2.

6.6 Procedure for fungal transmission

6.6.1 Isolation: After having indication that a fungal vector is involved in the diseased plants, soil samples are collected and bait plants are used to recover both the virus and possible fungal vector. Soil sampling procedure is the same as has been described in case of nematode transmission tests. It is better to collect the soil sample around the roots of the infected plants. Soil samples are usually mixed with sterile sand and placed in sterile pots. Infectivity of soil is usually greater if soil is sampled after over-wintering and or air drying, before testing, due to the increased release of zoospores from resting bodies. Generally the plants which are infected under field conditions are also used for greenhouse tests. It is better to grow plants from seeds to avoid root injury during transplanting.

6.6.2 Maintenance of vector culture: Isolation of specific vector species and vector (or non-vector) strains is more difficult and require several different approaches. One approach is to maintain culture at different temperatures in order to eliminate or decrease unwanted organisms at non-permissible temperatures. The methods are to establish cultures on selective hosts and dissect out root pieces.

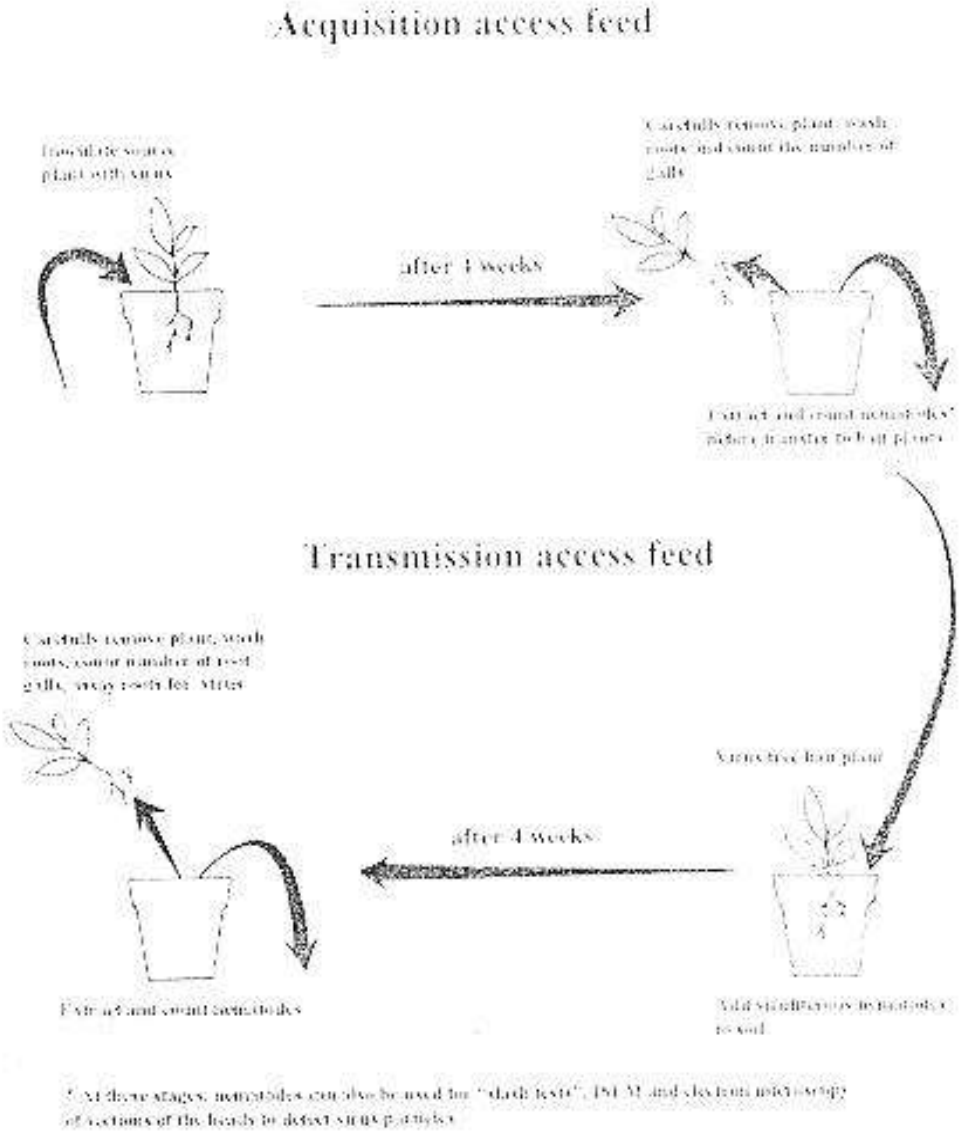

Figure 6.2: Outlines of a procedure for detecting virus transmission by nematodes (Matthews, 1993).

containing the desired fungal species. In order to establish fungal vector culture, in 3-4 weeks, the roots of bait plants are washed free from soil and used to establish further cultures. Fungal species can be identified with the help of a Mycologist.

Once the fungal culture has been obtained, they can be maintained by transferring zoospores suspension to fresh bait seedlings. The growing media for such purpose are sterile sand, malachite, or small glass beads. Active plant growth is maintained by watering with nutrient solution.

When fungal vectors are cultured from naturally infected plants, the culture will also contain the virus. A virus-free culture of the fungus is necessary to demonstrate virus transmission. Such a culture may be obtained from soil samples taken from outside the diseased area. If the virus is carried on the zoospores externally, virus can be eradicated by chemical treatment such as hydrochloric acid or detergents, ribuvirin or incubation with virus antiserum. Alternatively, serial culturing of the fungus on a non-host of the virus for some weeks can be effective. Once fungal cultures are obtained, they should be preserved for reference and future use. Fungal vectors are easy to preserve as they produce stable resting spores. Root pieces infected with fungus, when air-dried and stored in sealed container, remain viable for many years.

6.6.3 Vector transmission tests: In fungal vector transmission tests, zoospores are used effectively. Zoospores suspension is obtained from culture of fungal vector. A flush of *Olpidium brassica* zoospores is released in 2-3 min after roots are immersed in water. For other vectors such as *Polymixa graminis* and *Spongospora subterranea*, release of zoospores occurs when roots are immersed in water following air-drying. Generally, zoospores loose their activity after 10-60 min in water. In order to maintain the activity of the zoospores it is better to use 2 % Knop's solution, 5 % Hoagland's solution, 0.01 M phosphate buffer or 0.05 M glycerin and bovine serum albumin.

After preparing suspension of virus-free zoospores, it is poured around the roots of virus source plants infected with virus either by mechanical inoculation or virus-containing sap or by graft inoculation. The plants are incubated for 2 to 4 weeks to allow time for fungal

colonization of the roots and virus acquisition. A zoospore suspension is collected from the roots of these virus-source plants and used to inoculate healthy virus-free seedlings bait plants growing in sterile medium in pots. Zoospore suspension can be mixed *in vitro* with preparations of partially purified virus particles or virus in sap extracts from infected plants. After a few hours, this mixture can be added directly to healthy bait plant roots.

The inoculated bait plants are kept for 3 to 8 weeks at about 20°C, depending upon the virus bait plant species involved, and wait for virus symptoms development in leaves and/or virus multiplication in roots. Test the inoculated plants for virus presence in roots and terminal parts of the plants separately. Virus can be detected either by infectivity assay or by serology (e.g. ELISA). In transmission tests, always inoculate the following control treatments (i) zoospore suspension without virus to confirm they were initially virus-free and the fungal infection is not the cause of disease symptoms (ii) virus suspension free from zoospores to demonstrate that no transmission occurs without the presence of the vector (iii) untreated bait plants initially virus-free.

The symptoms expression of the inoculated plants is generally influenced by high temperature (greater than 20°C), and temperatures of 14° or 18° C are considered optimum for most viruses.

6.7 Transmission through seeds and pollen Seed infection plays a major role in both the survival and perpetuation of viral pathogens. Seed infection is not only important in local spread of virus, it is instrumental in long distance dissemination during international exchange of commercial seed or germplasm. At present, more than 200 viruses are reported to be seed-transmitted. The percentage of infected seed produced by an individual plant varies greatly depending on the virus, host plant involved, and a number of other factors. Seed transmission rate is usually very low except in few cases.

In most seed transmitted viruses, the virus seems to originate mostly from the ovule of the infected plants, but several cases are known in which the virus is introduced via the pollen during fertilization. In nature viruses transmitted most frequently in seeds tend

to be confined to a few plant virus genera such as *Cryptoviruses*, *Hordeiviruses*, *Harviruses*, *Nepoviruses*, *Potyviruses*, *Tobamoviruses* and *Tobraviruses*. All *Cryptoviruses* are seed-transmitted with a high frequency. Detection of seed transmission in particular hosts of the virus under study is helpful in eliminating the likelihood of the virus belonging to certain groups.

6.7.1 Detection of seed-borne viruses: Correct identification of seed-borne viruses is a fundamental step in any method adopted for control of a virus disease or for certification purposes. Significant progress has been made for rapid identification of viruses in leaf and seed tissues. Most of these diagnostic methods depend on the detection of virus antigens (i.e. coat protein), whereas hybridization tests permit the detection of specific viral nucleic acid by the use of isotope or non-isotope labeled complementary DNA (cDNA). Various methods which are commonly used for detecting seed transmitted viruses are discussed below:

Testing of progeny seedlings is the only way of determining if seed transmission occurs. However, infectivity assays or serological tests are conducted directly on seeds are often used to detect seed-borne infections.

6.7.1.1 Grow-out test: This test is based on virus symptoms appearance in the seedlings emerging from virus-infected seeds. In this test, called "controlled environment room test", seedlings are grown in a controlled chamber or greenhouse and the presence of virus is determined after 2-3 weeks, when the seedlings express virus symptoms. The major disadvantages of this test are the following.

1. It takes a long time to standardize grow-out test in order to obtain reproducible symptoms.
2. A lot of controlled room/greenhouse space is required.
3. The symptoms may be confused with deficiency symptoms.
4. The possibility of having a latent infection (symptomless).

In this test the seeds to be tested are grown in pots with sterile soil in an insect-free greenhouse. The seedlings are observed for virus

symptoms. The presence of virus is confirmed either by serological or infectivity tests.

6.7.1.2 Infectivity test: In this method the presence of virus is assayed by inoculating extracts of seed or seedlings on indicator plants such as *Chenopodium amaranticolor, C. quinoa, Phaseolus vulgaris, Vigna unguiculata, Datura stramonium* etc. This method could also reveal the symptomless or latent infection of plants as in grow-out test. However, this test has the following limitations:

1. It needs a span of time to standardize the indicator hosts and to record the symptoms.
2. A lot of controlled environmental chamber or greenhouse space is required.
3. The test is laborious and time consuming.

6.7.1.3 Serological tests: These tests are based on the specific reaction between antibodies and antigens. Serum containing antibodies is called "antiserum". Antiserum is produced by injecting purified virus preparations into a rabbit. The antiserum is used to perform serological tests, which are the most reliable and practical methods for the detection of seed-borne viruses.

The important serological methods which are used for detecting viruses in seed are agar gel double diffusion test, microprecipitin test, dot-immunobinding assay (DIBA), immunoelectron microscopy (IEM) and enzyme-linked immunosorbent assay (ELISA).

6.7.1.3.1 Agar gel double diffusion test: In agar gel double diffusion test the antigens and antibodies are added to wells punched in agar or agarose. They diffuse through the agarose and form a thin precipitin line where they meet in optimum proportions. This test is less sensitive, requires more antiserum and difficult to perform with elongated viruses. Details of this test are discussed in Chapter 8.

6.7.1.3.2 Microprecipitin test: The microprecipitin test involves placing of small drops of antiserum on plastic or plastic-coated petridishes or in small wells in plastic trays. Plant or seed extract is

added to the antiserum and the drops are viewed under the microscope to observe precipitation. Different antiserum antigen ratios can be tested. Drops in petri dishes are covered with liquid paraffin to prevent evaporation. This test was initially tried for routine testing of pea seed-borne mosaic virus (PSbMV) in seeds.

6.7.1.3.3 Dot-immunobinding assay (DIBA): The basic principle of this assay is similar to that of the plate ELISA. In this assay antigens are first immobilized on nitrocellulose membranes. Since these membranes have a high affinity for proteins, therefore, it is essential to block the free protein binding sites of the membrane. This step is usually done with non-fat dry milk, or bovine serum albumin or gelatin. This process is called "blocking". Immobilized antigens is then exposed to solutions of unconjugated virus specific antibody. Usually crude antiserum is used for this purpose. Trapped antibody is probed with alkaline phosphatase (ALP), or horseradish peroxidase (HRP)-labeled protein A, or anti IgG. Finally the conjugate treated membrane is exposed to substrate solution i.e. naphthol phosphate for ALP. Naphthol phosphate in the presence of ALP is converted to phosphoric acid and naphthol. Naphthol is detected by adding the diazonium salt, which together form a coloured, insoluble product which can be detected visually.

The main advantages of DIBA over ELISA are:

1. It permits to detect virus in extremely small volumes such as in seed and insects.
2. Membranes are cheap and easy to transport.
3. Membranes are easier to process than ELISA plates.

The details regarding procedure of this test are given in Chapter 8.

6.7.1.3.4 Immunoelectron microscopy: In this method virus and antiserum are reacted together and the results are viewed under the electron microscope (EM). Carbon coated copper grids are first coated with virus specific antiserum, and then with virus containing extract of seed or seedlings. Viruses trapped with the antiserum remain attached on surface of the grid and then are detected by negative staining and viewing under the electron microscope (EM). This method can give 1000 fold or more increase in sensitivity over conventional electron

microscopy in detecting viruses. It is the most sensitive method of all serological tests. Results are rapid, and a very small amount of antiserum is required. The only limitation is the high cost and availability of electron microscope. The details of this procedure are given in Chapter 9.

6.7.1.3.5 Enzyme-linked immunosorbent assay (ELISA): The basic principle of ELISA is to immobilize the antigen on the solid surface and probing with specific immunoglobulin carrying an enzyme label. There are two main categories of ELISA procedures, "Direct ELISA (DAS-ELISA) and "Indirect ELISA (DAC-ELISA). The DAS-ELISA is highly strain specific, whereas the indirect ELISA (DAC-ELISA) is more sensitive but less specific than direct ELISA. Mostly, indirect ELISA is used to detect viruses from seeds.

In "indirect ELISA" (DAC-ELISA) procedure the plates are first coated with antigen. The immobilized antigen is then targeted by unconjugated virus specific antibody in crude antiserum. The trapped antibody is detected by an enzyme labeled secondary antibody e.g. goat anti rabbit IgG. The main advantage of the DAC-ELISA is that one enzyme conjugate can be used for all systems. This form of ELISA is particularly suitable for virus detection during disease surveys, for testing virus presence in seeds and to establish serological relationships among viruses. It is also more economical than DAS-ELISA. Details of ELISA procedures are discussed in Chapter 9.

Chapter 7

VIRUS PURIFICATION

7.1 Introduction: In order to identify a new virus disease, the properties of the virus particles need to be determined. Some of these properties can be determined using crude sap, which can lead to a tentative virus diagnosis, or of assigning the causal virus to a virus family or genus. To determine the properties of the purified virus is an essential part of virus characterization.

7.2 Purification procedure: Purification of plant viruses is most commonly obtained by ultra centrifugation of plant sap. This involves differential centrifugation: 1 to 3 cycles of alternate high (40,000 - 100,000 g or more) and low (3000 - 10,000 g) speed centrifugation. Ultra centrifugation separates the virus from the host constituents. Several modifications of the ultra centrifugation techniques particularly the use of density gradient centrifugation are commonly used. In all these methods, the virus is finally obtained as a colorless pellet in a tube and may be used for different purposes.

The purification procedure involves the following steps: (a) extraction (b) clarification (c) concentration and (d) further purification

7.2.1 Preparation of buffers: Different buffer systems are used depending upon the virus or virus strain and its stability at different pH values. The most common buffers used for virus purification, and the procedure of their preparation is outlined below. Buffers should be stored at 4°C. It is better to prepare fresh buffers for each purification.

Potassium phosphate buffer: Phosphate buffers are usually used between 0.01M and 0.5M, and between pH 7.00 and 8.00. To prepare 0.5M phosphate buffer at 7.0 pH, dissolve the following in one liter distilled water:

Potassium phosphate (monobasic) KH_2PO_4	2.4 g
Potassium phosphate (dibasic) K_2HPO_4	5.4 g

Check pH with pH meter, it should be 7.0, store at 4° C

Sodium borate buffer: Mostly used between 0.05 and 0.5M and between pH 7.5 and 8.5, 0.5M borate buffer at pH 7.5 is prepared as follows:

1. Dissolve 61.8 g of boric acid (H_3 BO_3) in 1 liter of distilled water. Add a small volume of 10 % sodium hydroxide (Na OH) solution to help dissolve the boric acid crystals.
2. Prepare 1 N sodium hydroxide solution by dissolving 40 g of NaCl pellets in one liter distilled water.
3. Add 1 N sodium hydroxide solution to boric acid solution until pH 7.5 is obtained.

Sodium citrate buffer: It is used between 0.1M and 0.5M and between pH 6.0 and 7.4. To prepare 0.5M citrate buffer at pH 6.5

1. Dissolve 105 g of citric acid in one liter of 1 N NaOH.
2. Add 0.5 M NaOH to the above solution until pH 6.5 is obtained (an almost equal volume of NaOH will be required to reach the correct pH).

Tris-HCl buffer: It is used between 0.1M and 0.5M, and between pH 7.2 and 8.4. To prepare 0.5 M Tris-HCl at pH 8.0

1. Dissolve 30.3 g of Tris (hydroxymethyl) aminomethane [$(NH_2.C\ (CH_2OH)_3]$ in distilled water (approximately 400 ml)
2. Titrate the Tris solution with 10% hydrochloric acid (HCl) (approximately 100 ml) until pH 8.00 is reached. Make up total volume of solution to 500 ml with distilled water.

Note: See Appendix-5 for preparation of buffers.

7.2.2 Extraction: Systemically virus infected leaf tissue is harvested and stored at 4^{o} C or chilled for one day before extraction. All the materials to be used for virus purification must be kept cold (1-5^{o} C). If it is not possible use ice bath for keeping the materials cold and use only chilled solutions. In some cases fresh harvested virus infected plant tissue can be used.

The first step in plant virus purification is extraction by homogenizing infected leaf tissue thoroughly in an electric grinder after adding an extraction buffer (phosphate, borate or citrate) at proper pH

and molarity and in the presence of stabilizing or neutralizing agents. Usually 1 g of leaf tissue is ground in 2 - 4 ml of extraction buffer.

7.2.3 Clarification: Contaminating host proteins are commonly denatured by using some organic solvents like chloroform, butanol, ether or carbon tetrachloride. The organic solvents also help to release virus particles attached to the cell membranes. The selection of organic solvents depends upon the virus and virus strain. Some viruses are denatured by certain organic solvents. The most commonly used organic solvent is chloroform, but this solvent should not be used for enveloped viruses like tomato spotted wilt virus (TSWV) and rhabdoviruses.

Sodium diethyldithiocarbamate (Na-DIECA) or ethylene diamine tetraacetic acid (EDTA) are commonly used during extraction which neutralizes the acid components of leaves, prevent aggregation and adsorption of virus particles on cell constituents and may even prevent virus degradation. Detergents like Tween-80, Triton X-100 may also be used during extraction to prevent aggregation of virus particles.

Clarification can also be accomplished by subjecting the homogenized extract to low speed centrifugation (about 5000 X g). The centrifugal extract is sieved through muslin cloth or glass wool.

7.2.4 Concentration: After clarifying the extract, the suspension is treated further for concentrating the virus. The most commonly used method is precipitation with polyethylene glycol (PEG) of molecular weights 6000 to 8000. Since this is hydrophilic agent it causes virus particles to aggregate. Addition of NaCl (to a final concentration of 0.1M or 0.2M) is essential to enhance precipitation. If PEG proved not to be effective, high speed centrifugation (over 100,000 g or above) or centrifugation through a sucrose cushion (30 to 40 %) may be suitable.

Ultra centrifugation: Ultra centrifugation is the sedimentation of virus particles under the influence of a centrifugal force. A particle will sediment if its density is more than that of the solution, but will float if its density is less.

Sedimentation rate: Sedimentation rate of a particle is the distance that it travels in a specified interval of time under a constant centrifugal force.

Sedimentation coefficient: It is denoted by "s" or " S20 W, and it is the velocity in centimeters per second at which a particle will sediment in a field of one dyne (1/981 g) in water at 20^{o} C. Sedimentation rates of virus particles during centrifugation depend on their shape, size and density, density of suspending medium, and on centrifugal force applied.

7.2.5 Further purification: Further purification of virus preparation is obtained by the use of density gradient centrifugation.

7.2.5.1 Rate zonal centrifugation: The virus sample is layered on the top of a linear gradient, the most commonly used 10-40% sucrose. The components of the virus preparation precipitate under the enhanced gravitational field at different rates to form layers (virus band) in the tube depending upon their shape, size and density. It is called rate zonal centrifugation. In this case centrifugation is stopped after a short time (1-3 hr), while virus particles are still moving down the tube. Rate zonal centrifugation is the most widely used method for elimination of contaminating host plant material and for concentration of virus preparations.

7.2.5.2. Isopicnic gradient centrifugation: In isopicnic gradient centrifugation separation of different particles in mixture is obtained straightway on the basis of their respective buoyant densities, and centrifugation is stopped only when all the particles have accumulated in bands of their respective densities. In this case a solution of a salt of high molecular weight (most often Cesium Chloride) is mixed with the virus solution in a centrifuge tube. The tube is centrifuged at high speed for a long time (24 - 48 hr.) which causes the salt to form a concentration gradient and hence density gradient. Virus particles accumulate in the form of a band(s) in the tube where their buoyant density equals to the buoyant density of medium. The band formed is

independent of size and shape of the virus particles. This technique is especially useful for separating the different components of a multicomponent virus and for nucleic acid characterization.

The virus bands formed in the tube during density gradient centrifugation are collected and again sedimented with high speed centrifugation. The final pellet is resuspended in a proper buffer and stored in a refrigerator at $4^{o}C$ provided that an anti-microbial substance such as sodium azide (0.1%) is added. Alternatively, sample can be lyophilized (freeze dried) and stored at -20^{o} C.

7.3 Determination of virus concentration: After getting purified virus preparation, its concentration can be measured by using a spectrophotometer. Viruses being nucleoproteins have characteristics absorption spectra. The absorption of ultra violet (UV) light by viruses is due both to their protein and nucleic acid components. Absorption by protein is mainly by the amino acids tryptophan, tyrosine and phenylalanine and shows maximum at 280 nm and minimum at 260 nm. The nucleic acid bases, particularly adenine and uracil absorb UV light at 260 nm (maximum) and 230 nm (minimum). Nucleic acid absorbance is about 20 times stronger than the equal concentrations of protein. The property of UV light absorption of viruses help to determine virus concentration in a purified virus preparation.

Most viruses show maximum absorption near 260 nm and minimum at 240 nm. The 260/280 ratio depends on the nucleic acid content, and therefore, this ratio can be used for estimation of nucleic acid contents of viruses. The virus concentration in a purified preparation can be determined from its UV light spectrum by using its specific extinction coefficient ($E_{1cm}^{1\%}$) at 260 nm.

7.3.1 Extinction coefficient: When the virus absorbance is measured for 1 cm layer of 1 mg/ml virus preparation, it is called extinction coefficient (E) and is written as ($E_{1cm}1\%$). The extinction coefficient of some viruses have already been determined and is given in Table 7.1.

Table 7.1: UV light absorbance characteristics of some plant viruses

Virus	Percent nucleic acid	$A_{260/280}$	$(E_{1cm}^{1\%})$
Tobacco mosaic	5	1.19	2.7-3.5
Potato virus X	6	1.20	2.97
Potato virus Y	6	1.21	2.4
Cucumber mosaic	18	1.70	5.0
Tobacco necrosis	19	1.70	5-5.5
Bean golden mosaic	29	1.40	7.7
Turnip yellow mosaic	35	0.81	0.96

(Stevenson, 1983)

Each virus has a fixed ratio of protein to nucleic acid. Most elongated viruses have 95% protein and 5% nucleic acid. Whereas, isometric viruses have about 90% nucleic acid and 10% protein. For example, TMV has 95% protein and 5% nucleic acid by weight. If it is scanned with UV light the absorbance at 280 nm will result from its protein content, and absorbance at 260 nm will determine RNA content. Thus the ratio of absorbance at 260 nm (A_{260}) to absorbance at 280 nm (A_{280}) has a value of 1.19 for TMV and is a fixed feature of this virus.

Example: If absorbance values of UV light of cowpea aphid-borne mosaic virus (CABMV, an elongated potyvirus) at 260 nm is 0.9372, calculate virus concentration of CABMV in 4 ml volume of virus preparation.

Solution

$$\text{Virus concentration} = \frac{\text{Reading at 260 nm}}{\text{Extinction coefficient}} \times \text{dilution factor} \times \text{volume}$$

$$= \frac{0.9372}{2.4 \text{ (for potyvirus)}} \times 1 \times 4 = 1.562 \text{ mg per 4 ml}$$

Three virus purification protocols have been described below. One for isometric ssRNA cucumber mosaic virus (CMV), one for an elongated ssRNA pea seed-borne mosaic virus (PSbMV) and the other for spherical dsDNA cauliflower mosaic virus (CaMV).

7.4 Purification of CMV (an isometric virus): CMV is easy to purify as it occurs in the host in high concentration. CMV purification protocol is as follows:

7.4.1 Virus propagation: Inoculate cowpea strain of CMV on fully expended primary leaves of a susceptible cowpea variety. Harvest the leaves 15-21 days after inoculation.

***7.4.2 Purification procedure*:** For virus purification follow the following steps:

1. Keep all the buffers to be used, rotors and homogenizer at 4°C overnight.
2. Harvest 200 g of systemically infected cowpea leaves and chill them at 4° C overnight.
3. Add 400 ml of 0.5 M sodium citrate buffer at pH 6.5, containing 0.4 ml thioglycolic acid, and add 400 ml of chloroform. All additives must be prechilled at 4° C. All operations should be carried out at 4° C and in refrigerated centrifuge.
4. Homogenize the mixture for 4-5 minutes in an electric blender until a fine homogenate is obtained.
5. Centrifuge the homogenate at low speed (4000 rpm , 500 X g) for 10 min. Discard the pellet, and filter supernatant through cheese cloth.
6. Add 10 % (W/V) polyethylene glycol (PEG: Mol Wt. 6000) to the supernatant and stir it to dissolve at 4° C for 40 min.
7. Centrifuge at 10,000 rpm (8000 X g) for 20 minutes, discard supernatant and save pellet.
8. Resuspend the pellet in 0.05 M citrate buffer at pH 7.0, containing 2 % Triton X-100, and using 35 ml of buffer to each 35 ml capacity centrifuge tube, leave overnight or dissolve while stirring at 4° C for 30 minutes.

9. Centrifuge at 15000 X for 20 minutes in an ultracentrifuge.Retain the supernatant and discard the pellet.
10. Centrifuge the supernatant at 75000 X for 150 min in an ultracentrifuge. Discard the supernatant and retain the pellet. Resuspend the pellet in 1 ml of .05 M citrate buffer at pH 7.0, leave over night at 4° C.
11. Centrifuge the resuspended pellet at 5000 X g for 10 min. Retain the supernatant and discard the pellet. The supernatant will contain the partially purified virus.
12. Prepare 10-40 % sucrose gradient. It should be prepared one day before it is used and kept at 4° C.
13. Layer 1 ml partially purified virus on 10-40 % sucrose gradient tubes and centrifuge in a Swingout rotor (28,000 rpm) SW-25 Beckman Rotor, for 150 min. Virus band will appear in the tubes.
14. Collect the virus band with syringe and dilute it with two-third of the buffer used for preparing sucrose gradient and centrifuge to sediment the virus. Save the pellet, and discard supernatant.
15. Resuspend the pellet in 0.01 M citrate buffer pH 7.0, and take reading using spectrophotometer, calculate virus concentration at 260/280 nm ratio, and do the electron microscopy and take photographs. The outlines for CMV purification have been given in Figure 7.1.

7.4.3 Preparation of sucrose gradients

1. Prepare sucrose solutions by dissolving the following quantity of sucrose in distilled water or of low molarity buffer (0.01 to 0.1M phosphate buffer, pH 7.0) suitable for virus concerned .

5 g sucrose in 50 ml buffer (10 %)
10 g sucrose in 50 ml buffer (20 %)
15 g sucrose in 50 ml buffer (30 %)
20 g sucrose in 50 ml buffer (40 %)

2. Add the sucrose solution to a 9 cm x 2.5 cm cellulose nitrate centrifuge tubes. Then slowly and carefully with the tip of the pipette in contact with the bottom of the tube allow 7 ml of the 20% solution to run out below the 10% solution (Figure 7.2). Then add, 7 ml of the

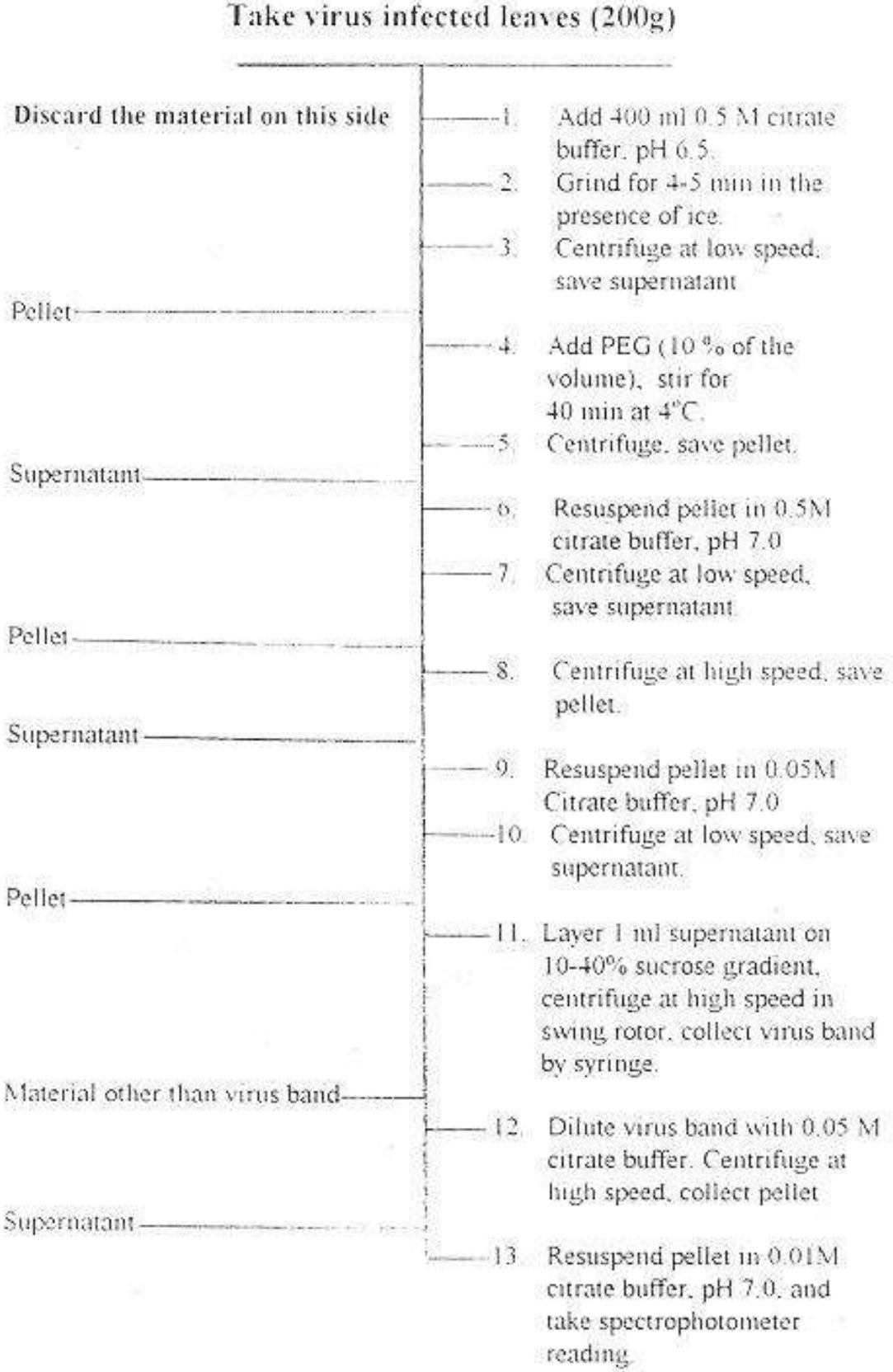

Figure 7.1: Outlines of purification procedure for CMV.

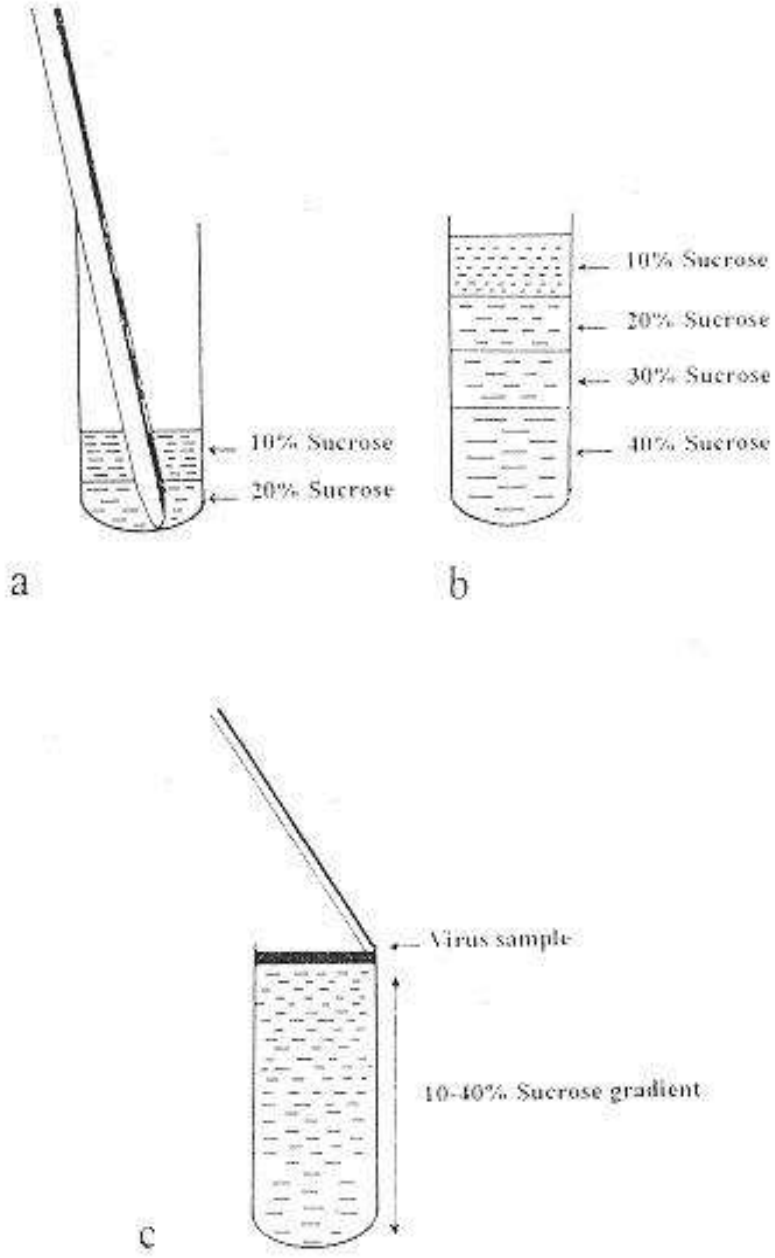

Figure 7.2: Preparation of a sucrose gradient for ultracentrifugation: (a) the lowest concentration is added to the tube first and the higher concentrations are added in order, at the bottom of the tube; (b) the 10-40% gradient is allowed to stand overnight; (c) the virus sample is layered onto the surface of the gradient.

30% and 15 ml of the 40% solution. Keep these tubes to stand at 4° C over night to enable the solutions to diffuse to form a gradient.

3. Carefully layer 0.5 ml of the partially purified virus on the top of the gradient, by slowly running the virus down the side of the tube above the surface of the sucrose.
4. Balance all the tubes, centrifuge the gradients in a swing out bucket rotor for 1 to 1.5 hr at the speed recommended for rotor concerned.
5. After centrifugation you will observe opalescent virus band in the tubes under narrow beam of light shown vertically down through the tube. The virus band may be removed with hypodermic syringe.
6. The virus may be separated from the remaining sucrose solution by dialysis against 0.01M citrate buffer over night at 4° C, or by diluting with the buffer. The diluted virus is then subjected to centrifugation at 75,000 g for 120 minutes and the resulting pellet is resuspended in a small volume of distilled water or of low molarity concerned buffer. The purified virus is then used for antiserum production or biochemical analysis.

7.5 Purification of PSbMV (an elongated virus): The purification procedure for PSbMV involves the following steps:

1. Prepare the following buffers before starting purification:

0.2M Borate buffer (extraction buffer), pH 8.0, in one liter distilled water.

Boric acid:	8.66 g
Sodium borate (decahydrated):	5.72 g
EDTA (ethylene diamine tetra acetic acid):	0.37 g
Beta-mercaptoethanol:	4 ml
Triton X 100:	10 ml

0.01M Borate buffer, pH 8.0, in one liter distilled water.

Boric acid:	0.43 g
Sodium borate(decahydrated):	0.29 g
EDTA	0.37 g
Beta-mercaptoethanol:	0.5 ml
Triton X 100:	10 ml

2. Grind one part well chilled virus-infected plant tissue in 3 parts of extraction buffer (0.2 M borate buffer, pH 8.0) with 1 % antifoam B and 20 % chloroform in electric belnder for 3-4 min.
3. Centrifuge the extract at 5000 rpm for 10 min, filter the supernatant through glass wool or cheese cloth.
4. Add 6 % polyethylene glycol (PEG) 8000, and stir for 45 min at 4°C.
5. Centrifuge at 10000 rpm for 10 min, save pellet, resuspend the pellet in 0.1 M borate buffer, pH 8.0.
6. Spin at low speed (5000 rpm) for 5 min and save the supernatant after filtering through wool glass or cheese cloth.
7. Layer 3 ml 30 % sucrose under the supernatant (25 ml high speed centrifuge tubes), and spin at 120000 g for 1.5 hr, save the pellets.
8. Resuspend the pellets in an appropriate quantity of 0.01 M borate buffer overnight at 4° C.
9. Spin at low speed (16000 g) for 5 min, save the supernatant and layer 1 ml crude preparation on to 10-40 % sucrose gradient prepared in 0.01 M borate buffer pH 8.0, centrifuge at 104,000 g for 2.5 hr.
10. Remove virus bands manually with syringe, sediment virus at 120,000 g for 1 hr, and resuspend in 0.01 M borate buffer without EDTA or Triton X 100.
11. Spectrophotometrically estimate the virus concentration.

The outlines of the procedure described above for PSbMV are given in Figure 7.3.

7.6 Problems of virus aggregation with elongated viruses:

Generally the viruses with elongated particles are difficult to purify because of their tendencies towards irreversible aggregation during extraction and concentration with consequent loss of virus during low speed centrifugation. Aggregation of virus particles, and virus and host components during purification reduces the amount of virus recovered. However, losses associated with low speed centrifugation due to aggregation of virus particles may be reduced by the addition of ethylene diamine tetra-acetate (EDTA) and Triton X-100 to grinding and subsequent resuspension buffers. Slightly alkaline phosphate buffer

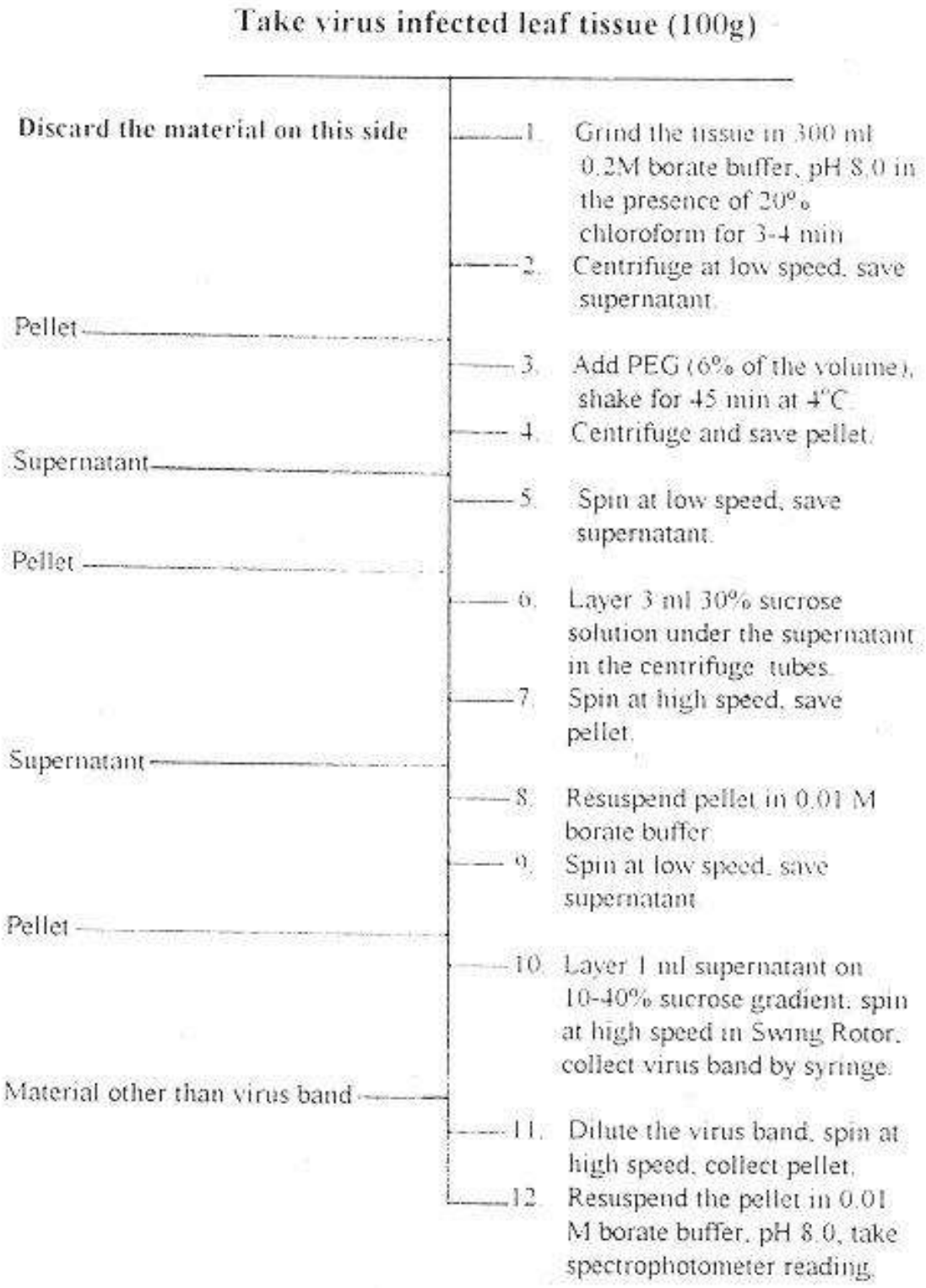

Figure 7.3: Outlines of purification procedure for PSbMV.

along with EDTA or any other salt is most commonly used for virus extraction. This neutralizes acid components of leaves, gives a favorable final pH of the extract, prevents aggregation, or adsorption of virus particles on cell constituents and may even prevent virus degradation. The net charge on virus particles at isoelectric point (the pH value at which total negative and positive charge on a virus particle are equal) is zero, and the virus precipitates at this pH. The pH 4.00 is the isoelectric point of most viruses which are best soluble and most stable at around pH 7.00. However, brome mosaic, cowpea chlorotic mottle and satellite tobacco necrosis viruses are more stable at pH 5.00, since their isoelectric point is around 7.00

7.7 Purification of CaMV an isometric dsDNA virus:

The purification of CaMV involves the following steps:

1. Grind 100 g of infected plant tissue in 0.1 M phosphate buffer, 1:3 (w/v), pH 7.2, containing 0.75% sodium citrate.
2. Squeeze through cheese cloth which has been washed in distilled water.
3. Add 6% urea, 2.5% Triton X-100 and 8% butanol. Stir while adding.
4. Shake overnight at 4^{o} C.
5. Centrifuge at 5000 rpm for 10 min in GSA rotor. Save supernatant.
6. To supernatant, while stirring, add 8 % PEG (MW 6000) and 1 % NaCl.
7. Stir in cold shaker for 1 hr.
8. Centrifuge at 10,000 rpm for 10 min.
9. Resuspend pellet in 0.01 M phosphate buffer, pH 7.2 (20 ml, 10 ml/bottle). resuspend slowly in the refrigerator overnight.
10. Put in smaller centrifuge tubes and centrifuge at 5000 rpm for 10 min.
11. Put the supernatant in ultracentrifuge tubes and centrifuge at 45,000 rpm for 60 min (add 25 min run up time). Balance the tubes with buffer (within 0.1 gm).
12. Decant the supernatant and resuspend pellets in 4 ml (total volume) of 0.01 M phosphate buffer, pH 7.2, slowly in the refrigerator overnight.

13. Thaw sucrose gradient (at room temperature: small tubes 1 hr., large tubes 1 hr 45 min).
14. Shake sample and then centrifuge at 5000 rpm for 10 min.
15. Load the supernatant gently onto the sucrose gradient tubes (10-40 % sucrose gradient) and balance the tubes. The size of tubes to be used depend on the volume of virus in virus preparation. 1-2 ml on the small tubes and 2-3 ml on the large tubes. Load the SW 40 Beckman Rotor and run at 29000 rpm for 138 min, or the SW 28 Beckman Rotor at 24000 rpm for 90 min (allow 20 min extra to get up to speed).
16. Warm up the ISCO density gradient fraction and UV monitor and run blanks and then the samples. Pool the virus containing peaks. Alternatively the virus fractions may be collected manually by syringe.
17. Balance the tubes and label. Centrifuge at 39 000 rpm for 2 hr in the TY 65 Beckman Rotor. Clean the ISCO equipment.
18. Pour off the supernatant and resuspend the pellet in 0.3 ml of 0.01 M phosphate buffer, pH 7.2, overnight. Estimate virus concentration using spectrophotometer.

The outlines of the purification procedure for CaMV are given in Figure 7.4.

7.8 Antiserum production: Once a purified virus preparation is obtained, it could be used for antiserum production, which is essential for serological tests. Antiserum is produced by injecting purified virus in a number of animals. Rabbits have been used most often since they respond well to plant viruses, are easy to handle and produce sufficient volume of serum. Generally, white New Zealand rabbit, chicken (Figure 7.5), mouse and small goats are used for antiserum production against plants viruses. Repeated intravenous or intramuscular purified virus injections are given at short intervals (1-2 weeks). For intramuscular injections the virus preparation is emulsified with an equal volume of Freund's Complete or Incomplete adjuvent (a mixture of mineral oil and surface active agent , which increases the yield of antibody and may prolong its production). Injection schedule vary from worker to worker depending upon the virus being used. Generally,

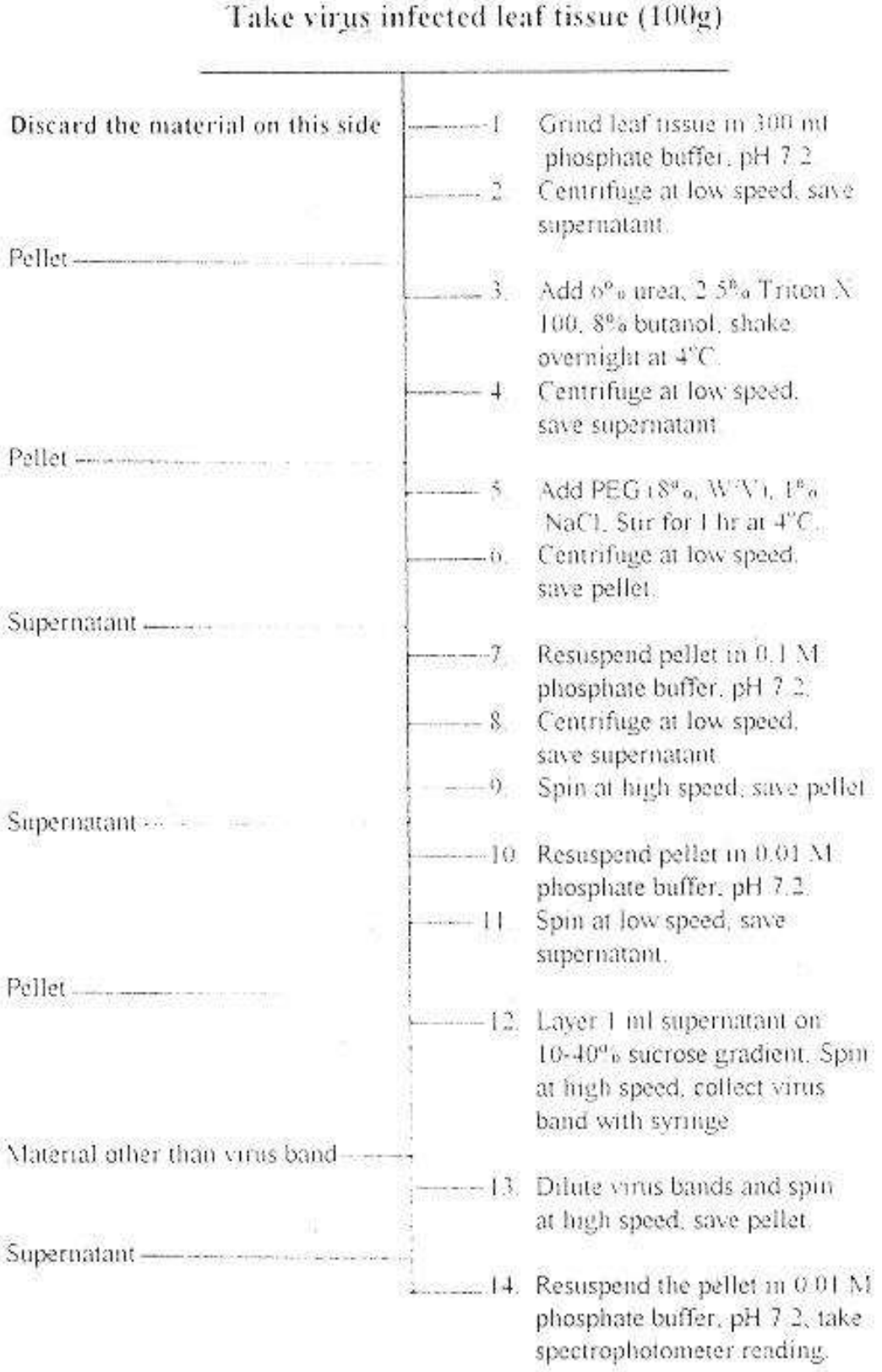

Figure 7.4: Outlines of purification procedure for CaMV.

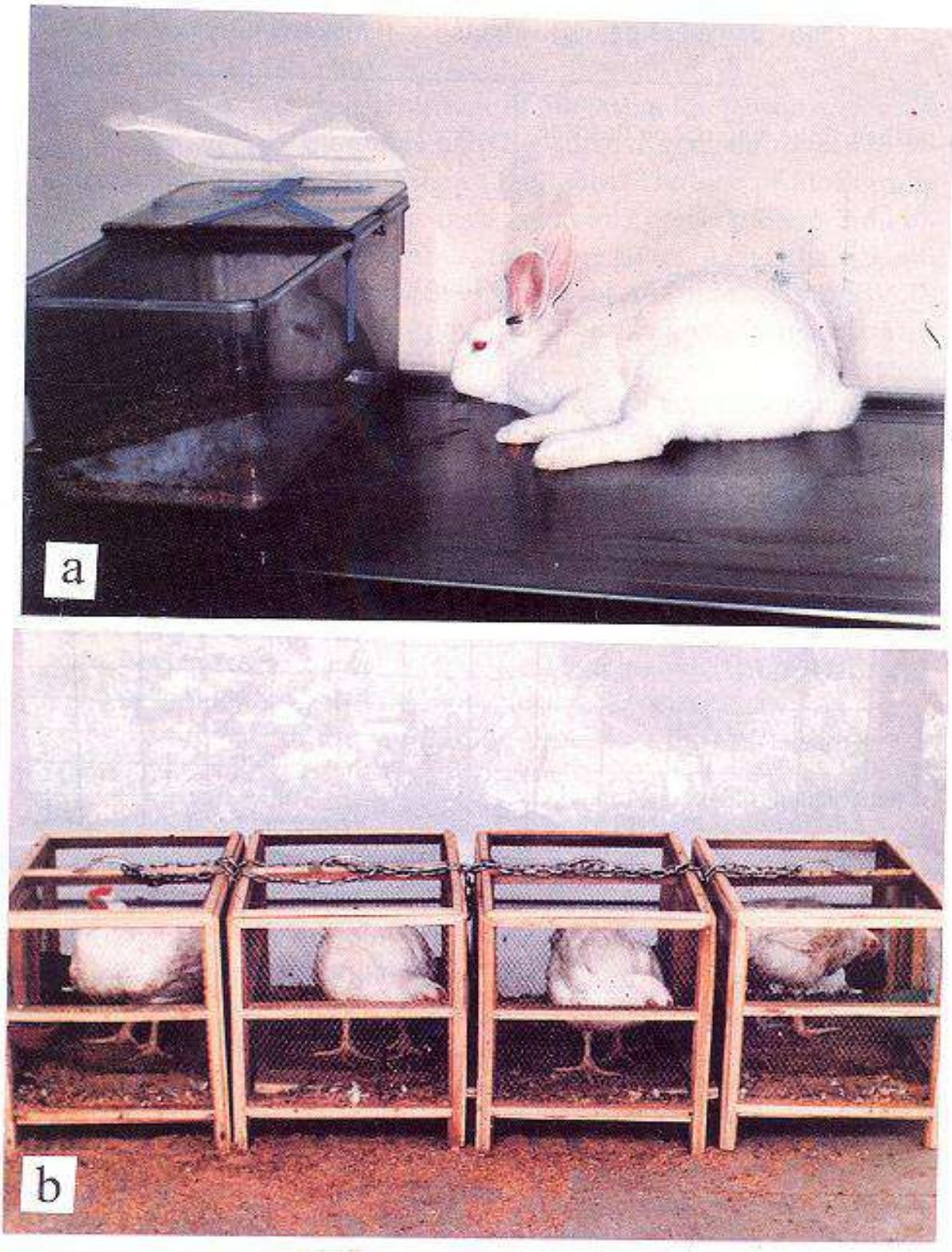

Figure 7.5: The most commonly used small animals for antiserum production (a) white New Zealand rabbit (b) broiler chicken.

antibody production increases progressively and reaches a maximum 4-6 weeks after the initial injection

7.8.1 Procedure for virus injection: The following procedure can be used to produce antiserum against a particular virus. The purified virus being injected into rabbit must be free of host protein contaminates to produce quality antiserum.

1. Take 1 ml of purified virus (0.500 mg/ml) and thoroughly mix it with 1 ml of Freund,s Complete adjuvant (a substance which allows slow release of the antigen within the animal). Inject mixture into the thigh muscle of the white New Zeland rabbit or other available animal.
2. Three to eight injections are usually administered at weekly intervals.
3. Take first bleeding from the immunized rabbit after 3rd or 4th injection (by drip bleeding approximately 15-20 ml from the outer vein of the ear or directly from heart) and then at two weeks intervals. Usually 3- 4 bleeding will provide sufficient antiserum.
4. A booster injection(s) 4 to 6 weeks after the fourth or fifth injection will often yield a high titer antiserum.

7.8.2 Separation of serum from blood: After taking blood from the rabbit, it is allowed to coagulate at room temperature for 2 to 3 hours or at 37° C for 1 hr. The clot is separated from the sides of the container by a sterile glass rod very carefully and then it is stored at 4° C overnight to release antiserum. The clear antiserum is separated from the clot with sterile pasteur pipette. If necessary the serum is further clarified by centrifugation in a bench centrifuge at 500 g for 5 minutes. The antiserum is stored by mixing an equal volume of glycerol (0.5 ml glycerol : 0.5 ml antiserum) or by freeze drying. One drop of 0.02 % sodium azide (NaN_3) is added to each vial of 1 ml to avoid bacterial/fungal contamination. Freeze drying in 1 or 2 ml ampules containing small amount of antiserum preserves the antiserum for long period. Store antiserum in the lyophilized form preferably in 1 or 1.5 ml volume at - 20° C.

7.8.3 Determination of antiserum titer: The highest dilution of antiserum (antibody) that reacts with its own homologous antigen (virus) is called titer of the antiserum. When antigens and antibodies are mixed they combine and form a precipitate. Historically, the titer of the antiserum is determined by precipitation tests. More commonly, tube precipitation test is used for titer determination. In tube precipitin test a small volume (0.25-0.5 ml) of virus suspension is mixed with an equal volume of antiserum in small glass test tubes (0.7 cm diameter). Various dilution's of each reactant are tested against each other. The samples are incubated at 37° C to observe precipitate. However, any other serological test can be used for titer determination, but since the sensitivity of different tests are not the same, it is essential to mention the type of test used along with the titer value.

Procedure

1. Take thin walled test tubes (75 x 9 mm) and make a series of antiserum dilution (the antiserum should be diluted with 0.9 % Na Cl) and mix with a constant dilution of the virus (antigen). Partially purified virus is the most suitable for this test when it is used at 1/5 dilution (with 0.9 % NaCl) or some preliminary tests need to be conducted to determine the optimum virus dilution.
2. Add 0.5 ml of virus to 0.5 ml of each dilution of antiserum, shake each tube for a few seconds to mix the reagents thoroughly, and then incubate tubes at 37° C partially in the water bath in order to from a precipitate.
3. Observe each tube at 5 to 10 min intervals against a black background and record the intensity of precipitation reactions, by a predetermined scoring scale (- = no precipitation, + = rarely visible precipitation, ++ = moderate, +++ = heavy, ++++ = very heavy precipitation). The reaction is usually complete after one hour.
4. It is necessary to include healthy purified host antigen and normal serum (serum taken without immunization of animal) to serve as controls.
5. Note the highest antiserum dilution beyond which no precipitation occurs. This will be the titer of the antiserum.

An example of the determination of antiserum titer has been given in Table 7.2.

Table 7.2: An example of the determination of titer of antiserum by tube precipitation test

Virus dilution	In Ti	Antiserum dilution						Normal serum dilution					
1/5 or		1/4	1/64	1/256	1/512	1/1024	1/ 2048	1/4	1/ 64	1/ 256	1/ 512	1/ 1024	1/ 2048
other proper	0	-	-	-	-	-	--	-	-	-	-	-	-
dilution	15	+	+	+	-	-	-	-	-	-	-	-	-
	30	+++		++	++	++	-	-	-	-	-	-	-
	40	++++	++++	+++	+++	+++	-	-	-	-	-	-	-
	50	+++++	+++++	++++	++++	++++	-	-	-	-	-	-	-

- : no reaction, + : slight reaction, ++ : moderate reaction, +++ : heavy reaction, ++++ : very heavy reaction
In. Ti. : Incubation time

Chapter 8

SEROLOGY IN VIRUS DETECTION

8.1 Introduction: Serological tests are the most important assays for identification of an unknown virus and important to study the relationships between related virus isolates and strains. Such tests are based on the binding capacity that individual antibody has for its own specific homologous antigen. When warm blooded animals are injected with a foreign protein (or virus) it stimulates the production of antibodies in animal blood. The antibodies thus formed are immunoglobulin (IgG) which combine with special part of virus at some specific amino acid sequence (the antigenic site) called *antigenic determinant* or *epitope*. Blood serum containing such antibodies is called "*antiserum*". Antibodies in this serum will bind with the homologous antigen to produce a precipitate and this is the basis of serological tests for viruses. Serological tests are very specific and reliable since antibodies will only react with their specific antigens.

8.2 Terminology

Antigen: An antigen is a substance particularly a protein that is capable of inducing an immune response with the production of antibodies when injected into an appropriate animal.

Antibodies: These are immunoglobulin (IgG) proteins which are produced in the animal blood in response to the injected antigen and have specific binding ability with the antigen.

Immunogenicity: The ability of an antigen to induce immune response is referred to as immunogenicity.

Immunogen: The substance which is capable to induce immune response in an animal is called immunogen.

Antigenic reactivity: The capacity of an antigen to react specifically with an antibody is referred to as antigenic reactivity, or antigenicity.

Antiserum: The serum containing specific antibodies is referred to as antiserum.

Polyclonal antiserum: It is the antiserum which contains a heterogeneous population of antibodies that react with more than one epitope on the antigen. Polyclonal antiserum is obtained from the serum of an animal inoculated with an antigen bearing many antigenic sites (epitopes).

Monoclonal antiserum: It is the antiserum which contains homogenous population of antibodies that react only with a specific epitope against which they are produced.

Epitope or antigenic determinant: The antibody binding sites on the antigen are called epitopes or antigenic determinants.

Paratope: Each molecule of antibody has two or more antigen binding sites which are referred as paratopes.

Homologous antiserum: An antiserum produced against a particular antigen (the virus) is called homologous with respect to that antigen.

Heterologous antiserum: If an antiserum produced against a particular antigen (a virus) also reacts with another similar antigen, then the serum is called heterologous antiserum with respect to another related virus antigen.

Normal serum: A serum separated from blood of an animal before immunization (without injecting a virus) is called normal serum. Such serum is used as a control in serological tests.

8.3 Serological tests: The most significant advances in virus testing have been made in the field of serodiagnosis. The specificity of the antigen/antibody relationship provides an extremely versatile tool for virologists for the valid identification of viruses. One problem in serodiagnosis is the supply or availability of antiserum and its

production which involves sophisticated techniques at high cost equipment.

Until recently, a range of serological tests are available for virus identification. Most serological tests are based on the precipitation produced when antibodies and antigens combine. The most commonly used serological tests for virus detection and identification are as follows:

1. Agglutination tests
2. Precipitin tests
3. Enzyme-linked immunosorbent assays
4. Immunoelectron microscopy

Agglutination and precipitin tests are discussed below, whereas the enzyme-linked immunosorbent assays and immunoelectron microscopy are discussed in Chapters 9 and 10 respectively.

8.3.1 Agglutination tests: In agglutination tests the antibody or virus antigen is adsorbed onto larger particles. A positive reaction causes those larger particles to clump and therefore, the antibody-antigen reaction becomes visible. Two types of agglutination tests are more important. Such tests are no more in use and are replaced by other more sensitive and reliable tests.

8.3.2 Slide agglutination: It is also called chloroplast agglutination test. It is a very simple test. One drop of the virus infected crude sap is mixed with one drop of antiserum on a microscopic slide, and is observed under the microscope. If the chloroplast and other sap debris clump together, this is an indication that the test is positive. The test is particularly useful for rapid detection of virus-infected potatoes in the field. This test is employed only with elongated plant viruses which occur in high concentration in sap such as tobacco mosaic virus (TMV) and potato virus X (PVX).

8.3.3 Precipitin test: The formation of a visible specific precipitate between the antigen and antibody is one of the most direct ways of observing the combination between antibody and antigen (virus). But in these tests relatively high concentration of reagents are needed. The

following three most commonly used precipitin tests in the past are now outdated and no more in use.

a. Tube precipitin test
b. Ring interface precipitin test
c. Microprecipitin tests in liquid and agar.

8.3.4 Ouchterlony double diffusion test: This is the most commonly used serological test. It is performed in a petridish, filled to a depth of 5 mm with agar (approx. 15 ml agar per dish). Wells are cut in agar with cork borer in a pattern such that a central well is surrounded by six or more peripheral wells. Antiserum of a known virus is added in the central well, and the antigens are added in the surrounding wells. When both antigen and antiserum diffuse from their wells into the agar, a visible precipitin line is observed. This test works best for spherical viruses. Virus particles of most of the elongated viruses do not diffuse readily through the agar medium, therefore, sodium dodecyl sulfate (SDS) is added in the sap, as SDS causes the elongated viruses to break down into smaller subunits which can diffuse easily in the agar.

Material

1. Flat bottom sterile standard size petridishes (plastic disposable dishes are preferred).
2. Sodium azide (NaN_3).
3. 0.01 M phosphate buffer, pH 7.0 containing 0.85 % NaCl.
4. Agar 0.8 % (Ion agar No 2, or agarose or Noble agar).
5. Pipettes, micropipette for dispensing and for diluting antigens or antibodies.
6. Moist chamber for incubating the plates (a plastic tray with cover with moist paper towels will be adequate for this purpose).
7. Water bath (50^{o} -55^{o} C)
8. Small 5.0 ml capacity (120 x 75 mm) tubes for making dilution.
9. Cork borer or gel cutter.

Procedure: Agar at a concentration of 0.8% will provide a satisfactory medium for diffusion. To prepare follow these steps.

1. Dissolve 0.8 g of purified agar or agarose in 0.01 M phosphate buffer containing 0.85 % NaCl. For elongated viruses prepare 0.8 % agar containing 0.5 % SDS and 0.01% NaN_3.
2. Autoclave for 10 min or microwave for 3 minutes.
3. Maintain agar at 50-55° C in a water bath.
4. Pour 15 ml agar with the help of glass cylinder into petri plates. Keep the petri plates on flat surface to ensure uniform spread of agar.
5. Allow the agar to solidify at room temperature.
6. Cut wells into the agar with the help of cork borer or a template and remove agar plugs. It is convenient to use a gel cullter with six circular cutters arranged around a central one of the same size (4 mm diameter), and a distance between the wells of 4 mm is suitable.
7. Add suitable dilution of antibody (antiserum) to the central well and antigen to the peripheral wells.
8. Incubate for 24 hr the petri plates at room temperature or at 37° C in a moist chamber.
9. Read the results in a dark room against dark back ground over box with slit or circular light source. Observe the dishes every day for one to two weeks.

If the antiserum is added to the central well, and sap (antigen) to the wells around it, different precipitation lines may be observed e.g. Figure 8.1.

Precautions

1. Avoid excess heating of agar.
2. Do not overflow the well with antigen or antibody dilution.
3. Use healthy plant and normal serum extracts as controls.

For certain viruses such as CMV, better results can be obtained if agar is dissolved in 0.05 M dipotassium hydrogen orthophosphate (K_2HPO_4) at pH 7 to 8, containing 0.005 M sodium EDTA (ethylene diamine tetraacetic acid, disodium salt) and 0.02 % NaN_3. This can be prepared by dissolving in one liter of distilled water, 8.7 g K_2HPO_4, 1.86 g Na-EDTA, 0.2 g NaN_3 and 7.5 g agar. The antiserum dilutions can be prepared with 0.9 % NaCl. The dilution required to give precipitin lines will depend on the titer of antiserum concerned,

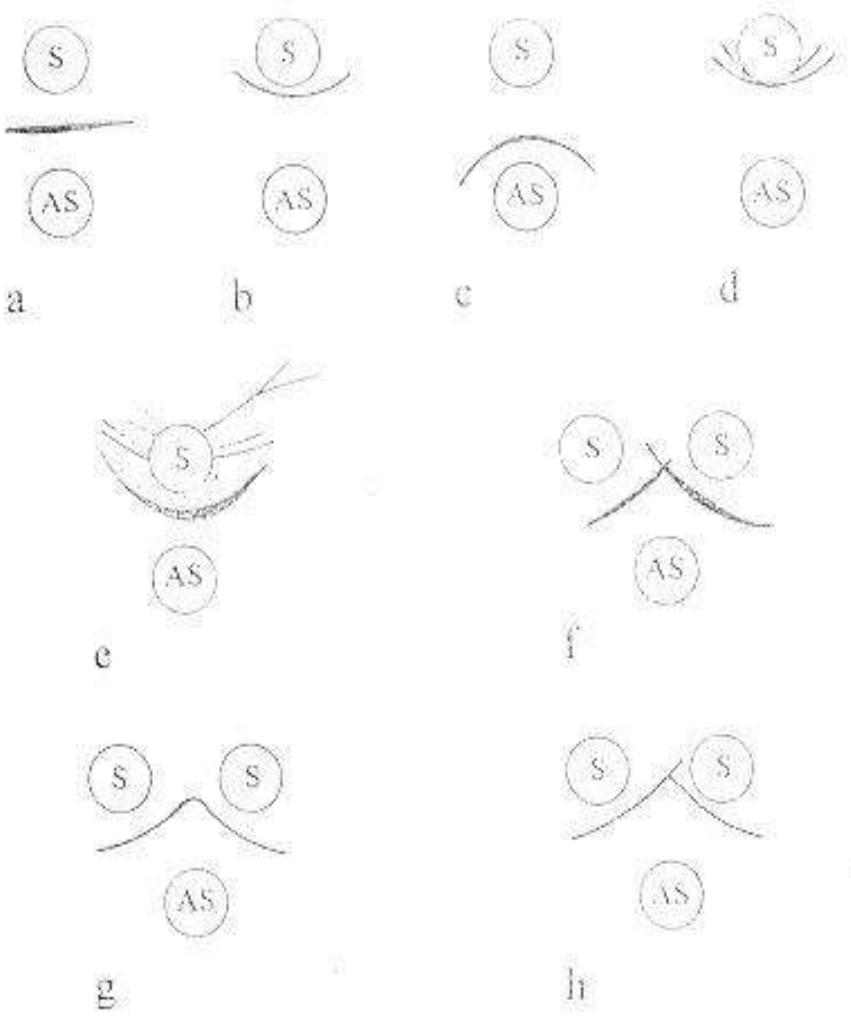

Figure 8.1: Different patterns of precipitation lines observed in double diffusion test. S: well with sap (antigen) AS: well with antiserum (antibodies).

antiserum used, undiluted and dilution. A dilution of 1:5 often gives satisfactory results.

Purified virus or infected fresh crude sap may be used, but later gives satisfactory results if virus is in high concentration. If purified virus is used it may be diluted upto 1/10 with 0.9 % NaCl. If crude sap is used, the infected leaves should be homogenized in proper buffer or in 1.8 % NaCl and the sap filtered before use.

8.3.4.1 Staining for photography: If it is necessary for photograph to make the precipitin lines more visible by staining with 0.1 M DOPA (3-4 dihydroxy phenyl-DL alanine). DOPA is prepared by dissolving 1.97 g DOPA in 100 ml of 0.1 M potassium phosphate buffer pH 7.4. The stain is applied to the surface of the agar gel above the precipitin line, by saturating a piece of chromatographic paper. Stain diffuses into the gel from the paper. Allow the lines to stain for 6 to 12 hr. During this period they could over-stain and then they may be destained to the required colour intensity by successive washing in distilled water.

8.3.4.2 Interpretation of results: Interpretation of results are based on a balanced system of antigen and antibody concentration. The development of spurs, crossed lines or blocked lines of precipitation can occur when the concentration of one reagent is out of proportion to the other, and therefore can give false results.

In ODDT test different pattern of precipitaion lines can be expected. In Figure 8.1 different precipitation lines observable have been shown. Their explanation is as follows: (in Figure 8.1 (a). A straight line is present just in the middle between the two wells. This arrangement frequently occurs when unabsorbed antiserum is used which contains antibodies against "protein 1", a protein present in healthy as well as in diseases plants. The concentration of this protein in diseased plants may be higher than in healthy plants. (b). Lines nearer to the antigen well curved around it. This type is characteristic of viruses, diffusing slower than antibodies. (c). Lines nearer, or one line which splits into two are present. It may be that two components are present in the sap and also in the antiserum well. (d) A blurred appearance of the lines may be an indication that only one component

is present, but that two lines are formed because of an excess of antibodies. (e): Different lines, blurred here and there sharp, are visible. This also may occur because of an excess of antibodies. (f): Two lines cross. This occurs when the two antigen wells contain two distinct antigens when react with two different antibodies in the antiserum well. (g): Fusion of lines occur, which indicates that the antigen in the two wells combine with the same antibodies. (h): Partially the lines are fusing, but one line shows the formation of a spur. Such pattern occurs in cross-reaction with two viruses, and gives rise a relationship between these viruses.

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Chapter 9

ENZYME-LINKED IMMUNOSORBENT ASSAY

9.1 Introduction: The enzyme-linked immunosorbent assay (ELISA) is one of the most suitable serological techniques that can be used in routine tests without expensive equipment. The most commonly used enzyme is alkaline phosphatase (ALP) and the solid phase is polystyrene microtiter plates. Horseradish peroxidase (HRP) or penicillinase (PNC) enzymes are also used. The ELISA method is one of the most widely used serological tests, originally developed in 1976. The test is particularly useful for testing a large number of samples, such as for virus surveys and for resistance screening of large populations or accessions. The test differs from the classical serological methods in which immunoprecipitation reactions are used. Immunosepecific reactivity is recognized through the action of the associated enzyme label rather than observing the formation of an insoluble antigen-antibody complex. There are numerous variants of ELISA, but the two main categories of ELISA procedures, which are most commonly used in virology laboratories are direct ELISA and indirect ELISA.

In direct ELISA the wells of the microtiter plate are first coated with immunoglobulin (IgG) purified from the antiserum. The test sample is then added to the adsorbed antibody. The enzyme-labeled antibody is then added to the trapped virus. The attached enzyme subsequently digests an added enzyme substrate which results in a colour change. In this case the antigen is sandwiched between two gamaglobulins and that is why this ELISA is also called as "double antibody sandwich ELISA", and is designated as DAS-ELISA. It is highly strain specific. One disadvantage of DAS-ELISA is that a specific conjugated antibody is required for each virus to be detected.

In indirect ELISA (e.g. direct antigen coated ELISA. DAC-ELISA) procedure, the immobilized antigen is the target for unconjugated specific antibody. The trapped antibody is detected by a secondary antibody (produced in a second animal against the antibody of the first animal) conjugated to an enzyme, called universal conjugate (goat anti rabbit). The main advantage of indirect ELISA is that one

single conjugate can be used with all systems. This type of ELISA is particularly suitable for virus detection in disease survey and for testing the presence of viruses in seeds. It is relatively more economical to perform than DAS-ELISA. Moreover, for the screening of a large number of field collected samples during surveys DAC-ELISA is more convenient and effective. DAC-ELISA is not highly specific, high background reactions are often encountered. In addition, it works well only in case of viruses that are present in plant tissues at high concentration.

9.2 Direct ELISA (DAS-ELISA): This ELISA procedure was first developed by Clark and Adams in 1977, and is still widely used with slight modifications. As already mentioned, DAS-ELISA is more specific as compared to DAC-ELISA, therefore it is mainly suitable to distinguish strains of a virus and to develop serological relationships among viruses. In particular, this test is not suitable for (a) virus detection in disease surveys (b) when adequate quantities of antisera are not available for gamaglobulin extraction and conjugation and (c) for probing a single antigen with several different antisera. To proceed for DAS-ELISA, first we should extract gamaglobulin (IgG) from crude antiserum.

9.2.1 Purification of IgG from crude antiserum: To conduct direct ELISA, immunoglobulins (IgG) for preparing conjugate and for coating plates are needed. Antibodies can be purified from crude antiserum with the following procedure:

Solutions required

1. Sodium sulfate (Na_2SO_4, anhydrous) 36 % solution. Dissolve 36 g Na_2SO_4 in 90 ml distilled water. Make volume up to 100 ml. Slight warming facilitates solubilization.
2. 18 % sodium sulfate (Na_2SO_4) solution (this can be prepared by mixing 36 % Na_2SO_4 with an equal volume of distilled water).

3. Phosphate buffer (PBS) with sodium azide (NaN_3). 1.4 g of Na_2HPO_4, 0.28 g of KH_2PO_4, 16 g NaCl, 0.4 g KCl, and 0.4 g NaN_3, dissolve in one litre distilled water and then make the volume up to 2 liters.

Extraction procedure

1. Combine 1 ml of crude antiserum with 1 ml of distilled water.
2. Add 2 ml of 36 % of Na_2SO_4 drop by drop to 2 ml antiserum water, stir this mixture and let it stand for 10 min at room temperature.
3. Centrifuge at 22° C for 15 min at 12000 g. Decant supernatant and save white precipitate.
4. Add 10 ml of 18 % Na_2SO_4, and vortex to mix the precipitate.
5. Again centrifuge at 2000 g for 10 min and save pellet, discard supernatant.
6. Repeat washing in 18 % Na_2SO_4, as above and save pellet after centrifugation (12000 g for 15 min).
7. Resuspend pellet in 1 ml of PBS (dilute 1:1 with distilled water and dialyze against one liter of 1 X PBS at 4° C changing buffer at 1 hr, 2 h and 16 hr.
8. Examine for visible precipitate, remove any precipitate if formed by centrifugation (12000 g, 15 min).
9. Estimate IgG concentration by absorbance at 280 nm in a spectrophotometer. Normally, preparation should be diluted as 1:10 for measurement. Optical density at $_{280}$ nm of 1.4 is considered to be equal to 1 mg/ml IgG. Concentration of IgG should be adjusted to 1 mg/ml.
10. Store purified IgGs in a refrigerator (4° C) in aliquot of 1 ml.

9.2.2 Conjugation of IgG with alkaline phosphatase enzyme: Two enzyme labels which are widely used for conjugation are alkaline phosphatase and horseradish peroxidase. Recently urase and penicillinase (B-lactamase) have also been used.

Procedure

1. In case of alkaline phosphatase (ALP) dilute IgG stock with 1 X PBS to produce 1 ml solution at a concentration of 1 mg/ml (A_{280}=1.4)
2. Add to it 2 mg of ALP, vortex gently, and then dialyze mixture against one liter 1 X PBS in a dialysis tube at 4° C. Change buffer at 1 hr, 2h, and 16 hr.
3. Measure volume of the dialyzed mixture and transfer it to a small glass tube.
4. While gently vortexing the mixture add 25 % glutaraldehyde to a final concentration of 0.02 % (i.e. add 8 ul of glutaraldelyde to a stock per ml of mixture). Vortex until homogenized.
5. Repeat dialysis as described at step 2.
6. When the dialysis is complete, remove the solution from the dialysis tube and store at 4° C. Alkaline phosphatase labeled IgG should not be frozen. Penicillinase labeled IgG can be stored at 70° C.

9.2.3 Conjugation of IgG with penicillinase enzyme: The penicillinase enzyme is more cheaper than alkaline phosphatase. The conjugation of this enzyme involves the following steps:

Material

1. Purified virus specific IgGs.
2. Phosphate buffer saline (PBS), make in 2 litre distilled water, adjust to pH 7.4.

Na_2HPO_4 :	2.38 g
KH_2PO_4 :	2.4 g
KCl:	0.4 g
Na Cl :	16 g

3. Dialysis bags.
4. Glutaraldehyde (25 % Sigma G 5882)
5. Penicillinase enzyme. Generally supplied as lyophilized powder from Sigma (P 0389) or from Hindustan Antibiotics Ltd. Pimpri, India. ELISA grade.

Procedure

1. Take IgG, 500 ug/ml (make appropriate dilution from stock solution) in a dialysis bag and add 250 ug of penicillinase. Solutions of IgG and penicillinase can be made at higher concentration and mixed to get 2 mg/ml and one mg/ml and mg/ml penicillinase.
2. Dialyze against PBS in a beaker at room temperature for 1 hr.
3. Transfer the dialysis bag into a beaker containing PBS with 0.06 % glutaraldehyde (mix 1 ml of 25 % Glutaraldehyde in 400 ml PBS to get 0.06 % Glutaraldehyde) and dialyze for 3-4 hr at room temperature.
4. Replace the buffer containing glutaraldehyde with 500 ml PBS containing sodium azide (0.02 %) and dialyze for 18 hr at 4° C with at least three changes of buffer at 1 hr interval (for each change replace with 500 ml PBS containing sodium azide).
5. Transfer the conjugate into a new glass or plastic vial and add bovine serum albumin at 5 mg/ml concentration. Do not freeze the conjugate. If stored properly the shelf life of conjugated globulin should exceed one year.

9.2.4 Double antibody sandwich ELISA (DAS-ELISA)

The following buffers must be prepared and stored at 4°C before starting direct DAS-ELISA.

1. Antibody coating buffer: Prepare in one liter distilled water

Sodium carbonate (Na_2CO_3) :	1.59 g
Sodium bicarbonate ($Na\ HCO_3$) :	2.93 g

First dissolve the chemicals in 900 ml distilled water, and check pH, it should be 9.6, make the volume up to one liter.

2. Virus (antigen) extraction buffer: (Also called virus buffer or conjugate buffer)

a. First prepare phosphate buffer saline (PBS) with 0.05 % Tween-20 (PBST) by dissolving the following salts in one litre distilled water.

Sodium chloride (NaCl) :	40 g
Potassium phosphate (KH_2PO_4, monobasic) :	2.0 g
Sodium phosphate (Na_2HPO_4, anhydrous dibasic) :	11.5 g
Potassium chloride (KCl) :	2.0 g
Sodium azide (NaN_3) :	2.0 g

First dissolve the chemicals in 800 ml distilled water, then make the volume up to one litre. It is called 5X PBS (stock solution).

b. Now take 200 ml 5 X PBS (stock solution) as prepared above and add to it

Polyvinylpyrrolidone (PVP: MW: 40,000):	20 g
Tween-20 (polyoxy ethylene Sorbitan monolaurate) :	0.5 ml/litre
Egg ovaalbumin :	2 g
Distilled water:	800 ml

Dissolve and make the volume up to one liter, adjust pH to 7.4.

Note: Extraction buffer is used both for grinding virus samples and for making conjugate solution.

3. ***Washing buffer (WB):*** In one liter distilled water

5 X PBS :	200 ml (prepared as above)
Distilled water:	800 ml
Tween - 20 :	1 ml

Stir well to mix. Store at room temperature.

*4. **Substrate buffer (SB):*** In one liter distilled water

Diethanolamine :	97 ml
Distilled water :	800 ml

Add slowly 97 ml diethanolamine to 800 ml distilled water on an electric stirrer. Add concentrated HCl drop by drop to adjust pH to 9.8, then make the volume up to one liter.

Note: Substrate buffer stored for a longer period, even in a refrigerator is not suitable. It is always better to use fresh buffer.

Material: ELISA plates, antiserum, micropipettes (10-100 ul and 100-1000 ul), 200 ul micropipette (fixed) or adjustable multichannel micropipette, ELISA reader, beakers, washing squeeze bottle, magnetic bars, electric stirrer, humidity chamber (plastic box with moist tissue papers), mortar and pestles.

Procedure

1. Add 200 ul of purified IgGs at concentration of 1 ug/ml (optimum concentration.of IgG can be determined by standardization trials, which may vary with each conjugate or IgG produced) to each well of ELISA plate with fixed volume micropipette.
2. Incubate antibody-coated plate at 37° C for 4-5 hr or over night at 4°C.
3. Wash plate three times, allowing 3 min soaking for each wash with washing buffer.
4. Add 200 ul antigen samples (crude plant or seed extracts, or purified virus preparation) prepared in virus extraction buffer (PBST containing 2 % PVP) to each well.
5. Incubate at 37° C for 4-5 hr or overnight at 4° C.
6. Wash plate as mentioned under step-3.
7. Add 200 ul alkaline phosphatase (ALP) labeled IgG diluted normally up to 1: 1000 in conjugate buffer pH 7.4.
8. Incubate at 37° C for 4-5 hr or overnight at 4° C.
9. Wash plate as before.
10. Prepare substrate solution by dissolving substrate tablets (1 mg of p-nitrophenyl phosphate/1 ml buffer) in 25 ml substrate buffer, pH 9.8. These tablets are available in 5 mg concentration and one tablet is adequate for each plate. Add 200 ul substrate solution in each well.
11. Incubate plate at room temperature for 1-2 hour. In case of positive reaction the substrate develops yellow colour (as a result of conversions of p-nitrophenyl phosphate to p-nitrophenol). Plates can be scored visually or if ELISA reader is available take absorbance reading at 405 nm in case of ALP. Test readings are considered positive if they were twice or more times greater than readings from healthy control plant extract.

The outlines of the DAC-ELISA are given in Figure-9.1.

9.2.5 DAC-ELISA: This is the simplest test of all ELISA procedures. Antigens prepared in 0.02 M carbonate buffer, pH 9.6, are used for coating ELISA plates. This is followed by the addition of crude antiserum (not purified IgG as in case of DAS-ELISA) with its optimal concentration. As majority of the virus antisera are produced

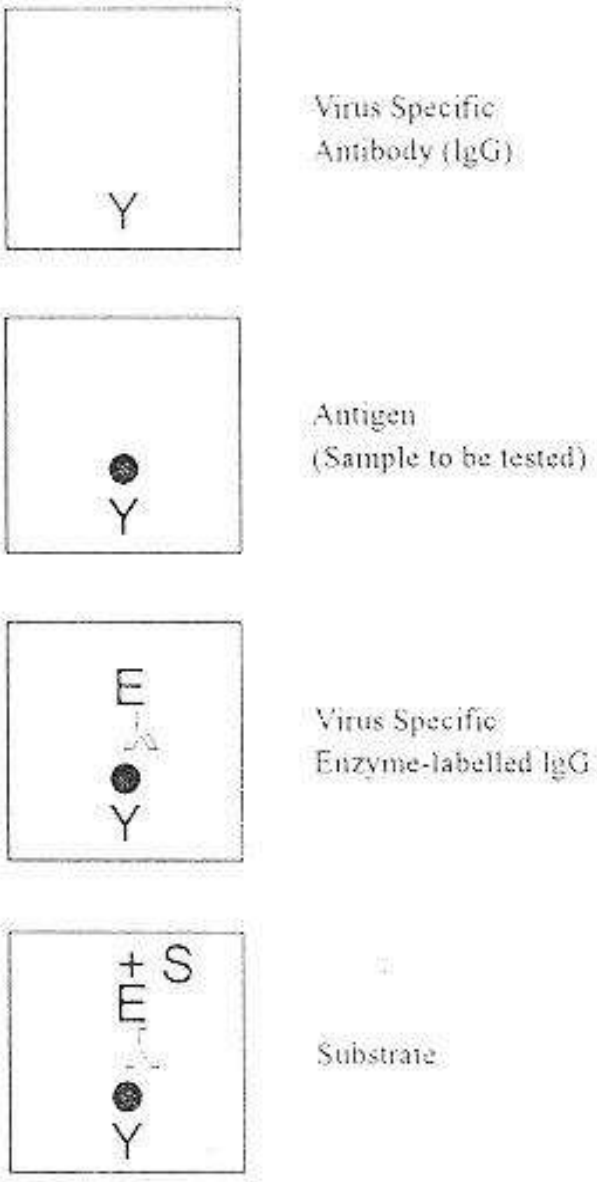

Figure 9.1: Outlines of double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Colour intensity in the final evaluation is proportional to virus concentration.

produced in rabbit therefore, the rabbit gamaglobulin attached to antigens are probed with enzyme labeled anti-rabbit gamaglobulins produced in goat. In place of gamaglobulins, the use of Protein-A conjugate is equally good as that of anti-rabbit conjugate.

Material

Same as listed under DAS-ELISA, the antigen buffer used in DAC-ELISA to extract sap from samples is prepared as follows. The other buffers are the same.

Carbonate buffer with DIECA, pH 9.6 : Prepare in one litre distilled water.

Sodium carbonate (Na_2CO_3)	1.59 g
Sodium bicarbonate ($Na\ HCO_3$)	2.93 g
Diethydithiocarbamate (sodium salt) Na-DIECA	1.71g

(0.01 M Conc) Store at 4°C.

Procedure

1. Prepare antigen (virus samples extracts) in 0.02 M carbonate buffer with pH 9.6 containing 0.01 M diethyldithiocarbamate (DIECA) while grinding in mortar with pestle. It is good to use antigen dilution of 1:50 or 1:100.
2. Using fixed volume micropipette add 200 ul antigen extract to each well of microtiter plate.
3. Incubate for 1-2 hr at 37° C or at 4° C overnight.
4. Wash plate with washing buffer as in case of DAS-ELISA.
5. Add 200 ul of cross-absorbed crude antiserum diluted in antibody buffer (also called conjugate buffer, the same used in DAS-ELISA). Antibody dilution used is 1:1000 or 1:5000 (or determined by standardization).
6. Incubate 1-2 hr at 37° C
7. Wash as above.
8. Add 200 ul goat-anti-rabbit conjugate diluted in antibody buffer, pH 7.2 (conjugate buffer) diluted 1:1000 (or other optimum as determined by testing)
9. Incubate at 37° C for 1-2 hr.
10. Wash plate as above.

11. Add the substrate p-nitrophenyl phosphate (PNPP) (similar to DAS-ELISA)
12. Keep the plate at room temperature and read plate after 15 min or 30 min at 405 nm

The outlines of DAC-ELISA are given in Figure 9.2

Note: This ELISA procedure is recommended for routine virus detection. The test can be completed within 3-4 hours.

9.2.5.1 Cross absorption: Sometimes the antiserum is not of good quality due to presence of antibodies against host plant components, and therefore such antiserum must be cross-absorbed before its use in DAC-ELISA. Cross absorption of crude antiserum is made with healthy plant extract. This can be achieved by grinding healthy leaves in conjugate buffer (antibody buffer) to give a 1:100 dilution, then filtering through cheese cloth. Prepare a suitable antiserum dilution in healthy plant extract suspended in conjugate buffer. Incubate at 37° C for 1 hr prior to adding it to microtiter plate. Cross absorbed antiserum substantially reduces non-specific reaction due to precipitation of antigens of plant origin.

9.3 Other variants of ELISA : In addition to DAS- and DAC-ELISA the other variants of ELISA are as follows:

9.3.1 Two step ELISA : Flegg and Clark (1979) developed a two step DAS-ELISA procedure in which samples and conjugate are mixed and incubated in one step. This eliminates one series of washing and long conjugate incubation steps. This ELISA procedure not only eliminates a long incubation step and a washing series from standard DAS-ELISA, but also increases sensitivity. The procedure for the two step ELISA is as follows:

Procedure

1. Add 200 ul aliquots of purified immunoglobulins (IgG) (with appropriate dilution, say 1000 X) diluted in carbonate buffer pH 9.6, to each well of microtiter plate. Cover the plate to prevent evaporation with plate cover or plastic envelope.

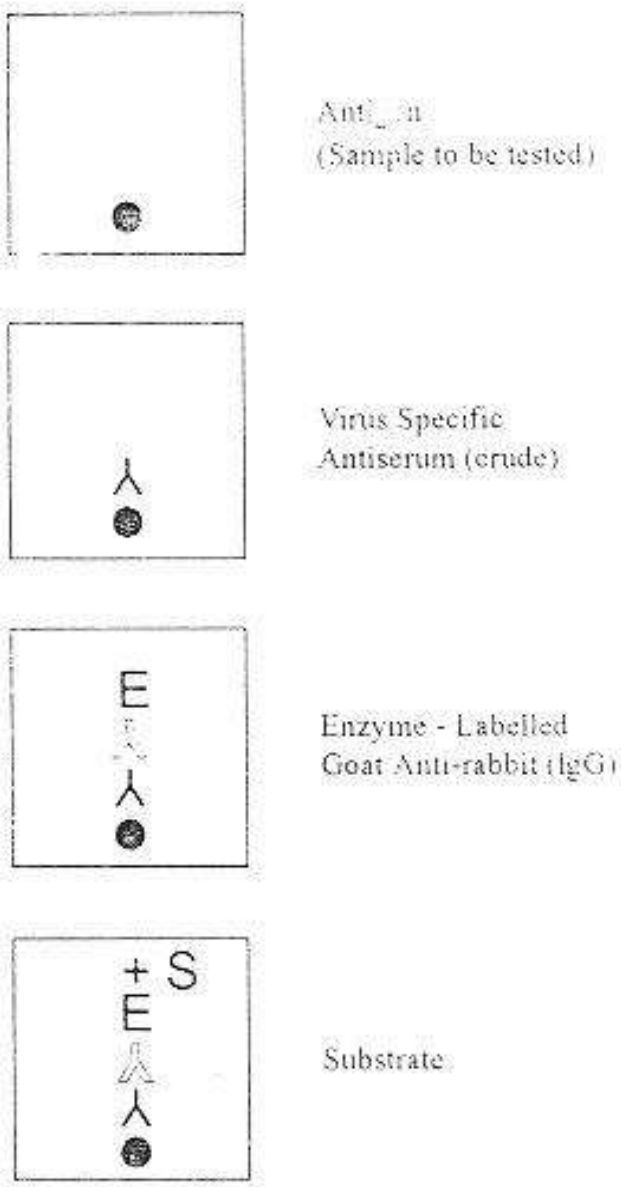

Figure 9.2: Outlines of the direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA).

2. Incubate at 37° C for 2 to 4 hr or at 4° C overnight.
3. Wash the plate with washing buffer (PBS-T) as in case of DAS-ELISA.
4. Add 100 ul aliquots of test samples (1:10 dilution) extracted (PBS-Tween+PVP+egg albumin) to duplicate wells, followed by 100 ul aliquots of specific conjugate (2 ug/ml) to each well. Agitate the plate to mix well contents thoroughly.
5. Cover the plate to prevent evaporation in humid chamber (plastic tray with moist tissue papers) at 37° C for 2-4 hr or at 4° C overnight.
6. Wash the plate with washing buffer.
7. Add 200 ul aliquots of appropriate dilution of enzyme substrate (PNPP) prepared in substrate buffer.
8. Incubate at room temperature for 1 hr or until colour is developed to desired intensity.
9. Stop reaction (if necessary) with 50 ul of appropriate stop solution (3N Na OH).
10. Measure absorbance at 405 nm with automatic ELISA reader take observations visually.

9.3.2 Protein-A coated ELISA (PAC-ELISA): The DAS-ELISA procedure has two disadvantages (a) it is highly strain specific and (b) it requires the preparation of a different enzyme-conjugate IgG for each virus to be tested. To avoid this, several indirect forms of ELISA have been developed in which the enzyme conjugate is an antispecies of IgG or Protein A.

The Protein A has a great affinity to bind FC portion of antibody. In Protein A coating ELISA (PAC-ELISA) procedure, first plate is coated with a solution of Protein A followed by specific antibody treatment. The Protein A binds to the immunoglobulins (IgG) at the FC region. The bound antigen is then detected by another layer of antibodies, which are in turn detected by enzyme-conjugated Protein A. This form of ELISA has been successfully used to detect small fruit viruses and to detect groundnut viruses.

Procedure

1. Prepare an appropriate dilution (1 ug/ml) of Protein A (Sigma Product No P 6650 in carbonate buffer, pH 9.6, and add 200 ul aliquots to each well of microtiter plate.
2. Incubate at 37° C for 2 hr or at 4° C overnight.
3. Wash plate with washing buffer.
4. Add 200 ul aliquots of cross-absorbed polyclonal antiserum (1:1000) prepared in antibody buffer pH 7.4 (PBST+PVP+ egg albumin).
5. Incubate at 37° C for 2h.
6. Wash plate with washing buffer.
7. Add 200 ul aliquots of test samples prepared in antibody buffer (1:10 w/v) in duplicate wells.
8. Wash plate as before.
9. Repeat step 4 (i.e. 200 ul cross-absorbed polyclonal antiserum dilution again)
10. Incubate at 37° for 2 hr.
11. Wash plate.
12. Add 200 ul aliquots of Protein A-alkaline phosphatase conjugate (Sigma Product No P 9650) prepared in antibody buffer with pH 7.4, at an appropriate dilution (1:1000).
13. Incubate at 37° C for 2 hr.
14. Wash plate.
15. Add 200 ul aliquots of p-nitropheny phosphate substrate solution (1 mg/ml) prepared in substrate solution.
16. Incubate plate at room temperature for 30 min and take reading at A 405 nm.

The outlines for PAC-ELISA are given in Figure 9.3.

9.4 Dot immunobinding assay (DIBA): Basically this serological test is similar to that of the plate DAC-ELISA. Nitrocellulose or nylon based membranes are used to immobilize the antigens. As these membranes have a high affinity for proteins, therefore it is essential to block the free-protein binding sites of the membrane. This step is called *"blocking"*. Blocking is done with bovine serum albumin, gelatin or non-fat dry milk. Non-fat dry milk is preferred because it is less expensive, easily available and gives equally

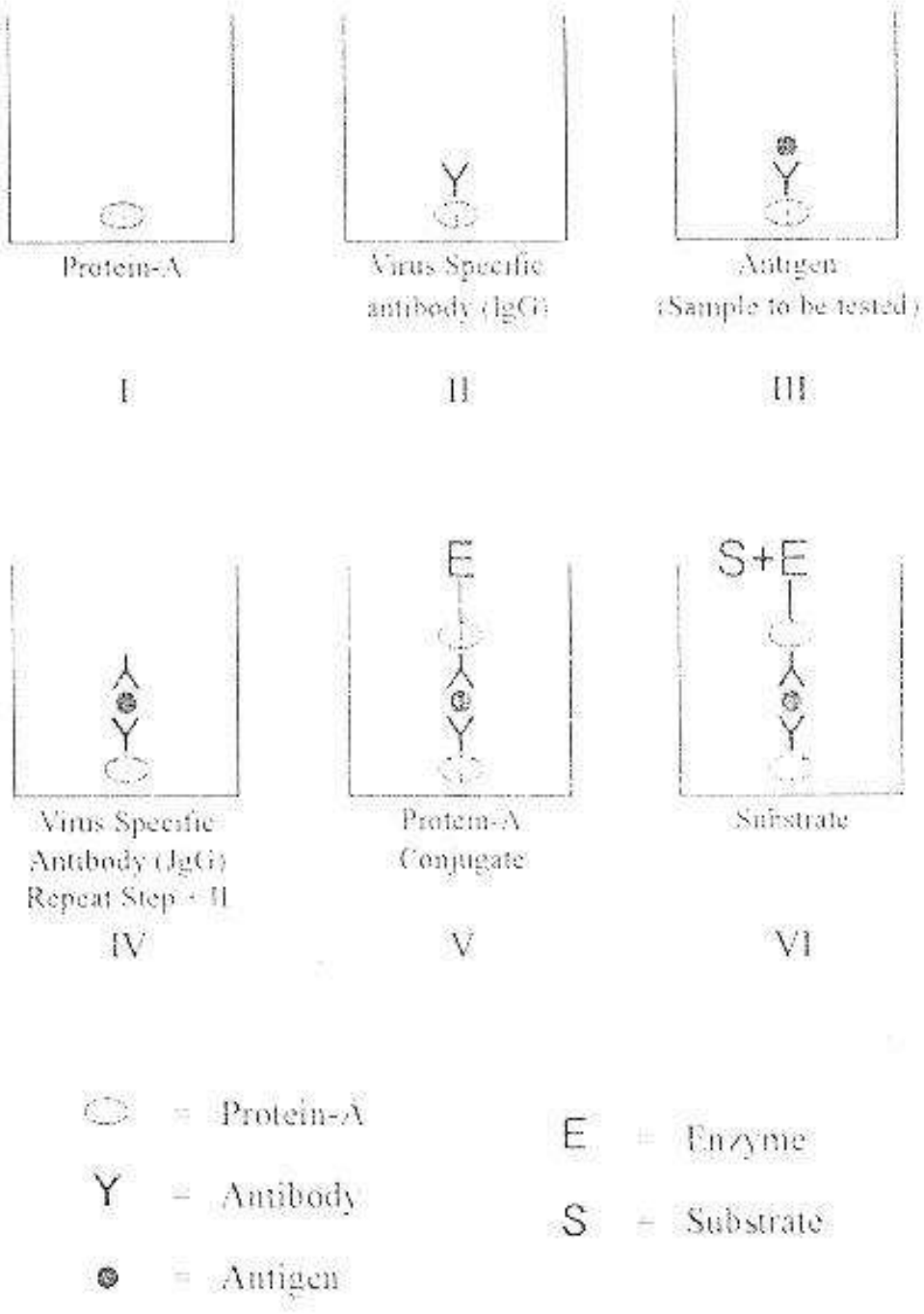

Figure 9.3: Outlines of the protein A coating enzyme-linked immunosorbent assay (PAC-ELISA).

good results as other blocking agents. The immobilized antigen is then exposed to solutions of unconjugated virus specific antibody. Mostly crude antiserum is used. Trapped antibody is detected with alkaline phosphatase (ALP) or horseradish peroxidase (HRP)-labeled protein A, anti-Fc, or anti-IgG. ALP is preferred because it is easier to prepare conjugates with ALP than HRP. For ALP, naphthol phosphate is used as a substrate, followed by the addition of a diazonium salt such as Fast Blue RR or Fast Red RR. Naphthol phosphate in the presence of ALP is converted to phosphoric acid and naphthol. Naphthol is detected by adding diazonium salt, which together form an insoluble product which can be detected visually.

9.4.1 Advantages of DIBA over plate ELISA

1. It permits virus detection in extremely small volumes such as in insect extracts.
2. It is more suitable for detection of viruses in samples during disease surveys due to its simplicity.
3. Membranes are easier to process than ELISA plates.

Materials needed: Nitrocellulose membranes, antiserum, micropipette, glass dish (approx. 10 x 12 cm) or disposable square plastic petri dishes (9.5 x 9.5 cm), shaker, blunt ends forceps.

Solutions required: Keep ready the following buffers before start. Store buffer at 4°C.

1. Coating buffer, pH 9.6

Sodium carbonate (Na_2CO_3)	1.59 g
Sodium bicarbonate ($NaHCO_3$)	2.93 g

Dissolve the above salts in 900 ml distilled water adjust pH to 9.6, make the volume to one liter.

2. Tris-buffered saline (TBS)

Tris	4.84 g
NaCl	58.48 g

Dissolve these salts in 1.9 liter distilled water, adjust pH to 7.5 and make up the volume to 2 liters. It is useful to make a 5X concentration stock solution and dilute this when required.

3. TBS-Tween (TBST)

TBS : 1 litre
Tween-20 : 0.5 ml
Mix Tween-20 in one liter TBS

4. Blocking solution

TBS : 1 litre
Non-fat dry milk powder: 10 g

5. Antibody buffer:

TBS : 1 litre
Non fat dry milk powder: 10 g

6. Alkaline phosphatase labeled goat anti-rabbit IgG or Fc-specific dilute in antibody buffer. Make appropriate dilution such as 1 : 1000.

7. ***Solution A*** : Add 50 mg of naphthol phosphate AS-B1: 20 ml of dimethyl formamide to 20 ml distilled water. Adjust pH to 8.00 with 0.1 M Na_2CO_3.

8. Substrate solution: Fast Red or Fast Blue RR salt, 50 mg; Tris HCl (0.2 M, pH 8.3), 18 ml; distilled water, 13 ml; solution A, 2 ml. Substrate solution should be prepared fresh each time and filtered through any ordinary filter paper (Whatman No.3).

9. Washing buffer: Prepare in 50 ml PBST

PBST :	50 ml
Bovine serum albumin :	1% (0.5 g)
Non-fat dry milk :	4 % (2 g)

First dissolve the reagents, then make the volume up to 50 ml, store at 4^{o} C.

Procedure: Always wear gloves when handling nitrocellulose or nylon membranes. A rotary shaker is needed to perform various steps in the procedure. As nitrocellulose membranes are fragile therefore, these should be handled with blunt tipped forceps. This procedure involves the following steps:

1. Prepare appropriate dilution of antigen (leaves, seeds, or insects etc.) in carbonate coating buffer. Usually 10^{-1} to 10^{-5} (10 to 5000 at 10-fold intervals) are used. Extract from healthy tissue should serve as control.
2. With the help of a micropipette apply gently one or 2 ul antigen dilution onto membrane to avoid excess spreading.
3. Air dry the membrane at least for 15 minutes.
4. Transfer membrane to a plastic or glass dish containing blocking solution so that the membrane is fully emersed.
5. Shake the membrane in blocking solution at room temperature for 1 hour.
6. Grind healthy plant tissue to make 1: 20 dilution in antibody buffer (1 g in 19 ml buffer). Filter through cheese cloth. Now use this healthy sap to make appropriate dilution of virus-specific antiserum. Normally a dilution of 1:250 or 1:500 (0.05 ml serum diluted in 12.5 ml (1/250) or 0.05 ml diluted in 25.0 ml (1/500) After incubating the diluted antiserum at 37° C for 45 minutes, it is now ready to use.
7. Remove the membrane from blocking solution and transfer to diluted antiserum. Shake at room temperature for 1 hr.
8. Pour off antibody solution. Wash the membrane in TBS-Tween milk powder thrice, shaking 5 minutes during each wash.
9. Dilute the alkaline phosphatase conjugate (goat antirabbit IgG or FC) to a dilution of 1:500 (0.05 ml in 25 ml buffer in antibody buffer and add to dish containing membrane).
10. Shake at room temperature for 1 hr.
11. Pour off conjugate solution, wash the membrane as in step 8.
12. Add substrate solution and shake till colour develops (15 -30 min are sufficient). Do not allow excess colour development.
13. Pour off the substrate solution and wash membrane in distilled water.
14. Dry the membrane with paper towels and record the results.

9.5 Tissue print ELISA: It is a form of DIBA where tissue is directly applied to membranes without extraction. This technique has been applied to cucumoviruses, luteoviruses, potexviruses, potyviruses and tospoviruses. In some cases it was shown to be more sensitive than that of plate ELISA. Background reaction due to pigments from tissue may interfere and detergents such as Triton-X or blocking agents such as sodium hypochloride are recommended to remove pigments. Once the tissue is applied to the membrane it can be stored for several weeks. Therefore, this procedure is ideal to be used during virus disease surveys.

Procedure

1. Harvest tissue to be tested, prepare thin tight roll, cut the tissue with sharp razor blade to obtain a single cut surface.
2. Immediately press the cut surface on to nitrocellulose membrane.
3. The rest of the procedure is the same as described under DIBA.

Chapter 10

ELECTRON MICROSCOPY

10.1 Introduction: Transmission electron microscope (TEM, Figure 10.1) is frequently used in plant virus research to detect virus in crude sap extract, because virus particles are only visible under the electron microscope. The visual observation of virus-like particles in crude sap extract indicates viral infection, and may even allow the plant virologist to assign them tentatively to a known virus group on the basis of particle morphology. Although some authors have been able to detect isometric viruses in leaf dips, the technique has usually been applied to the detection of elongated viruses. Electron microscopy (EM) of viruses may be carried out on purified preparation, which is sometimes essential to study virus structure in detail. Examination of crude sap preparation provides quickly information on virus morphology. Due to the high resolution power of the TEM, virus particles, protein and nucleic acid molecules can be easily seen under the electron microscope. Magnifications of an object up to 80,000 or more can be obtained by TEM.

10.2 Procedure for particle examination: Specimens which are to be observed under EM should be first mounted on a rigid support. Usually copper grids (3 mm in diameter) containing a number of apertures e.g. 60-160 mesh/cm onto which a thin film of plastic e.g. formvar or carbon is placed to mount samples. Such support films should be transparent to electrons, but strong enough to withstand irradiation.

10.3 Preparation of support films for negative staining: A variety of methods are used to prepare support film for attachment to electron microscopic grids. Such films are prepared from parlodion or formvar using very simple techniques.

10.3.1 Collodion or parlodion: These are brands of purified pyroxylines (nitrocellulose). Solid or liquid, powder, chip or sheet,

Figure 10.1: Transmission electron microscope (Model JEOL TEM 100 CX 11 Japan) used for electron microscopy.

the plastic is diluted or dissolved in absolute amylacetate at the rate of 0.25-1.0g per 100 ml, depending on thickness of support film desired.

10.3.2 Formvar: This is a polyvinyl form, solid or powder to dissolve in 1, 2-dichloroethane (ethylene dichloride) or absolute chloroform at the rate of 0.25 to 0.5g per 100 ml. Solution of ethylene dichloride tends to give films with greater likelihood of defects due to its greater tendency to mix and dissolve atmospheric moisture. These solvents are volatile, must be kept tightly sealed, when stored.

10.3.3 Procedure for preparing plastic support films: The plastic support films are prepared as follows (Figure 10.2).

1. Prepare 0.4 % solution of formvar in ethylene dichloride in a beaker covered tightly with aluminum foil, and stir slowly overnight on a magnetic stirrer. This concentration can be further diluted with solvent to have desired thickness of film.
2. Dip two-third of a new clean glass microscopic slide in a beaker, containing formvar solution. Drain excess solution from slide onto a paper towel or blotter and allow slide to completely air dry (usually 2-3 minutes).
3. Fill a bowl or tray with distilled water. Clean dust from the water surface by placing a tissue paper on water at one side of the bowl and drawing it across the water so that entire water surface is swept clean.
4. Score the edges of the plastic coated slide with a razor blade so that the film will be free to float off on the water surface.
5. Breath on the film, the moisture and heat will expand the film, breaking the seal between the film and the glass, allowing easier stripping of the film. Lower the slide slowly into the water, letting film float off on the water surface. Film should be uniformly gray colour. If the texture of the film is not uniform, discard the film and make another one.
6. Place grids with their bright side against the formvar film. Leave a 2.0 cm margin at one end of the film to aid in picking up the film.

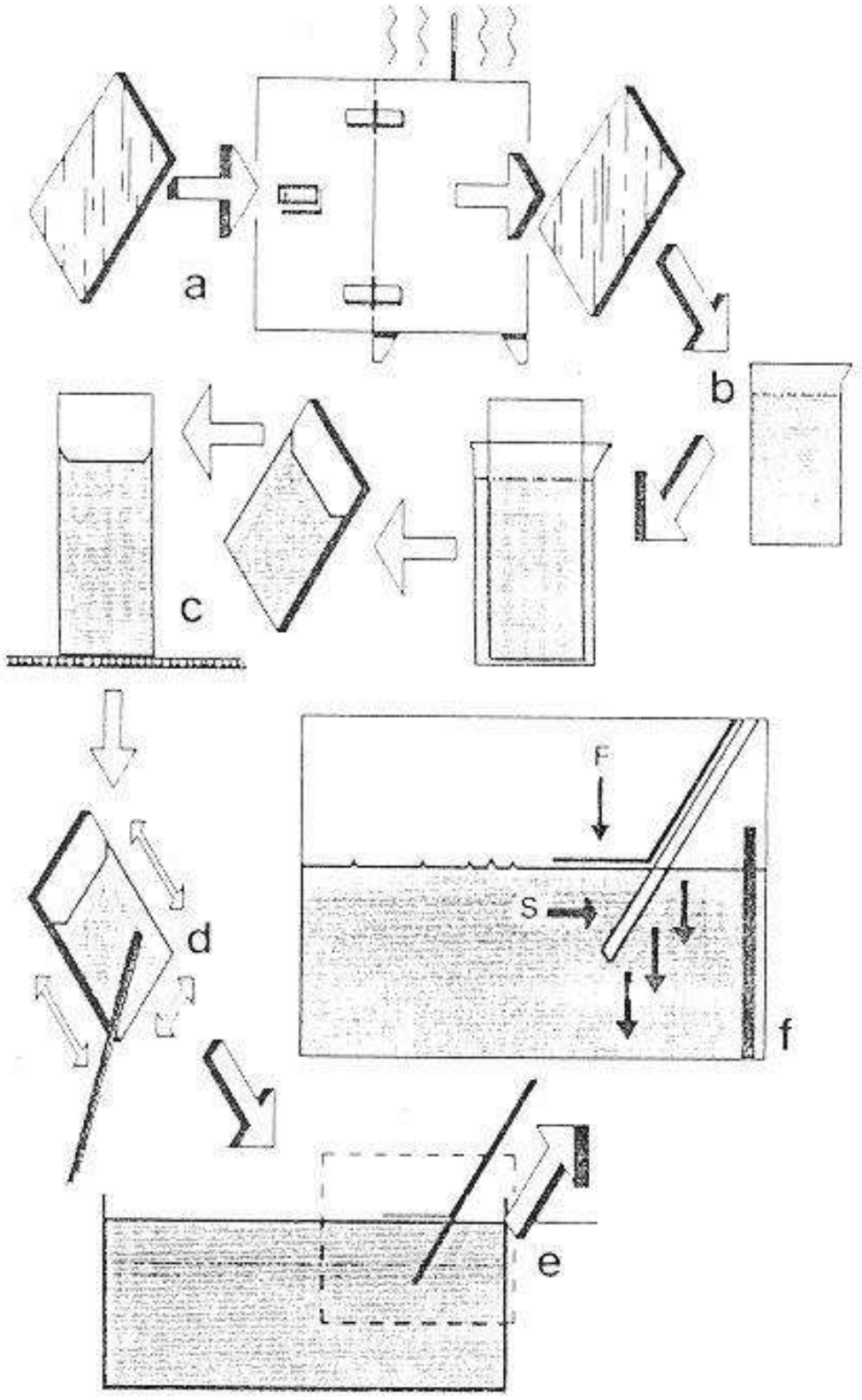

Figure 10.2: Step-by-step procedure for the production of Formvar substrates for electron microscope specimen grids.

7. Pick up the grids and the film on a clear slide by pushing down on the film with the end of the slide and allowing the film to fold back on both sides of the slide. Use a smooth consistently forward "bombing run" motion.
8. Allow slide to completely air dry in a dust free environment before removing grids from the slide with a forceps.

10.3.4 Preparation of carbon support films: Carbon support films are made by vacuum evaporation of spectrographically pure carbon rods or by sputtering pure carbon from carbon electrode in vacuum. To get carbon evaporate, it must be extremely hot (over 4000^{O} C), and this is achieved in a special machine (Figure 10.3) made for such purpose. Carbon films are easily casted on water from freshly cleaved mica sheets. The procedure is as follows:

1. Split a mica sheet length wise by pushing of two pairs of forceps into an end of the sheet and putting them gently apart. Avoid touching the freshly exposed mica surface.
2. Put mica, freshly cleaved surface up, in an evaporation or sputtering device. Set up carbon source, close the system, get proper vacuum, and evaporate or sputter carbon. Recover carbon coated mica sheet from the vacuum chamber.
3 Place a 5 cm of fine mesh screening in a container of distilled water. The screening should be about 2-3 cm below the water surface. Place grids to be filmed with carbon, bright side up on the screen.
4. Breath on the carbon film. The moisture and heat will expand the film, breaking its seal on the mica, and allowing easier stripping of the film. Lower the mica slowly into the water letting the carbon film float off on the water surface. A film should be uniform, gray in colour and have smooth texture.
5. Position the carbon film over the grids by blowing it or pushing it gently with the tips of the forceps. When it is properly positioned, slowly and gently drain water from the container.

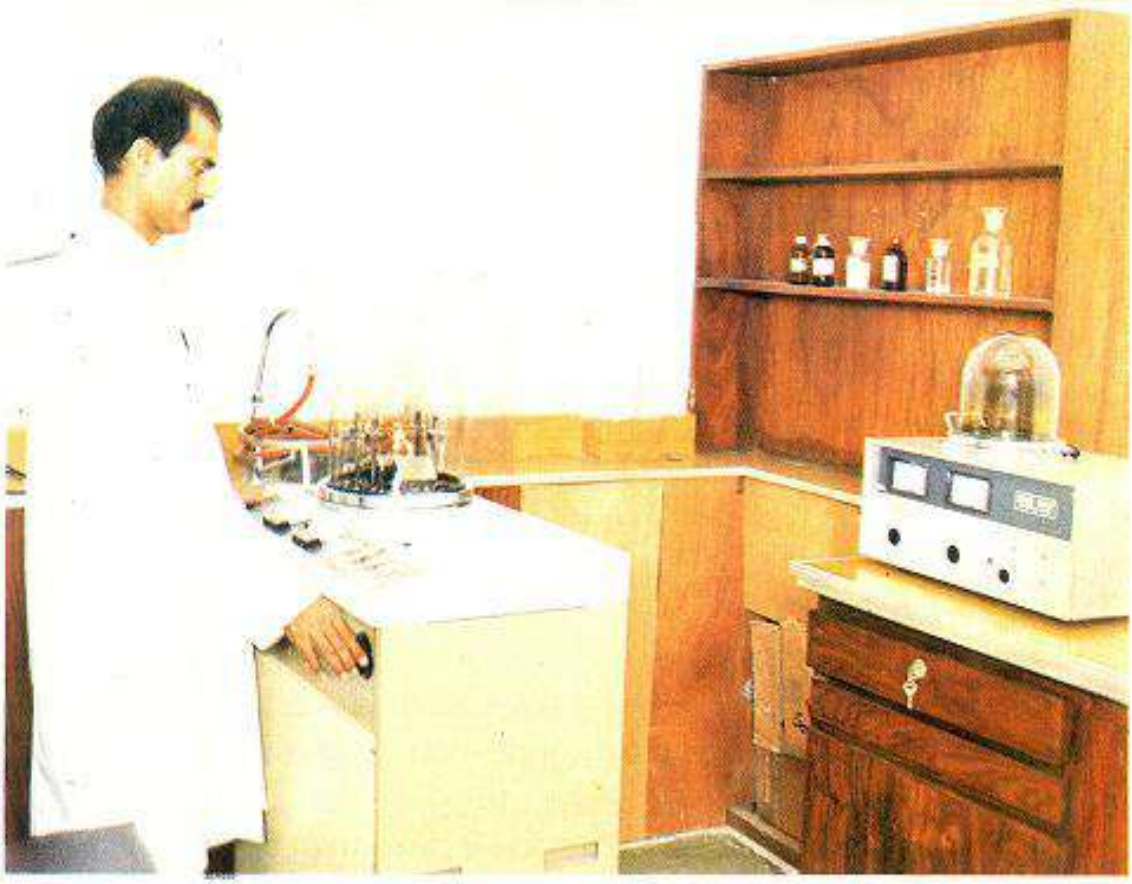

Figure 10.3: A special machine to get carbon evaporation to coat electron microscopic grids.

6. Allow water to completely air dry from grids and screen at room temperature and dust free environment. Remove grids from screen with a forceps. Store grids in a grid box or on clean filter paper in a closed petridish.

10.4 Negative staining: This is the most widely used technique for EM. The basic principle of this technique is that the visibility of particles of low scattering power can be increased by surrounding rather than impregnating them with dense material. Mostly phosphotungstic acid (PTA: 1 to 5 %, pH 6.5 to 7.5), uranyl acetate (UA: 2 %, at pH 4.8) and ammonium molybdate (AM: 1 to 3 %, pH 6-8) stains are used to surround the virus particles. These stains provide good contrast to see the virus particles. Negative staining is more simple, rapid and gives more resolution of the material than obtained by other methods. It can tolerate more impurity than other methods, because PTA penetrates most of the contaminant molecules and makes then invisible.

Procedure

1. Place grids, filmed side up, on a taped microscope slide. Place one drop of specimen suspension on a grid using a small micropipette or a syringe. Allow particles to settle out of suspension within an appropriate length of time (2-3 min.).
2. Gently blot off the liquid away with a filter paper. Leave the surface of the grid wet.
3. Place one drop of any negative stain on the grid. This drop should be about equal in size to the drop of specimen suspension originally applied.
4. Immediately blot the grid with a fresh clean triangle of filter paper. Use another triangle of filter paper to pass under the grid in case any fluid has leaked beneath the grid. Grid is now ready for observation.

10.5 Preparations of negative stains: A negative stain should give high contrast and high resolution while supporting the particles against flattering. It should be easy to prepare and use. The procedure

for the preparation of commonly used negative stains is outlined below:

10.5.1 Phosphotungstate (PTA): Prepare 2% aqueous neutral PTA solution. It is stable, easy to use and is tolerant to various buffers or the presence of sap in the preparation. When used as an acid, at a pH values of 3 to 5, a 2% PTA gives low contrast with low resolution, but it causes little or no damage to labile specimens. When the pH is brought to 7.0 with KOH, it gains high contrast but destroys ribosomes, damages host membranes, and destroys a significant number of plant viruses, unless these are first fixed with glutaraldehyde.

10.5.2 Uranyl acetate (UA): It is a poisonous and slightly radioactive stain, therefore, it should be handled with care. It is not commonly used, because it precipitates in the presence of even small amount of sap constituents, phosphate or buffers above pH 5.5. Care should be taken to avoid contamination of the foreceps holding the grids. UA gives a stain of high contrast and fine grain. Majority of the viruses are not damaged by this stain except rhabdoviruses. A 1% aqueous solution of UA with pH 4 to 5 is a good routine stain.

10.5.3 Ammonium molybdade (AM): It is a stain which is easy to use and does not cause damage to virus particles. The problem of this stain is that it does not give good contrast. A 2% aqueous solution, pH 5.5 can be adjusted with HCl or ammonia below 4 and 9. Most often pH 4.0 is the best. The stain is stable and can be mixed with or added to preparations without previous washing.

10.6 Positive staining: Positive staining differs from negative staining in that the goal is to density-stain particles and examine them against a transparent background. Particles appear dark against a bright light background by electron microscopy. Positive staining solutions are aqueous solutions of salts of heavy metals which generally have the same properties as negative staining substances. Positive staining tends to be more cumbersome than negative staining in that more time and

processing is required for the sample preparation before the results are seen by microscopy. Positive staining is less commonly used technique for particle studies, although results from positive staining are also useful. The following positive stains are used: phosphotungstic acid (3-5%, pH 6.5-7.5), uranyl format (1%, pH 4.00-5.5), uranyl sulfate (1-3%, pH 5.5-6.0) and osmium tetraoxide (2%, pH 6.5-7.0).

Procedure

1. To avoid staining residue in the back ground, it is best to positive stain particles before applying them onto grids. Stain particles as dispersions in vials or centrifuge tubes. How long to stain, must be determined empirically: 5-10 min., 60 min., or overnight time frames are generally used.
2. Completely separate stain solution from particles by centrifugation, filtration, dialysis, or other suitable techniques. Repeat washing to assure complete removal of all unincorporated stain. When washing is complete, concentrate particles to an appropriate density.
3. Apply particles onto grids and examine under the EM.

10.7. Examination of virus particles: The virus particles are examined under EM either in crude virus infected plant sap or in purified virus preparation. It is better to examine virus particles in crude plant sap as the virus particles do not rupture and break, and represent true particle length as compared to purified virus preparation.

10.7.1 Leaf dip preparation: In this method the virus particles are observed in crude sap extract. It does not extract particles very efficiently, but it does so rapidly, without breakage of virus particles, which often happens in purified virus preparation. Essentially, a drop of negative stain such as PTA (2%, pH 6.5) is placed on a carbon coated grid, and the freshly cut edge of a virus infected leaf tissue is touched with the drop of stain. The drop is then drained with the help of a blotting paper and allowed to dry. The grid is now ready to observe under EM. PTA sometimes causes rupture and disappearance of virus

particles. To avoid harmful effect of PTA, fixation for 20 min in 10% formaldehyde stabilizes most of virus particles.

10.7.2 Purified virus preparation: The purified virus preparation is first diluted to 1:10 or 1:100 in a proper buffer (phosphate, citrate, borate buffer etc.). Take a grid and place it on a microscopic slide. Place a drop of virus dilution on the grid. After a minute drain off the excess liquid with the help of a piece of triangular filter paper. Now put the grid on the filter paper in a petridish. Put a drop of 2% PTA or other proper stain and eventually a small drop of 0.03% egg albumin to a small drop of the virus solution. After a minute, drain the excess liquid with filter paper. The grid is ready to observe under the EM.

10.8 Measurement of virus particles: The size of the particle is a distinctive character of plant viruses. It is therefore, essential to measure the particle size when characterizing a new virus. The size of particles may vary with a homogenous preparation of the same virus, especially for elongated viruses due to several reasons e.g. breakage during purification and other factors which may influence the particle size such as presence of Mg^{++} ions. So at least 100 particles or more must be measured, preferably in crude preparation if possible, where particle breakage is less likely to occur than in purified preparations. Magnification of not less than X 200,000 are required for accurate measurement of particles, and the method and chemicals used to prepare virus preparation for EM examination should be given when a new virus is described.

Rod shaped viruses may vary in particle length, but the length of most particles of each virus is within a narrow range. The mean length of the 100 particles within such range is called *"normal length"*. Measurement is usually done with a binocular microscope using a micrometer eyepiece or with a ruler if projected on a screen or wall or if viewed in photographic prints. Sizes are usually classed in intervals of 10 or 25 mm. The data are presented in the form of a histogram, and the overall particle length is quoted as either the modal length (i.e. the value that occurs most frequently) or the arithmetic mean.

The histogram in Figure 10.4 represents the length distribution of blackeye cowpea mosaic (BlCMV, PI-26B) and cowpea aphid-borne mosaic virus (CABMV, RN-7C) particles measured on a photograph with a magnification 30000 X . The figure shows a maximum between 700 to 800 nm, and a small number of particles with shorter lengths which are unspecified. The mean maximum is used for calculation of normal length which is 725 nm and 742 nm in case of BlCMV and CABMV respectively. Usually only particles considered for calculation are those which exceed the level of unspecified lengths. In this example the particles of 500 to 900 nm were used. The modal length of the particles was determined to be 725 nm and 742 nm respectively.

10.9 Immunosorbent electron microscopy (ISEM):

Immunosorbent electron microscopy (ISEM) involves a combination of electron microscopy and serology. This technique was first used by Larson *et al* (1950) and is still widely used with various modifications by the plant virologists for virus identification. This rapid and sensitive technique offers a diagnostic procedure based on two properties of the virus, serological reactivity with the antiserum used and particle morphology. This technique has the great advantage of being applicable to tissue homogenates and of requiring very low quantities of virus and antiserum. The sensitivity of ISEM is generally comparable to that of ELISA, and it may detect 0.1-10 ug/ml of virus in volumes of a few ul. Different authors have used different names for this method such as serological specific electron microscopy (SSEM), immunosorbent electron microscopy (ISEM), solid phase immune electron microscopy (SIEM) and electron microscope serology (EMS). In these techniques virus and antiserum are reacted together and the results are viewed under the electron microscope. Three main methods that combine serology and electron microscopy are discussed as follows:

10.9.1 Clumping: In this method, virus-containing sap is mixed with antiserum and a grid is touched to the mixture. The grid is washed

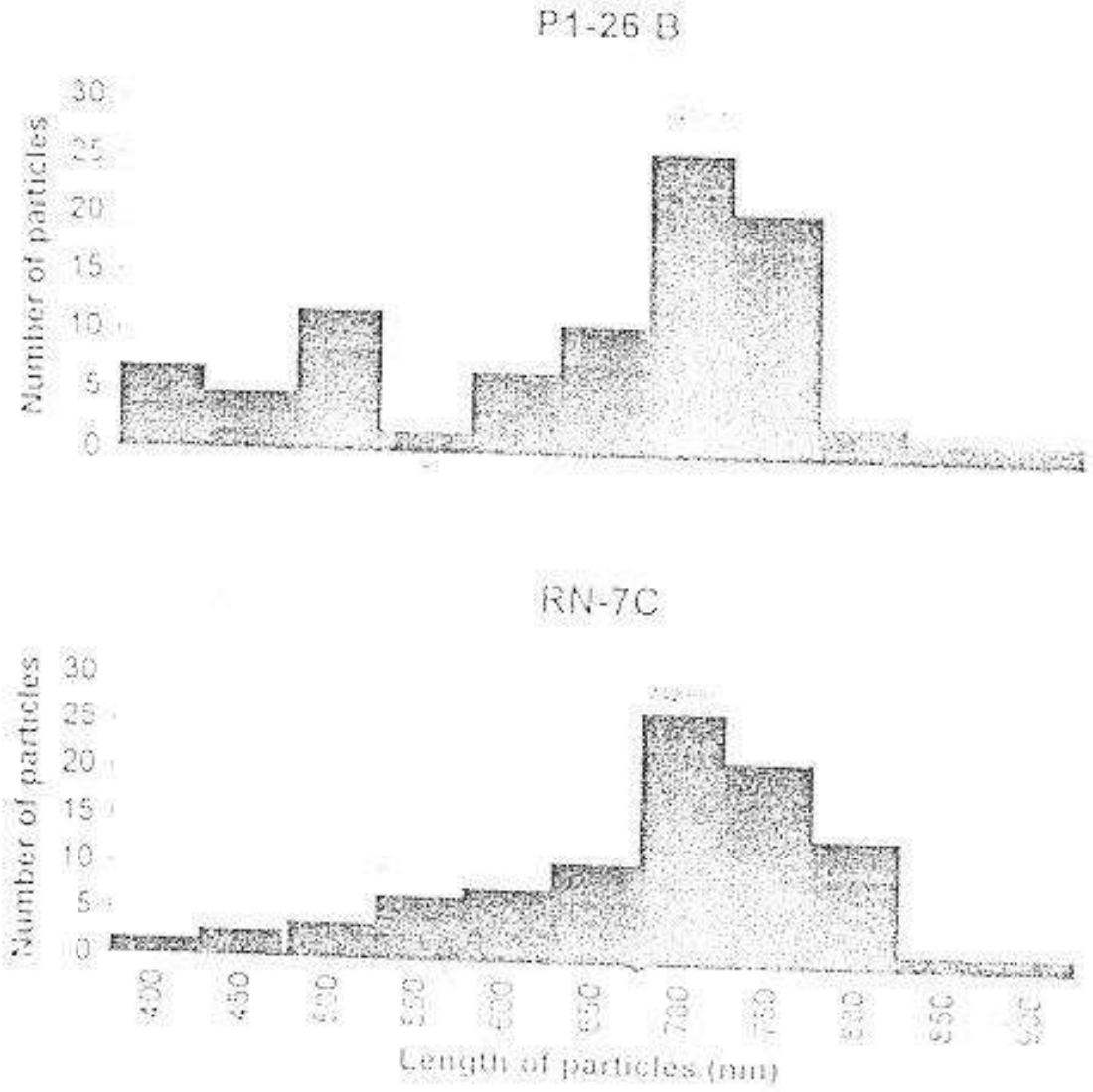

Figure 10.4: Histogram of particle lengths of BICMV isolate PI-26B with modal length of 725 nm and of CABMV isolate RN-7C with modal length of 742 nm.

and then negatively stained (Figure 10.5). This method produces clumps (aggregates) of virus particles and also an approximately ten fold increase of particles on the grid. Virus particles not reacting with the antiserum are not coated with antibody molecules. This method is used for the sensitive detection and identification of virus particles.

Material needed

1. Clean glass petridish or waxed petridish.
2. Parafilm.
3. Filter papers.
4. Carbon coated copper grids.
5. Adjustable micropipetts.
6. Negative stain (e.g. 2 % PTA pH 6.8 or 2 % AM, pH 4.0).
7. Antiserum, diluted in phosphate buffer, but not less than 1/16,dilution should be made freshly each time they are used.
8. Virus sap preparation should be extracted from virus infected leaf tissue in a mortar and pestle and let it settle for 1 hr to remove plant debris.
9. Fine tip foreceps.

Procedure

1. Take 40 ul of antiserum onto squares on a waxed petridish (or onto parafilm)
2. Add 20 ul of diluted virus preparation to each antiserum drop.
3. Mix drops by carefully drawing up and expelling out liquid from the micropipette.
4. Cover petridish with lid to avoid evaporation and incubate either at 37^{o} C for 2-3 hr or at 4^{o} C overnight. The former gives rapid results for heat stable viruses. Shorter incubation will speed up the test.
5. After incubation touch surface of the drop with carbon filmed grids (coated side down)
6. Remove immediately and wash with 10 drops of stain (2 % PTA, pH 6.8) and observe under the EM.

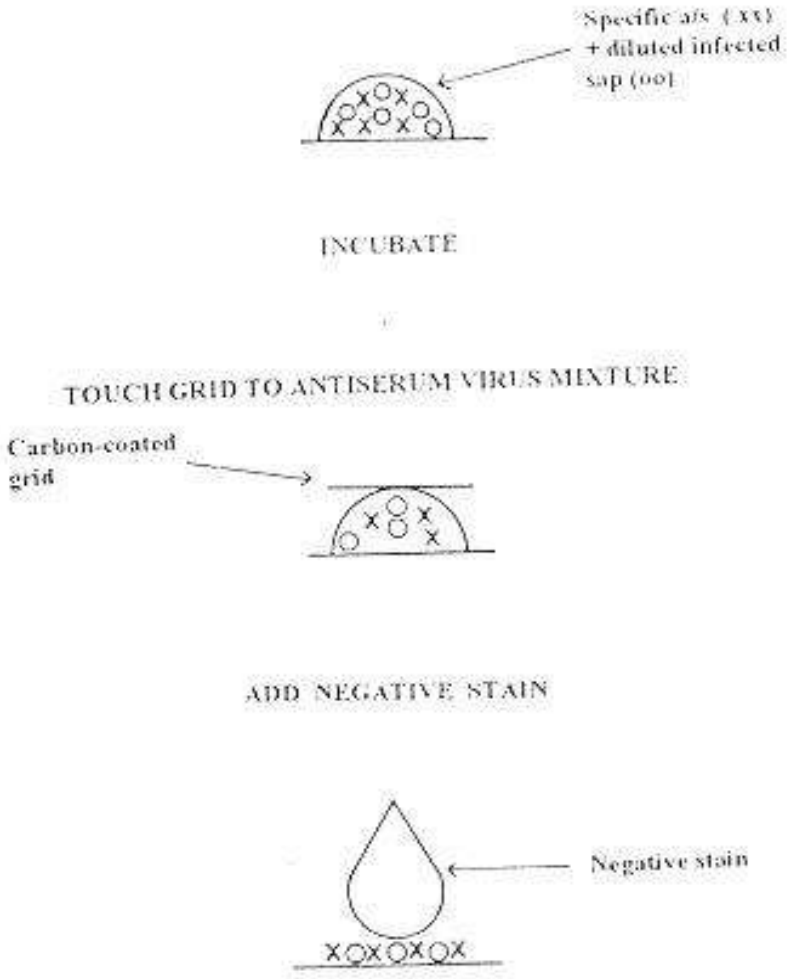

Figure 10.5: Immunosorbent electron microscopy: clumping procedure (Hill, S.A. 1984).

10.9.2 Decoration: Decoration method has become a popular method of identifying plant viruses, mainly because the result is direct. Of all serological methods it is one of the most easily and safely interpreted because we can see the virus particles attached to antibodies. This method involves the decoration or coating of virus particles already on the grids with a layer of specific antiserum. It is most frequently used for the specific confirmation of the serological identity of unknown viruses or separating morphologically identical viruses in mixtures.

In this method the virus is detected by *"decoration"*. Virus containing extract is applied to carbon coated grid (Figure 10.6). The grid is treated with antiserum which produces a clump of antibody molecules around the virus particles that can be visualized by negative staining. This procedure offers a convincing demonstration of specific combination between virus and its antibodies. Viruses devoid of decoration are not antigenic to the serum used. This technique allows viruses in mixtures of similar shaped and sized particles to be distinguished.

Materials needed: The material needed for this method is the same as is mentioned under clumping method, except that crude antiserum or purified immunoglobulins (IgG) may be used. For diagnostic purposes dilution between 1/10 and 1/100 are used.

Procedure

1. Prepare virus extract from virus infected leaf tissue by grinding in proper buffer (0.06 M phosphate buffer, pH 6.5 - 7.00) in a mortar with pestle. Pour the extract in a test tube and let it settle for one hour at 4^{o} C.
2. Take the sap on the bottom of petridish in the form of a drop, and touch carbon coated grid to a drop of extract for a few seconds, then raise the grid with 20 drops of phosphate buffer, and then drain (but do not dry).
3. Float grids (with virus) support film downwards on diluted antiserum drops in a waxed petridish (or on parafilm).
4. Incubate for 15 min at room temperature in the petridish covered with lid to avoid evaporation.

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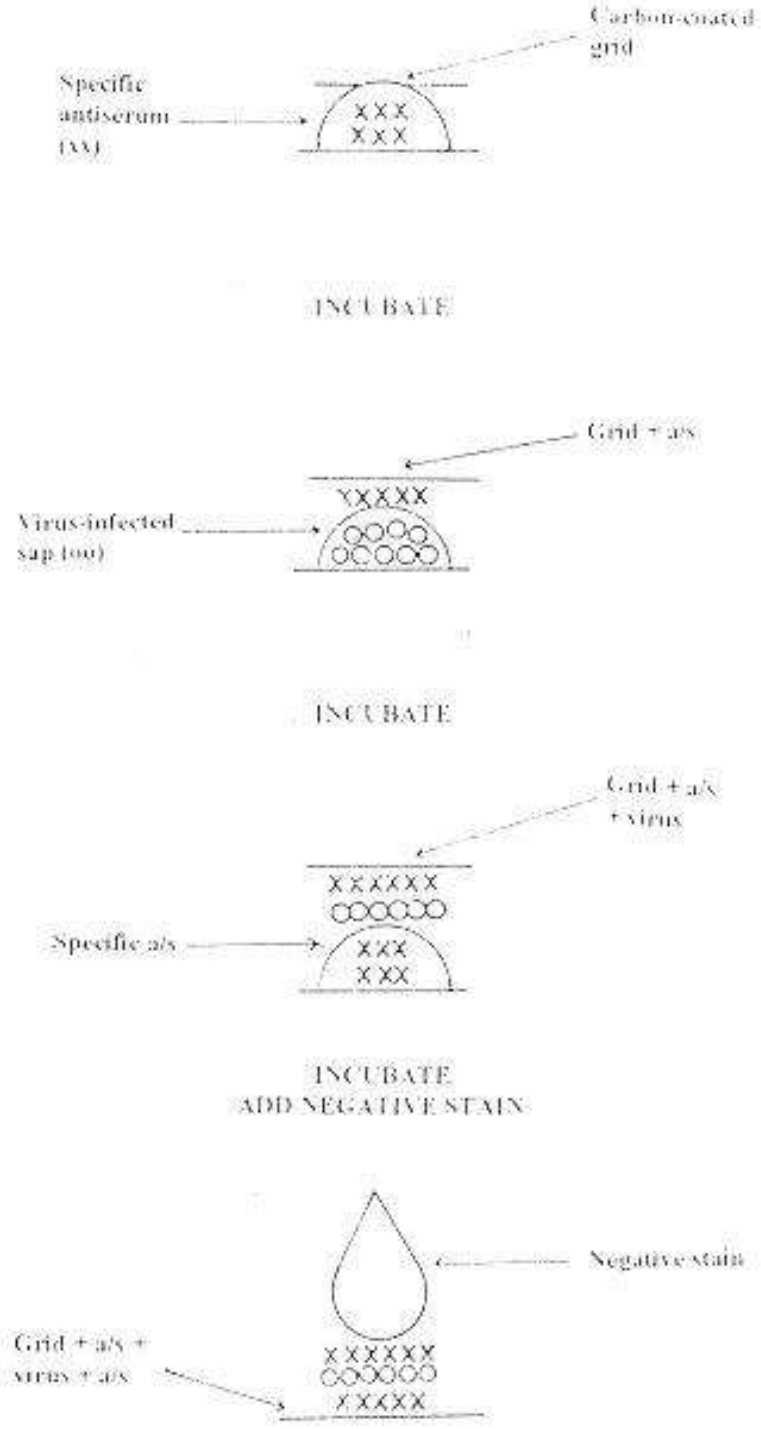

Figure 10.6: Immunosorbent electron microscopy: decoration procedure (Hill, S.A. 1984).

5. Wash the grids with 10 drops of distilled water in squeeze bottle.
6. Stain the grids with six drops of an appropriate stain (e.g. PTA 2 %, pH 6.8 or AM 2 % pH 4.0).
7. Drain and allow to dry and examine under the electron microscope.

10.9.3 Specific trapping: In this method the grids are first coated with specific antiserum (Figure 10.7). The antiserum coated grids are then placed on drops of virus containing purified suspension or crude sap extract. The trapped virus particles are detected by negative staining. This method is usually used to detect viruses present in host plant sap in concentration too low to permit detection in conventional EM preparations. This method can result in 1000 fold or more increase in sensitivity over conventional EM in detecting viruses. It is the most sensitive method of all serological tests. Results are quick (1-4 hours) and use only small volumes of antiserum. This technique can be used with mixed antiserum (to trap more than one viruses at a time) and can be used to study serological relationships between virus strains, since the efficiency of trapping is related to the degree of homology between antiserum and virus.

Materials needed

1. Clean glass petriplates.
2. Parafilm or parafilm wax.
3. Carbon coated grids.
4. Filter papers.
5. 0.06 M phosphate buffer, pH 6.5-7.0.
6. Adjustable micropipettes (5-100 ul).
7. Negative stain e.g. 2 % PTA, pH 6.8 or 2 % AM, pH 4.0.
8. Antiserum (try to use antiserum which does not contain glycerol as a preservative as it reduces staining). Freshly prepared 0.06 M phosphate buffer is suitable for diluting antiserum. For rod shaped viruses antiserum should be diluted to 1/1000 and for spherical viruses to 1/2000.

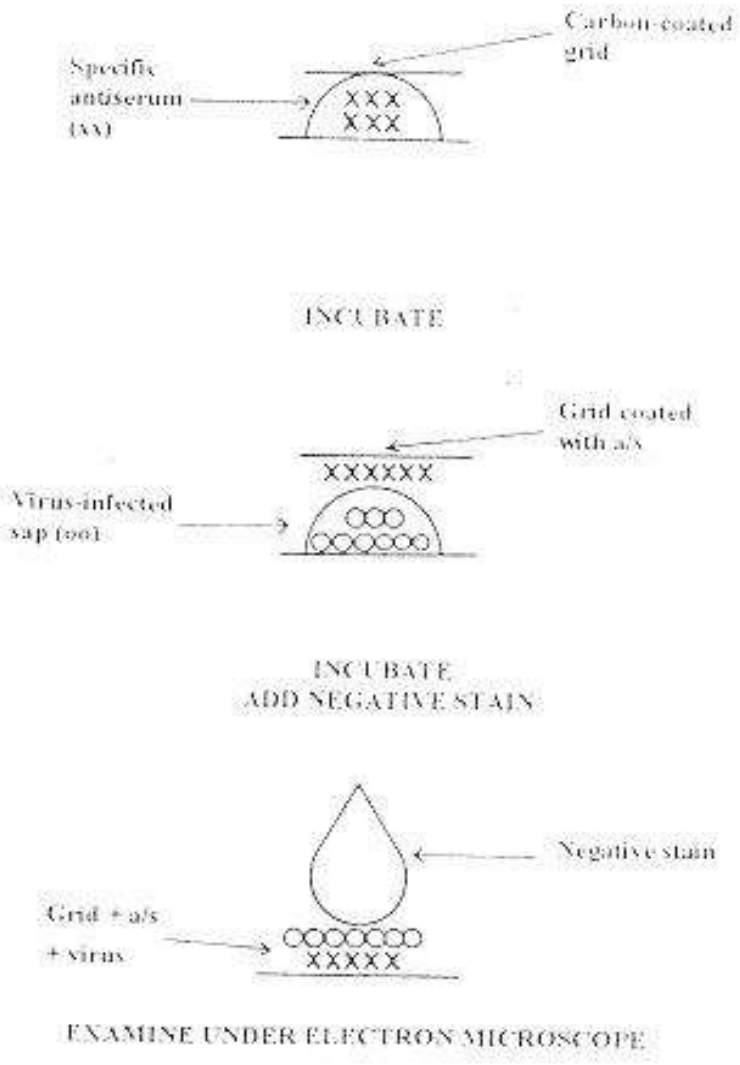

Figure 10.7: Immunosorbent electron microscopy: specific trapping (Hill, S.A. 1984).

9. Virus preparation either from purified virus or from virus infected leaf tissue. Grind the tissue in proper buffer in mortar with pestle. Let it settle in a test tube at 4° C or 1 hr before use.
10. Fine tip forecep.

Procedure

1. Place parafilm on the base of the petridish or coat with a thin layer of paraflim wax.
2. Draw 1 cm squares on the petri plates if different preparations are to be examined.
3. Pipette separate 20 ul drops of diluted antiserum onto base of petridish.
4. Carefully place a carbon coated grid on each drop so that it floats on the drop.
5. Cover petri plate with lid to prevent evaporation and incubate at 37° C for 15-30 min.
6. Wash grids by flooding drop wise with buffer from a squeeze bottle, holding grid with forceps to remove excess antiserum.
7. Float each antiserum coated grid, coated side downwards on a 20 ul drop of virus extract on parafilm or on wax surface of a petriplate.
8. Incubate grids plus virus in a covered petriplate for 3-36 hr at 4° C or at room temperature for 1-5 hr.
9. After incubation drain and blot the grid to dry, and stain the grid with proper stain by placing face downwards on one drop of stain. Drain and blot after 30 seconds.
10. Now the grid is ready to observe under the EM.

Note: For routine tests an increase in incubation time from 3-36 hr may give a five fold to ten fold increase in the amount of virus trapped. Incubation at lower temperature (e.g. 4° C for 3-36 hr) gives more uniformity of trapping and avoids evaporation of the extract. Incubation at room temperature gives more rapid reaction.

Chapter 11

MONOCLONAL ANTIBODIES IN VIRUS DETECTION

11.1 Introduction: Monoclonal antibodies (McAbs) have emerged as important tools in diagnostic work of plant pathogens including viruses, bacteria, fungi, spiroplasma and mycoplasma-like organisms (MLO). Since 1981, McAbs have been produced against more than 100 plant viruses. McAbs are now available for many viruses of economically important crops. These reagents are specially valuable for detecting viruses that can only be purified with difficulty and for which adequate supply of polyclonal antisera have been difficult to obtain.

When McAbs are produced for diagnostic work, it is important that they should detect the widest range of viral serotypes. This can be achieved by collecting as many serotypes of the virus as possible and selecting those McAbs that recognize the largest number of serotypes. Although McAbs specific for a single virus strain can be readily obtained, it is possible to select for McAbs that recognize a wide range of different virus species within a plant virus group or genus.

Monoclonal antibodies are used not only for the detection and identification of plant pathogens but also for characterization and analysis of variants and strains of the pathogens for studying structure and their gene products.

11.2 Advantages of monoclonal antibodies

1. An unlimited quantity of antibody can be produced from a small quantity of antigen.
2. Pure antibodies specific for single epitope can be obtained, even when antigenic mixture is used as immunogen.
3. Hybridomas can be preserved by freezing in liquid nitrogen for continuous supply of antibody over time.
4. The use of monoclonal antibodies eliminates the qualitative and quantitative variability in specific antibody content in different batches of polyclonal antiserum.

11.3 Disadvantages of monoclonal antibodies

1. The production and characterization of a collection of monoclonal antibodies for strain and epitope specificity can take over a year.
2. This technology is expensive and needs more sophisticated facilities than conventional production of antiserum.
3. Screening and cloning of hybridomas is a laborious and time consuming process.

11.4 Basic requirements for the production of monoclonal antibodies: Hybridoma production requires various major and minor equipment and cell culture wares. In order to produce hybridoma it is better to have a specific laboratory to obtain good results. The following equipment should be near the laboratory and easily accessible.

11.4.1 Equipment

1. Vertical laminar air flow hood with ultraviolet light.
2. Humidified, temperature controlled carbon dioxide (CO_2) incubator.
3. Inverted microscope.
4. Bench top clinical centrifuge.
5. Deep freezer (- 80° C and - 20° C).
6. Autoclave.
7. Liquid nitrogen container.
8. Haemocytometer.
9. Adjustable multichannel pipettes (8-, 12-channel, 10-400 ul)
10. Adjustable micropiptte (0-10 ul, 20-100 ul, 200-1000 ul).
11. Filtering apparatus.
12. Refrigerator.
13. Sucking pump for pipettes.
14. Oven (400° C)
15. Microplate reader.
16. Spectrophotometer.
17. Cell counter.
18. Mouse strain BALB/C

11.4.2 Cell culture wares

1. 96-, 24-, and 6-well flat bottom sterile cell culture plates with cover.
2. 96-well flat bottom microtiter plates.
3. 1-, 5-, and 10-ml pipettes.
4. Cryopreservation tubes.
5. 5-, 15-, and 50-ml sterile screw-cap centrifuge tubes.
6. Protective gloves.
7. Glass petriplates (small, medium and large size)
8. Sterile glass and plastic syringes with needles (1 ml, 5 ml, 10 ml, and 20 ml).
9. 16, 20, 23, 25, and 27 gauge syringes and surgical scissors.
10. Small and large size forceps.
11. Glass bottle with screw-cap (100 ml, 250 ml and 500 ml).
12. Centrifuge tubes (glass and plastic).

11.5 Materials and media: The hybridoma not only requires special attention but also quality reagents, as it involves the growth of vulnerable cells that are sensitive to extremes in cell densities requiring continuous monitoring and nursing of cells. All preparations should be made with tissue culture grade, deionized, glass double distilled water under extreme sterile conditions.

11.5.1 Media: The most commonly used media for hybridoma production are Iscove,s Modified Dulbeco,s Medium (IMDM) and Royal Park Memorial Institute (RPMI) medium. Both media are usually buffered with carbonate/bicarbonate. Both media are equally good. Media are commercially available both in liquid and powdered form, but the later is preferred because of longer shelf life. It is advisable to use media that are less than three months old. Prepared media can also be stored at 4° C in the dark to prevent the production of highly toxic photo products.

11.5.2 Antibiotics: During the process of monoclonal antibody (McAb) production the most common cell contaminants are bacteria, yeast and fungi. Fungi are more dangerous than others. In order to

avoid bacterial contamination penicillin and streptomycin (Gibco: 100 X, 10000 units/ml) is used. Gentamycin (100 ug/ml) is a broad spectrum antibiotic. Fungizone (Amphoterin) at 5-10 ug/ml is useful against fungal contamination.

11.5.3 Sera: Fetal bovine serum (FBS) or new-borne calf serum is used nearly in all hybridoma work. The serum can be kept at -20° C at least for 1-2 years. For use, the FBS is thawed at room temperature then stored at 4° C.

11.6. Preparation of media and working solutions: It must be ensured that all the equipment used is in sterile conditions before starting to prepare media and other solutions.

11.6.1 IMDM medium

1. Take two bags of IMDM medium (Iscove,s Modified Dulbeco,s Medium) and dissolve in one liter distilled water in two liter capacity beaker (each packet of IMDM medium weighs 17.7 g powder, and is stored at 4° C under dry conditions).
2. Add 6.02 g of sodium bicarbonate and dissolve while stirring with magnet bar.
3. Add 20 ml Penicillin-Streptomycin (it is kept frozen, thaw before use).
4. Add 20 ml Amphotericin (it is an antibiotic).
5. Make up the volume two liters with distilled water.
6. Filter the two liter solution through a special filter paper under laminar air flow hood. Collect 500 ml IMDM medium in each sterile bottle
7. Add 2.5 ul 2-Mercaptoethanol (2-ME) in each bottle containing 500 ml IMDM medium. This can be added as follows: first take 5 ul 2-ME in a sterile petriplate containing, add 1 ml IMDM medium, then by pipette add 0.5 ml of this solution in each of the two bottles containing 500 ml IMDM medium, store at 4° C.

11.6.2 HAT medium

1. Take 20 ml FCS (fetal calf serum) in a sterile bottle.
2. Add 4 ml of HAT liquid.
3. Make the volume up to 200 ml while adding IMDM medium containing 2-Mercaptoethanol.

11.6.3 HT medium

1. Take 4 ml of HT liquid (it is kept at -80° C, thaw before use).
2. Add 20 ml FCS (fetal calf serum) to 4 ml HT.
3. Make the volume up to 200 ml while adding IMDM medium containing 2-Mercaptoethanol to the above mixture.
4. Store at 4° C.

11.6.4 Normal medium

1. Take 20 ml FCS in a sterile bottle.
2. Add 180 ml IMDM medium containing 2-ME.
3. Store at 4° C. This medium is used to grow myeloma cells for fusion.

11.6.5 Complete culture medium

Dulbeco Modified Eagle Medium (DMEM), high-glucose formula (4.5 g glucose/liter, GIBCO # 430-2100) supplemented to the indicated concentration with the following additives:

2.8 g/l sodium bicarbonate (33.3 mM)
4.8 g/liter HEPES (20 mM)
10% fetal calf serum (vol/vol)
10 ml/ liter L-glutamine (2 mM)
10 ml/liter sodium pyruvate (1 mM)
10 ml/liter penicilline (50 IU/ml) and streptomycin (50 ug/ml)

The last four additives are available as 100X solutions from GIBCO and other major suppliers of cell culture media. Penicilline and streptomycin are combined in one solution. Store at 4° C.

11.6.6 Polyethylene glycol (PEG) solution: Take 1 g of PEG 4000 (PEG should be very fine grade, e.g. gas chromatographic grade (Merck), and add 1 ml double distilled water. Autoclave in the morning

and keep it at room temperature till it is used in the afternoon. Just before use add 100 ul dimethylsulfoxide (DMSO) $(CH_3)_2$ SO_2: MW:78.13), thoroughly mix with pipette.

11.7. Myeloma cell lines: The choice of animal species as immune spleen donor depends mainly on myeloma cell line to be used for fusion. Mouse and rat lines are readily available, with mice as the most commonly species used. The BALB/C strain of mice (Figure 11.1) is preferred for monoclonal antibody production. Other inbred strains are used when low immune response is encountered in BALB/C. The LOU/C and LOU/M strains of rat are recommended when using rat-derived myelomas.

Myeloma cell lines are mutant deficient in either the enzyme hypoxanthene-guanine phosphoribosyl transferase (HGPRT) or thymidine kinase (TK). Such cells die in the presence of aminopterin, which blocks the main DNA (deoxyribose nucleic acid) synthesis pathway (called Salvage Pathway). Selection of antibody-secreting-hybridomas is possible, because myeloma-spleen cell hybrids can survive in aminopterin when hypoxanthene and thymidine (components of HAT medium) are present for use by the Salvage Pathway.

Several cell lines currently in use yield high frequencies of hybridomas include P3/NS1/1.Ag.4.1(NS), P3X63/AG.663(P3), P3.X63Ag.Ag8.3UI and SP2/O/G14.(Sp2). All these cell lines have been used successfully in Plant Pathology. Rat myeloma cell lines include YB2/0 and IR983/F. Rats are good to produce a large volume of ascitic fluid, but their myeloma efficiency is lower than murine myeloma. Most of the mouse and rat myelomas are available from different companies such as Flow, Gibco and ATCC.

Myeloma cell lines are normally maintained in 1 ml aliquot in cryotubes in liquid nitrogen. Freezing is accomplished by pelliting a 24 hr culture grown in normal medium containg 20% FCS, resuspending in fresh normal medium with 20% FCS and 10% DMSO (dimethyl sulfoxude) at a concentration of 3×10^6 cells/ml, and distributing into cryotube (Nunc Plastic, 1 cc/vial).

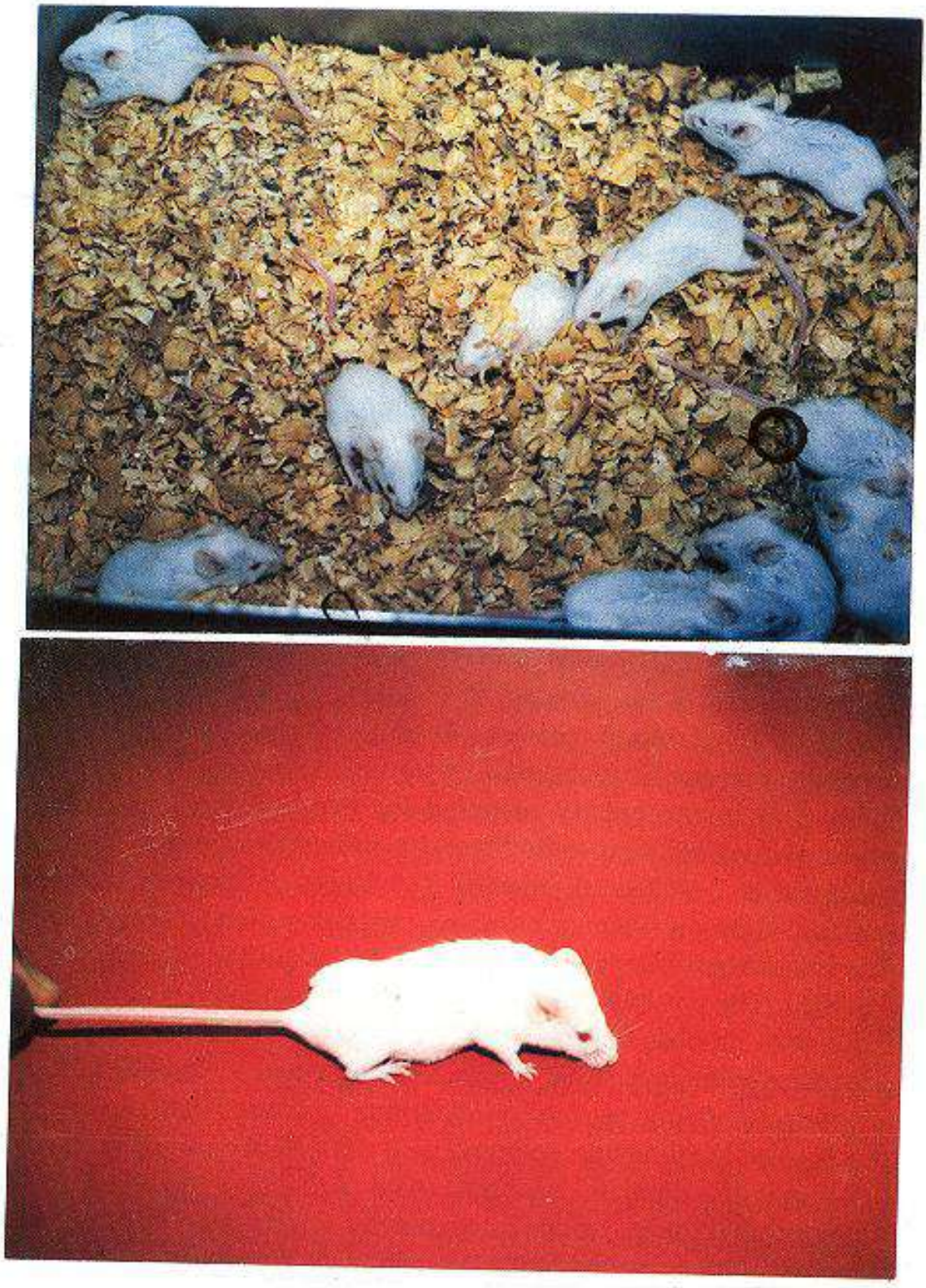

Figure 11.1: Mice BALB/C strain commonly used for the production of monoclonal antibodies.

11.8 Procedure for production of hybridoma cells: For the production of cell clones secreting antibodies, spleen cells of mice immunized with antigen i.e. plant pathogen (viruses, bacteria or fungi) to be investigated are fused with mouse myeloma cells. The important element in the production of McAbs includes the immune state of the spleen donor animal, the ability to prepare and fuse myeloma and spleen cells and obtain viable hybrids to clone and cryopreserve cells, and preservance through hundreds of hours over a period of 3-6 months.

11.8.1 Preparation of antigen: Before immunizing a mouse, one must have antigen in hand. For example the virus against which monoclonal antibodies are required, is first to be purified from the infected plant tissue to inject into the mouse. The protocols for purification of different viruses have been described in Chapter 7.

11.8.2 Immunization of mouse: The immune state of the animal from which the spleen is taken is very important in determining the success of a hybridoma project. A protocol that works well for a membrane proteins (e.g. bacteria) may not necessarily be good with soluble protein. Antigen is prepared for injection by emulsifying an antigen (virus) solution with Freund's complete adjuvant. Mice are immunized at 2 to 3 week interval. The bleeds are collected 3-7 days after each booster immunization to monitor serum antibody levels. Mice are chosen for hybridoma fusion when a sufficient antibody titer is reached. The protocol for immunization is as follows:

1. Inject 4-5 mouse (strain BALB/C), 8-10 weeks old with 20-100 ug purified virus in PBS homogenized with equal volume of Freund,s complete Adjuvant (an oil-detergent mixture that is used to stimulate the immune response (e.g 100 ul of virus solution and 100 ul of complete adjuvant) with a 2-CC glass syringe. An emulsion is most readily prepared by linking two locking glass syringes, one loaded with antigen and the other loaded with adjuvant, using a double-ended locking connector. Press syringe barrels back and forth, transferring the contents from one syringe to the other, for 5 to 10 min until a stable emulsion is obtained. A

stable emulsion will not spread when dropped into water. This is a useful check for the emsulfication endpoint.

2. Four weeks later give a booster injection intravenously with the same dose of the antigen as was injected first time. This time do not mix adjuvant. Prepare virus solution in PBS and inject in the tail intravenously (Figure 11.2a). If you fail to inject intravenously then inject into the peritoneal cavity of the mouse.
3. Three to 4 days after booster injection take the serum samples from individual immunized mouse while cutting a 0.5 cm of tail with a sterile sharp scissors or razer blade (Figure 11.2b). Collect 100 to 200 ul blood into a separation serum tube. Centrifuge (1000 rpm for 10 min) and collect serum by micropipette.
4. Determine the antibody titer in the serum by ELISA (the procedure has already been described).
5. If the antibody titer is considered too low (i.e. less than 1/1000) for cell fusion, mice can be boosted every 2 weeks until an adequate response is achieved. Bleed the mice and test serum with an ELISA.
6. When the antibody titer is sufficient (i.e. more than 1/1000), boost mice by injecting 10 to 50 ul antigen intraperitoneally (200-400 ul), or intravenously (50-100 ul) via the tail veins, three days before fusion but at least 2 weeks after the previous immunization. In general, the higher the serum antibody titer, the more antigen-specific antibody-producing hybridomas are obtained per fusion.
7. Remove spleen from the immunized mouse with the highest serum titer and prepare spleen cells for fusion. This should be done 3 to 4 days after the last (booster) injection.

11.8.3 Determination of antiserum titer: Usually the same assay which is adopted for screening hybridomas is used to test the titer of the antibodies in the immunized mice. Mostly enzyme-linked immunosorbent assay (ELISA) is used for this purpose. Two types of ELISA i.e. indirect and direct are commonly used to select monoclonal antibody secreting hybridomas. In case of indirect ELISA the microtiter plate is first coated with an appropriate dilution of antigen concerned. Whereas, in direct ELISA the antigen is captured

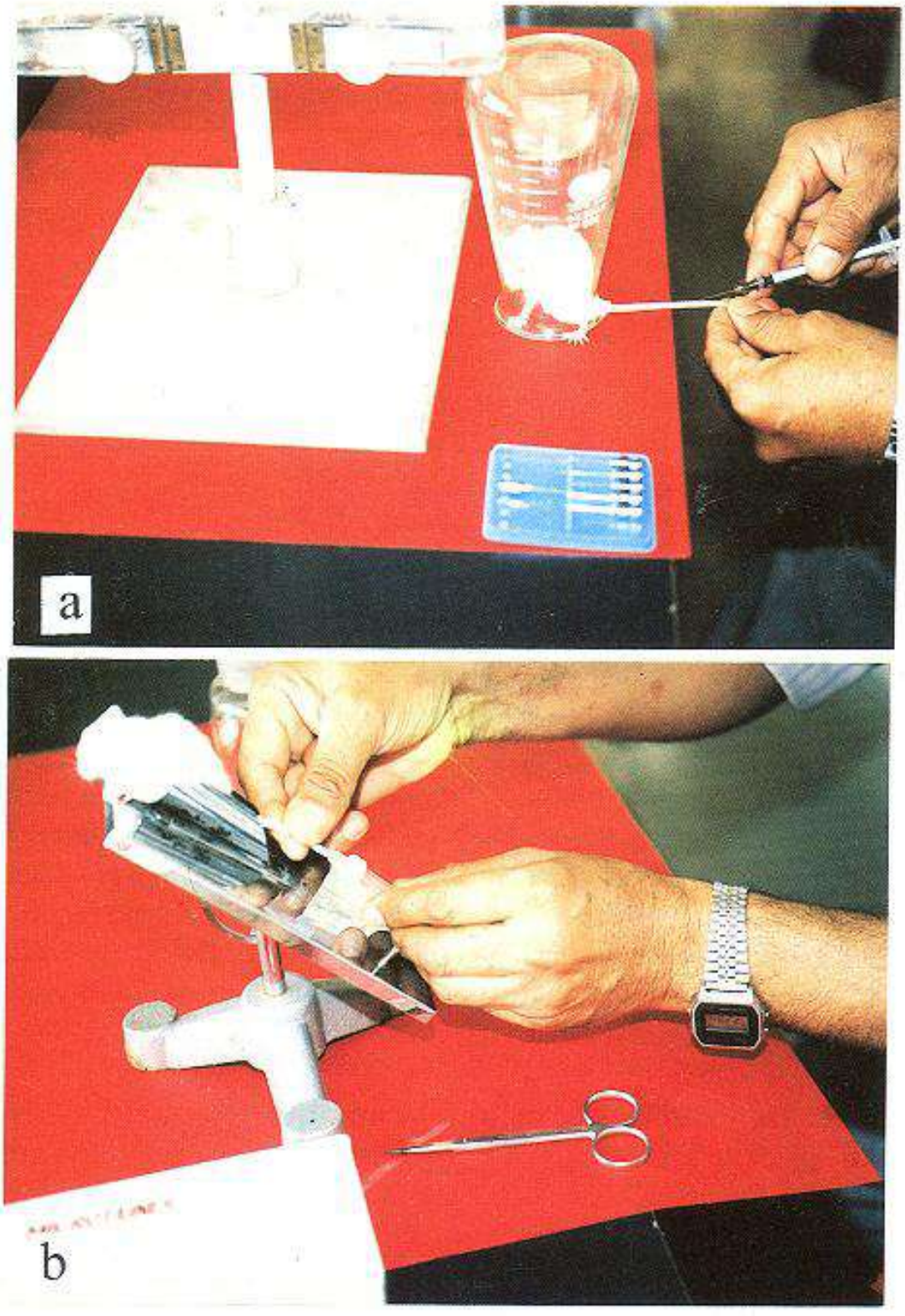

Figure 11.2: (a) Intravenous immunization of BALB/C mouse through tail (b) collection of serum sample from immunized mouse while cutting 0.5 cm of tail with sharp blade.

by coating the plate with antigen specific polyclonal antibodies produced in rabbit. In this case we get sandwich of the antigen.

Coating buffer, pH 9.6 (10 X stock solution), in one liter distilled water

Sodium carbonate: 14.3 g
Sodium bicarbonate: 30.4 g

Dissolve and make the volume up to one liter. For further use, take 100 ml from this stock solution and dilute to one liter with distilled water.

10 X PBS stock solution

Potassium chloride (KCl):	10 g
Potassium biphosphate (KH_2PO_4):	10 g
Sodium chloride (NaCl):	400 g
Sodium phosphate (dibasic: Na_2HPO_4):	145 g

First dissolve in 4 litre distilled water then make the volume to 5 litres. This will be stock solution. To use take 100 ml stock solution and add 900 ml distilled water. It is ready to use. Store at room temperature.

Substrate for horseradesh peroxidase enzyme system

1. Take a small quantity of ABTZ (2, 2-Azinobis (ethyl benzothiazole 6-sulfonic acid) with a small spatula and dissolve in 5 ml PBS containing 100 mM citric acid pH 5.3, it is solution A.
2. Take 5 ml distilled water in a glass tube and add to it 5 ul hydrogen peroxide (H_2O_2). This is solution B.
3. Mix solution A and B. It is ready to use. Prepare fresh substrate each time.

Determine the antiserum titer as follows

1. Coat the microtiter plate with an appropriate dilution of antigen (e.g. in case of purified virus it will be 1.0 - 0.5 ug/ml buffer) in sodium carbonate buffer (50 mM pH 9.6). Add 100 ul per well. Incubate plate at 4° C overnight.
2. Wash plate three times with washing buffer (PBS: phosphate buffer saline).

3. Make antiserum dilution as 100 X, 200 X, 400 X and 800 X in sodium carbonate buffer. Add 100 ul/well. Add buffer in duplicate wells as a negative control. Incubate 2 hr at 37° C.
4. Wash plate three times with PBS washing buffer.
5. Add two drops/well from bottle 'a' from Vector Laboratory ABC Kit Inc., 30 Ingold Road, Burlingame, CA 94010, USA. This step is called blocking to reduce non-specific reaction. Incubate for 2 hr at 37° C.
6. Repeat step 2.
7. Add one drop/well from bottle 'b' from ELISA Kit. Incubate for 15 min at room temperature.
8. Repeat step 2.
9. Add one drop/well from bottle 'c' from ABC kit. Incubate for 15 min at room temperature.
10. Repeat step 'b', this time wash 5 times.
11. Add one drop/well substrate. Blue colour will be developed and take reading by microplate reader at $A_{415\ nm}$.

* **Note:** Vector Laboratory ABC kit has three large size and four small size bottles. Large size bottles are marked as a, b and c. The small size bottles are marked as A, B, C and D. The bottle 'A' contains normal horse serum used for blocking. The bottle 'B' contains biotinylated antibody taken from horse against mouse IgG. The bottle 'C' contains Avidin. The bottle 'D' contains biotinylated peroxidase. The solution in bottle 'a' is prepared by dissolving 3 drops from bottle'A' in 10 ml PBS. The solution in bottle 'b' is prepared by dissolving 3 drops from bottle 'A' and one drop from bottle'B' in 10 ml PBS. The solution in bottle 'c' is prepared by dissolving 2 drops from bottle 'C' and two 2 drops from bottle 'D' in 10 ml PBS. The solution of bottle 'C' must be prepared 30 min before use. This kit is stored at 4° C.

11.9 Preparation of parental cells for fusion: The most important part in the production of hybridoma is the preparation of cells for fusion. Both spleen B-lymphocyte cells and myeloma cells need to be in optimum cell cycle stage and conditions for successful immunization and subsequent hybridoma production.

11.9.1 Preparation of spleen cells

1. Take two beakers, one with 30 ml 70% ethanol and the second with 30 ml 99% ethanol. Dip two pairs of forceps and two pairs of scissors in 99% ethanol and sterilize on gas burner.
2. Take immunized mouse which gave the highest titer, and draw some quantity of blood with syringe from the heart to be used as polyclonal antiserum for positive control in ELISA.
3. Kill the mouse by cervical dislocation with forceps and then dip into a beaker with 70% ethanol.
4. Put the mouse on the dissecting board so as the face should be on the left side. Give a cut to the skin with a scissors to expose the abdominal wall (Figure-11.3).
5. Cut through abdominal muscles very gently to remove the spleen with sterilized forceps and scissors. Put the spleen in a petriplate containing 10 ml IMDM medium. Remove fats and connecting tissue under aseptic conditions.
6. Transfer the spleen to another petriplate containing 10 ml IMDM medium. Wash spleen thoroughly.
7. After washing transfer the spleen to the third petriplate which contains 10 ml IMDM medium and a fine mesh (60-100 mesh metal sieve). With the help of a fine needle (23-G) with syringe, make holes in the spleen (make at least 100 holes). Suck the IMDM medium into the 10 ml syringe and pass this medium through the spleen while holding the spleen with forceps in the left hand. Repeat this step 2-3 times so that the spleen look pale white and all the spleen cells are removed.
8. Sieve the medium containing spleen cells on the mesh with a pipette to remove bigger cells.
9. Collect the medium containing spleen cells in a glass centrifuge tube and spin at 1000 rpm for 10 min. Resuspend the pellet in 10 ml IMDM medium and count the cells using Trypan Blue with the help of haemocytometer under the microscope.

11.9.2 Preparation of myeloma cells

1. Take out the frozen cells (myeloma cells are preserved in liquid nitrogen or at -80° C) and thaw under mild hot water. Centrifuge at 1000 rpm for 10 min and collect pellet.

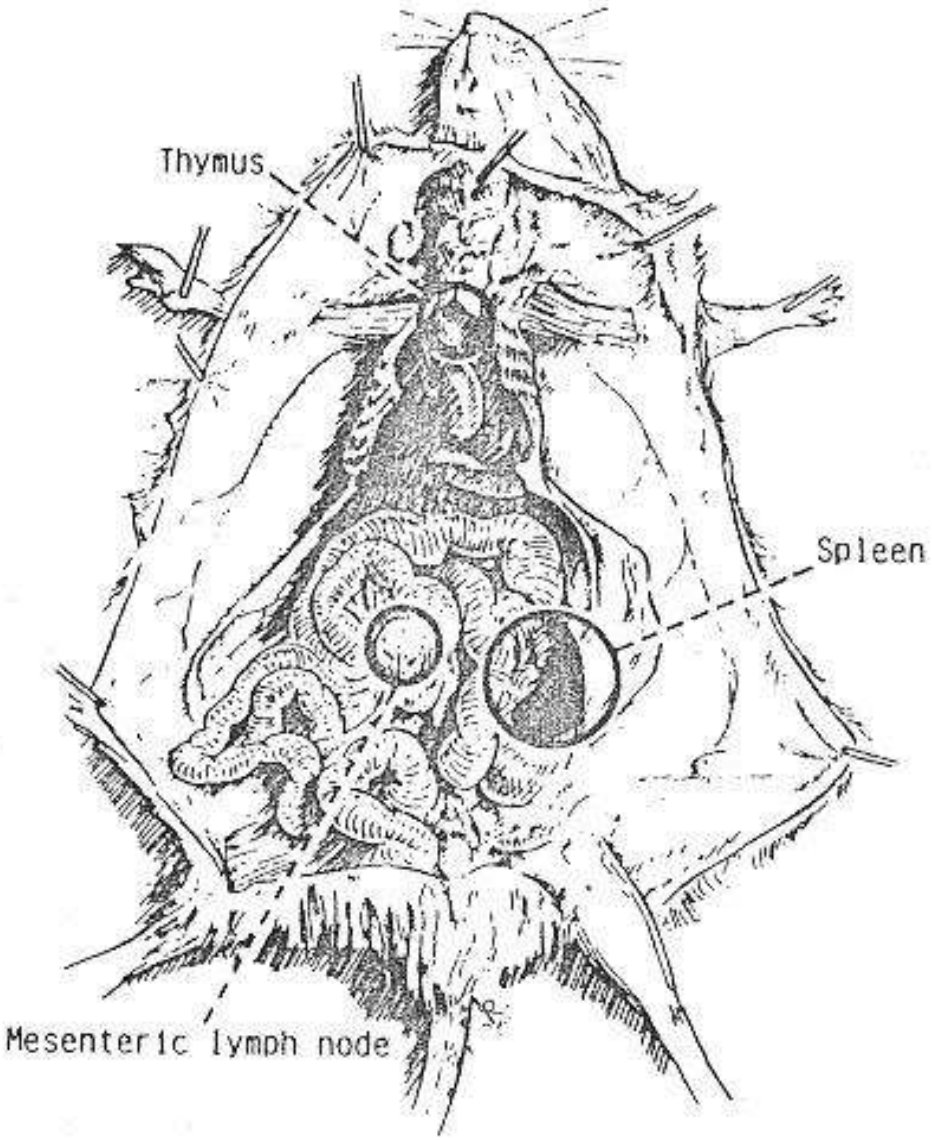

Figure 11.3: Removal of spleen from an immunized mouse for the preparation of spleen cells.

2. Resuspend the pellet in 1 ml normal medium. Put 5 ml normal medium in each of two wells of 6-well culture plate. Put 0.5 ml cell suspension in each well. Incubate at 37° C with 5% CO_2 supply and 98% humidity. Also put 2 ml cell suspension in a sterile petriplate to check for contamination.
3. When the cells will grow the colour of the medium will turns yellow due to change in pH. Replace fresh normal medium after every 2-3 days.
4. After 6-7 days harvest cells. Centrifuge at 1000 rpm for 10 min and collect pellet.
5. Resuspend the pellet in 10 ml IMDM medium and count the number of cells with the help of a haemocytometer. A total of 1 x 10^7 myeloma cells (i.e. 1 : 10 ratio to immune spleen cells) is used for fusion.
6. Save excess of cells. Preserve the cells in 1 ml preservation stock solution. For stocking myeloma cells, the cell density should be 3 x10^6 cells/ml in IMDM medium plus 20% FCS and 10% dimethylsulfoxide. First preserve at -80° C for 24 hr then transfer to liquid nitrogen container.
7. Prepare myeloma and spleen cells at the same time for fusion.

11.9.3 Preparation of macrophage cells (feeder cells)

Macrophages are probably the best feeder cells to be used for hybridoma production immediately after fusion as they are active phagocytes. In the week after fusion there is a massive cell death of the parent myeloma cells due to the presence of aminopterin in the medium, and the emerging clones may be very difficult to see. Macrophages help the hybridoma cells to grow. Macrophages remove much of the cell debris. Macrophages are prepared one or two days before fusion. Macrophage cell preparation involves the following steps:

1. Take a male mouse of bigger size (not immunized) of the same strain and kill by cervical dislocation. Pin its legs on the dissecting board.
2. Saturate the abdomen of the mouse with 70% ethanol, and inject 10 ml HAT medium into the abdominal skin with a syringe with needle of 23 G.

3. Shake the mouse for 5 min to release cells into the fluid.
4. Give a cut to the skin with a scissors and expose the abdominal wall to take out fluid by injecting syringe into the peritoneal cavity. Try to take 4-5 ml fluid.
5 Count the number of cells under the microscope with haemocytometer while staining with Trypan Blue. If you find 100 cells in the grid of haemocytometer then proceed further (3×10^6 cells/ml) for 6 plates.
6. Dilute the cells while adding 40 ml HAT medium and add 100 ul/well of the six 96-well plates.
7. Incubate at 37° C with 5% CO_2 supply and 98% humidity.
8. Next day check plates for contamination. These plates can be used after two days for growing fused cells.

Note: Usually 5000 macrophages per well are sufficient for growing hybridoma. This cell requirement can be achieved if you find 100 cells under the haemocytometer.

11.9.4 Staining and counting of cells

1. Prepare 0.75 sodium chloride (NaCl) solution. It is solution A.
2. Prepare 0.2% aquous solution of Trypan Blue (a kind of dye). This is solution B.
3. Take 100 ul of solution A and 400 ul of solution B and mix. This is solution C.
4. Take 100 ul of solution C, and add 100 ul of cell suspension (spleen cells, macrophages or myeloma cells). Mix and take 50 ul from it and count under the microscope with the help ofhaemocytometer and calculate cell number per ml.

11.9.5 Calculation of cell number

Example:

Suppose the number of cells in 25 squares counted at two places are 150 and 180. Calculate the average, it will be 165. The number cells in one ml will be as follows:

$165 \times 10^4 \times 2$ (1 : 1 dilution factor) = 3.30×10^6 cells/ml

Usually the ratio for spleen to myeloma cells should be 10 : 1 (i.e. for each one cell of myeloma there should be 10 spleen cells for fusion purpose).

11.9.6 Cell viability test by trypan blue exclusion

This procedure is used to determine the number of viable cells present in the cell culture. A non-viable cell will have a blue cytoplasm; a viable cell will have a clear cytoplasm. This test is performed as follows:

1. Centrifuge 1 ml cell suspension at 1000 rpm for 5 min.
2. Resuspend the cell pellet in 1 ml PBS or serum-free culture medium.
3. Mix one part of Trypan Blue solution and one part of cell suspension (1/2 dilution).
4. Using a binocular microscope or inverted microscope, count the unstained (viable) and stained (dead) cells separately in a haemocytometer. Each of the four corner squares (composed themselves of 16 small squares) have 1 mm sides and are 0.1 mm deep mm^3). Count all cells within each of the four squares, including those that lie on the bottom and left-hand perimeters, but not those that lie on the top and right-hand perimeters. Count any clumps of cells as one cell. Calculate the mean number of the cells per 0.1 mm^3 volume. Multiply by 10^4 to obtain the number of cells/ ml (i.e. cells/cm^3). Apply dilution factor for Trypan Blue (2X) to obtain the number of cells per milliliter of culture.
5. Calculate the percentage of viable cells as follows:

$$\text{Viable cells (\%)} = \frac{\text{Number of viable cells}}{\text{Total number of cells (dead and viable)}} \times 100$$

11.10 Fusion of myeloma cells with immune spleen cells:

Freshly harvested spleen cells and myeloma cells are pelted by centrifugation and fused by addition of polythelene glycol (PEG 4000) solution to the pellet. Cells are diluted by HAT medium and aliquoted into six 96-well plates microtiter plates. Hybridomas are grown in HAT

medium and then assayed for production of antigen specific antibody. The fusion procedure is as follows:

1. When the immunized spleen cells and myeloma cells in appropriate dilution are ready, mix them in one tube (keep some portion of both kind of cells to be used as control). Centrifuge at 1000 rpm for 10 min, and remove all the supernatant by careful aspiration. For mixing, the appropriate ratio of spleen cells to myeloma cells should be 10 : 1. Adjust this ratio before mixing.
2. Loosen the pellet gently while tapping the tube on the table to get a fine layer of the cells along the tube wall.
3. Using a 1-ml pipette, gently add 0.5 ml of PEG solution containing DMSO (dimethyl sulfoxide: 1 ml PEG + 100 ul DMSO) dropwise while rotating the tube within 30 seconds. Let it stand for another 30 seconds.
4. Add 10 ml IMDM medium containing 2-ME (2- Mercaptoethanol) very slowly and shake the tube very gently and let it stand for 10 min at room temperature.
5. Transfer the cell suspension to 90 ml HAT medium in a bottle, and add 2-3 drops per well of the six plates containing macrophage cells, prepare two days earlier. Also add parent cells as control. Incubate the plates in CO_2 incubator at 37° C with 5% CO_2 supply and 98% humidity.
6. Next day add HAT medium (100 ul/per well) in all wells of six plates. Also check for contamination, aspirate the contaminated wells and treat with concentrated NaOH.
7. Grow the hybrid cells in HAT medium for two weeks after fusion.
8. After two weeks, replace HAT medium by HT medium.
9. The hybrids are grown in the HT medium until the completion of two cloning procedures.

Note: If the plates with macrophage cells are not ready or if you find that all the plates have been contaminated then you can use BrioClone as 5% in the final fused cell suspension. BrioClone serves the same purpose as that of macrophage cells. It has been prepared from human cell line, a product of Bio Research Ireland, Glasnevin Dublin 9, Ireland. This product is in liquid form and is kept at -20° C.

11.11 Post fusion care of hybridoma cells: After fusion the hybridoma cells are placed into the HAT medium that allows hybridoma cells to grow and prevents the growth of unfused parent cells. The cultures are observed daily under the microscope to access the progress of the hybridoma and to see any contamination. If contamination is found remove the contents of the contaminated cells and treat it with concentrated NaOH (add 200 ul/well). Supernatant fluid secreted by fused cells (hybrids) is tested for the presence of the specific antibody. This stage in the production of monoclonal antibodies is difficult and time consuming. Hundreds of clones at various times after fusion will be ready for screening. Once antigen positive antibody secreting hybridomas have been identified, they are transferred to larger wells, prescreened, expanded, again screened, and bulk cultures for antibody production.

Laboratory protocols about early feeding after fusion and assay of fused cells (hybridomas) vary widely. However, in principle it should kept in mind that aminopterin inhibits many normal cell functions and should be removed from the medium as soon as possible. The time to do this can be judged by monitoring the myeloma control wells. In mouse system, myeloma cells are frequently dead within 10-14 days and it may never be necessary to feed a fusion with medium containing aminopterin at all if the control cells are dead. It is therefore, recommended that when parent cells are completely dead, the feeding medium should not contain aminopterin. Replace HAT medium with HT medium. It is important to monitor the control wells at all times. After the aminopterin is removed, hypoxanthene and thymidine (i.e. HT medium) should still be supplied for at least one week. However, the following points must be kept in mind while taking care of the cells after fusion:

1. Fast growing cells are indicated by the change in pH (i.e. colour of the medium turns yellow, Figure 11.4). These wells should be fed with HAT medium.
2. Early-tissue-culture operation increases the risk of contamination and may disturb the growing clones. The feeding should be as infrequent as possible.

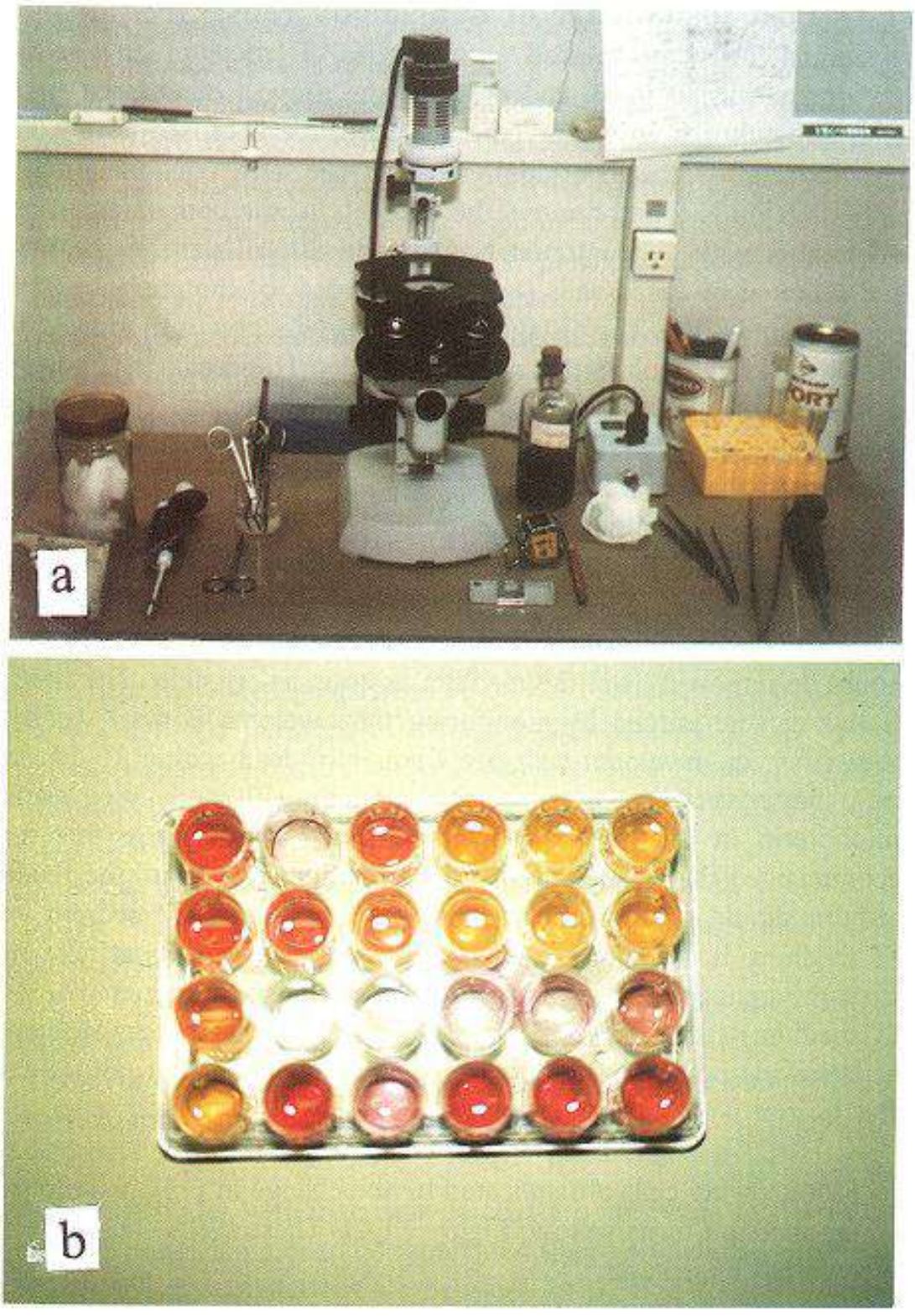

Figure 11.4: (a) Inverted microscope to observe hybridoma cells (b) the fast growing hybridoma cells are recognized by change in pH of the medium into yellow colour.

3. All clones should be assayed and sub-cloned as early as possible.
4. During change of medium to feed cells the secreted antibodies are also removed. Therefore, at the initial stage of the cell cloning, the amount of antibodies produced may be so small to detect by the assay system.

After fusion follow the following steps

1. Next day of fusion feed the cells with HAT medium. Add 100 ul/well HAT medium.
2. After three days examine the plates for contamination. Individual contaminated well can be aspirated and treated with concentrated NaOH. In case of many contaminated wells particularly with fungi, better to discard the whole plate. Feed all wells first by aspirating half of the medium in each well using pipette and then replace with fresh HAT medium (100 ul/well). Do not disturb the cells during taking out half of the medium from the wells.
3. On days 17-14: after two weeks of fusion, the hybridoma colonies are very easily recognized. They look dividing bigger cells. Begin screening assays. Aseptically with a sterile micropipette remove culture fluid for screening when bottom of the well is 30-50% covered with hybridoma cells and the colour of the medium turns yellow. Replace with fresh HAT medium. Screen and feed every 3-4 days. Plan to spend all the day working with the cells and screen. Transfer the positive cell lines to 24-well plates by gently aspirating the media and cells up and down in the well with a sterile glass pipette (about 3-4 times to mix the cells with media), and adding this suspension to 1ml fresh HAT medium in the new well.
4. On days 14: check control cells (spleen and myeloma) they must have been dead after two weeks. If the control cells are completely dead then start feeding hybridoma cells with HT medium instead of HAT medium. Continue feeding every 3-4 days.
5. On days 14-30: allow sufficient time for proliferation of transferred cells and screen. Transfer positive cell lines to 24-well plate. This time use HT medium to feed hybridoma. Continue to monitor 96-well plates, screen and transfer the hybridoma to large volume plates e.g. 6-well plates.

6. On days 30-45: about 80-90% of all the antigen specific hybridomas will have been selected in the next 2-3 weeks.
7. When all the antigen specific antibody-secreting hybridoma have been placed in frozen storage, retained final culture supernatant fluids are evaluated in detail in a variety of assays to identify antibodies of interest and to ascertain which cell lines to carry through the cloning process and antibody production stage.

Note: If fusion shows no growth of hybridoma cells after 14 days, it should not be discarded but may just be slow growing hybridoma. It should be fed by changing half of the medium and maintain up to 6 weeks. If it shows no growth by that time, it probably never will. Discard the plate.

11.12 Cloning of hybridomas: Hybridomas are usually not stable cell lines, i.e. they tend to lose the ability to produce antibody. Cells which have lost the ability to produce antibody can rapidly overgrow the cells which have retained antibody production. This may occur while antibody levels in the culture medium remain high. Therefore, valuable cultures may be lost. To avoid this loss, useful hybridomas should be cloned to select the antibody-producing cells from the mixture of producing and non-producing cells. This should be done as soon as the antigen positive hybridoma is recognized. After the cloning has been repeated several times, the cell line may be more stable. However, in most cases, enough antibody can be obtained from the first, not yet fully stabilized clone to complete a reasonable research project. If clones have been saved in liquid nitrogen they can be thawed out to produce a large amount of antibody that may be required for extensive projects.

There are several methods to clone hybridomas cell lines but cloning by limited dilution is more common than others. All methods of cloning require that hybridomas be growing actively and be in excellent condition. Thus, the culture to be cloned should be split and given fresh medium with 20% FCS on the day before cloning. Cloning of hybridoma cell lines which are positive for a specific antigen by limited dilution method is done as follows:

1. Two days before cloning, isolate mouse feeder cells (macrophages) and prepare 96-well plates with feeder cells in HAT medium as described under preparation of macrophages cells.
2. Transfer all of the cells from each well containing antigen-specific antibody in its hybridoma supernatant (as determined by ELISA) into a separate well of a 24-well plate that has been preincubated with 0.5 ml of an appropriate culture medium e.g. HAT medium (choice of medium depends on the current stage of the cloning process. In first cloning use HAT medium, and the second cloning use HT medium. In other cloning use normal medium) and culture overnight at 37° C, CO_2-in-air, and 98% humidity in a CO_2 incubator. It is better to clone 6 cell lines at a time, as it is easy to manage.
3. Perform cell viability count using Trypan Blue exclusion method on the overnight culture in 24-well plates. This procedure is used to determine the number of viable cells in the culture. A non-viable cell will have a blue cytoplasm, a viable cell will have a clear cytoplasm.
4. Using a 6-well plate, make dilution of cells from overnight cultures in HT medium.In the first well, make a 1: 1000 dilution in a total of 3 ml; in the second well, dilute an aliquate of the first dilution to 80 cells/ml in 5 ml; in the third well, prepare 8 cells/ml in 10 ml (i.e. a 1 :10 dilution from the second well).
5. With a multichannel pipette, fill the upper 50 wells of the inner 60 wells of the 96-well plate from step 1 with 100 ul of 8 cells/ml dilution (i.e. 0.8 cells/well) and the 10 wells of the bottom row with 100 ul of 80 cells/ml dilution (i.e. 8 cells/well).
6. Incubate at 37° C in a CO_2 incubator in 5% CO_2-in-air with 98% humidity (day 1).
7. On day 6; feed the culture with the addition of 100 ul/well of fresh medium, using a multichannel pipette. Thereafter, if necessary, refeed the culture every other day by removing 100 ul media from each well and adding 100 ul fresh media.
8. When the colour of the medium turns yellow, assay for specific antibody in the hybridoma supernatants using an ELISA.
9. Transfer 2 to 3 selected positive subclones from each plate into a 24-well plate.

10. Expand the subclones and freeze one aliquot for each subclone in a cryotube. This is done as a precaution in case one fails to recover positive clones.
11. Repeat cloning procedure from the beginning until a stable and single hybridoma cell is established.
12. Once established as stable cell lines, hybridomas are maintained in normal medium in a similar manner to the myeloma cell line. Cells are then propagated for liquid nitrogen storage and for antibody production in ascites fluid.

Note: Hybridomas that yield more than 90% antibody-positive culture upon recloning are considered to be stable. Those that yield less than 90% positive cultures are subjected to further cloning. When clones become stable, reduce 20% FCS (fetal calf serum) level in the HT medium to 10%, gradually reduce HT level, and finally remove HT from the medium entirely. Some clones are more sensitive to this HT wearing process than others.

Recloning of established hybridoma lines may become necessary when they are cultured for longer than 30 days. Somatic mutation or chromosome loss may occur during an extended culturing, which could lead to a loss of antibody production.

11.13. Freezing and recovery of hybridoma cell lines

Hybridoma cells are suspended in dimethyl sulfoxide and fetal calf serum and frozen in a dry ice-ethanol and glycerol bath (Figure 11.5) followed by transfer to liquid nitrogen storage. Cells are recovered by thawing rapidly at 37° C, with immediate replacement of freezing medium by culture medium. The composition of freezing medium has been given below. The hybridoma cells to be frizzed should be in the log phase of growth.

11.13.1 Freezing of hybridoma cells

1. Perform a cell viability count using Trypan Blue exclusion procedure.

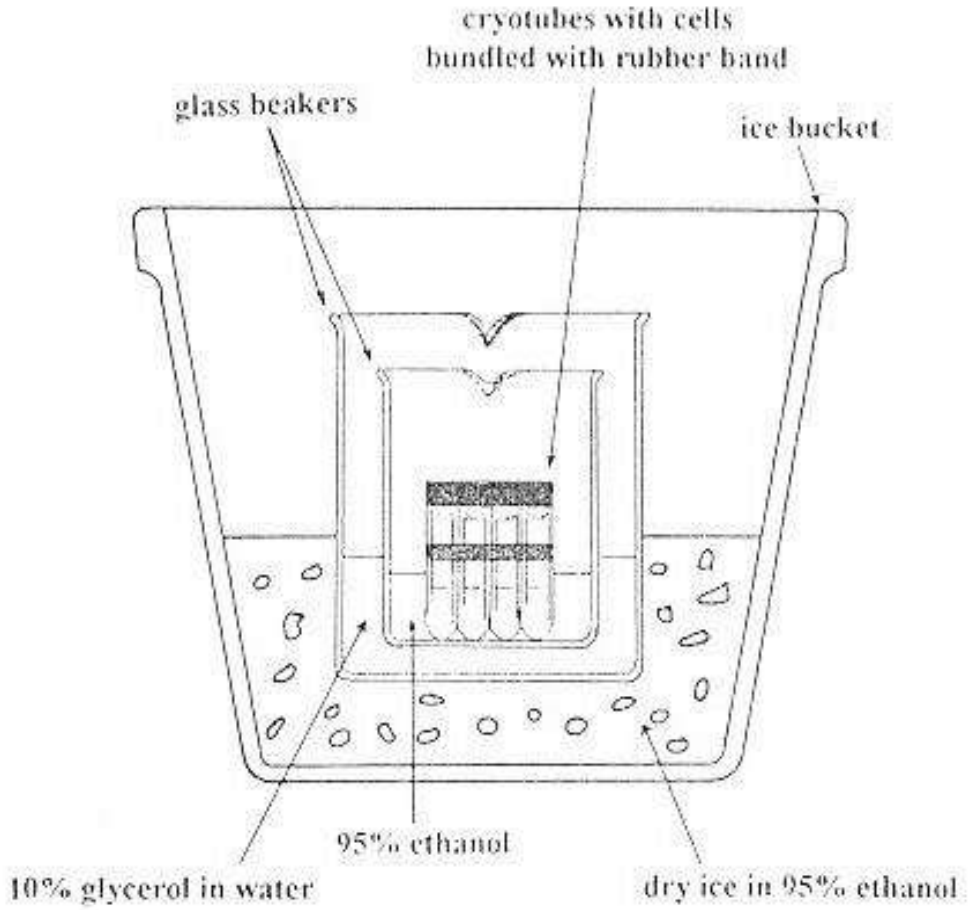

Figure 11.5: A devise for freezing hybridoma cell lines in dry ice ethanol and glycerol bath.

2. Using a pencil, label cryotube with identification and date.
3. Centrifuge cell suspension at 1000 rpm for 5 min at room temperature
4. Aspirate the supernatant very carefully.
5. Resuspend cell pellet in freezing medium to give a cell density of 1 x 10^7 viable cells/ml.
6. Aliquot 0.5 ml/cryotube (i.e. 5 x 10^6 cells /tube).
7. Freeze in dry ice/ethanol and glycerol bath for 60 min (Fig.11.5). Alternatively the cryotubes are first stored at - 80° C for 24 hr and then are transferred to liquid nitrogen.
8. Transfer cryotubes to liquid nitrogen.

Freezing medium:
10% (vol/vol) dimethyl sulfoxide, analytical grade.
90% (vol/vol) fetal calf serum(FCS).
Medium is prepared on day of use and chilled to 4° C before use.

11.13.2 Recovery of frozen cell lines

1. Thaw cryotubes completely at 37° C in water bath. Thawing should be completed within 1 min. Immerse tube only to level of contents.
2. Wipe top of cryotube with alcohol swab, transfer cells to 15-ml centrifuge tube and add 5 ml of normal culture medium warmed in 37° C water bath.
3. Centrifuge at 1000 rpm for 5 min.
4. Aspirate supernatant.
5. Resuspend cell pellet in 5 ml warm complete culture medium (37° C).
6. Transfer suspended cells to a 25-cm^2 tissue culture flask.
7. Incubate overnight at 37° C in a CO_2 incubator in 8 % $C0_2$-in-air with 98% humidity. Keep flask upright.
8. Add 5 ml warm complete culture medium (37° C). Lay flask flat.
9. Propagate cells in complete culture medium.

11.14. Characterization of monoclonal antibodies: The characterization of monoclonal antibody can be one of the most exiting aspects of McAbs production. It can be a long and complex procedure

depending on the objectives of the McAbs production. Most often McAbs are characterized for their specificity, class, sub-class, specific titer, storage ability, stability, purification property and affinity.

After recognizing hybridomas in the culture medium their secretion is assayed to determine the hybridoma positive to specific antigen. Mostly not all are antibody secreting hybridomas, because not all the spleen cells are always immunized. Therefore, some un-immunized spleen cells may also fuse, which are not useful. The secretion of hybridoma cells is usually tested by ELISA.

11.14.1 ELISA for detection of antibodies in hybridoma secretion: The ELISA described in this protocol can be used for detecting antibodies in serum or hybridoma supernatants. Antigen is first adsorbed on the well surface of microtiter plate. Serum samples from the test bleeds or hybridomas supernatants are incubated in separate antigen-coated wells. Antigen-specific antibody present in the samples will bind to the antigen. Unbound serum or supernatant components are washed away, and horseradesh peroxidase-antimouse IgG conjugate is then bound to the mouse antibodies already bound to the antigen. Excess conjugate is washed away, and substrate solution is added to each well. The amount of colour develops as the substrate is acted upon by the enzyme of the conjugate cleaved by the enzyme in the enzyme-IgG conjugate.

Protocol for ELISA

1. Dilute antigen to 10 ug/ml in carbonate coating buffer, pH 9.6. Add 100 ul/well antigen solution of 96-well microtiter plate. Cover the plate with cover and incubate 2 hr at 37° C or 4° C overnight.
2. Wash plate three times with wash buffer (PBS-T).
3. Add 100 ul/well of each dilutes mouse serum or undiluted hybridoma supernatants. Incubate plate for 1-2 hr at 37° C.
4. Repeat step 2.
5. Add 100 ul/well of horseradesh peroxidase-antimouse IgG diluted 1 : 1000 in diluting buffer (without 0.1% Na N_3). Incubate at 37° C for 30 min.
6. Repeat step 2.

7. Add 100 ul/well peroxidase substrate solution. The method for preparing substrate has already been described.
8. Incubate at room temperature and record observation by microplate ELISA reader at $A_{415\ nm}$. Colour change is from colourless to blue-green.

Conjugate diluting buffer:

Tween-20:	0.5 ml
Bovine serum albumin (BSA):	2.5 g
Na N_3 (Sodium azide):	1.0 g
PBS (phosphate buffer saline):	1 liter

Note: Do not add sodium azide if the horseradesh peroxidase -IgG method is being used.

11.14.2 Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis is widely used in characterization of both monoclonal antibody and its antigen. It is used to determine the class of secreted antibody, for proof of monoclonality and for immunoblotting. Only slab gels are suitable for most of these procedures.

11.14.2.1 Slab gel apparatus: Slab gels are poured into two glass plates typically 18 cm x 13 cm clamped vertically together with spacers holding the plates apart. Thicker gels hold more protein and may be more suitable for immunoblotting of minor proteins. When gel is poured a comb made of plexiglass of the same thickness as spacer is inserted between the two glass plates while the gel sets. The comb is removed before sample application into the slots it creates in the gel. The number of teeth in the comb is a matter of choice relating to the number of samples to be analysed, then volume and expertise of the operator.

The gel is poured in liquid form and there is a possibility of leakage out of the bottom of such an apparatus unless care is taken. Leakage can be stopped by greasing the spacers with petrolium jelly at the bottom, or by using a sticky tape at the bottom. The two plates are

firmly clamped together with foldback office clips. Such apparatus is commercially available with several companies.

11.14.2.2 SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis): SDS-PAGE separates proteins on the basis of size of the proteins. SDS denatures the proteins to give a uniform tertiary structure and binds to most proteins to the same extent. The PAGE has molecular sieving effect so that small proteins move more raspidly through the gel. Gel concentrations are usually in the region of 10-15%. Lower percentages are used for large proteins and higher for small proteins. Gels of 5% polyacrylamide and below are extremely difficult to handle.

The gel mould is prepared and the ingredients for the gel are made up according to the information given in Table 11.1. Acrylamide and bis-acrylamide should be handled with gloves. Before starting electrophoresis, keep ready the following solutions/buffers.

11.14.2.3 Solutions and buffers for SDS-PAGE

Solution A: (30% acrylamide):

Acrylamide:	29.2 g
N, N-Methylenebisacrylamide:	0.8 g

First dissolve in 50 ml distilled water then make the volume up to 100 ml. This solution is stable for at least one month at 4°C.

Solution B: (Separating gel buffer, 1.5 M Tris-HCl pH 8.8):

Tris:	18.2 g
SDS:	0.4 g
HCl:	2 ml (Concentrated HCl to adjust pH).

This solution is stable for at least one month at 4°C.

Solution C: (Staking gel solution, 0.5 M Tris-HCl buffer pH 6.5):

Tris:	6.1 g
SDS:	0.4 g
HCl:	4.2 ml
(Concentrated HCl to adjust pH)	

After dissolving the ingridients make the volume as 100 ml with distilled water. This solution is stable for at least one month at 4°C.

Solution D: (10% ammonium persulphate):

Ammonium persulphate:	100 mg
Distilled water:	1 ml

Make this solution just before use.

Electrode reservoir solution (or running buffer):

Tris:	1.5 g (25mM)
SDS:	0.5 g (o.1%)
Glycine:	7.2 g (192mM)

Make total volume as 500 ml with distilled water. This solution should be prepared fresh for each electrophoresis run.

Dye solution:

PBB (Bromophenol Blue):	1.0 g
Glycine (Glycerol):	0.1 ml
Distilled water:	0.1 ml

Staining solution:

CBB (Coomassie Brilliant):	0.1 g
Methanol:	30 ml
Acetic acid:	10 ml
Distilled water:	60 ml

Filter through filter paper.

Destaning solution:

Methanol:	300 ml
Acetic acid:	100 ml
Distilled water:	600 ml

Sample solution:

SDS:	3 g
2-Mercaptoethanol:	3 ml
0.5 M Tris-HCl pH 6.8	30 m (take fom solution C)

Make the volume as 100 ml with distilled water. This solution should be prepared fresh each week and stored at 4°C.

Sample preparation:

Virus infected plant tissue: 0.1 g
Sample solution: 1 ml

Dilute solution three times before homogenizing the plant tissue in mortar with pestle. Grind the plant tissue, and then take 10 ul sample and add to it 5 ul 3X sample solution. Boil the sample for 5 min in boiling water, and then add 2-3 ul dye solution to the sample. Now the sample is ready for loading.

Table 11.1: Gel concentration for SDS-PAGE.

Solution	Separating gel						Stacking gel
	5%	7.5%	10%	12.5%	15%	20%	4.5%
A	3.0	4.5	6.0	7.4	9.6	12.0	1.0
B	4.5	4.5	4.5	4.5	4.5	4.5	-
C	-	-	-	-	-	-	-
D	0.08	0.08	0.08	0.08	0.06	0.06	0.02
TEM	0.01	0.01	0.01	0.01	0.01	0.01	0.01
D.W.	10.5	9.0	7.5	6.0	4.5	1.5	3.6

Note: For protein and virus proteins mostly 15% gel is used.

11.14.2.4 Protocol for electrophoresis

1. Assemble the gel cassettes using clean, dry glass plates according to the manufacturer,s instructions for the particular apparatus being used.
2. Prepare a sufficeint volume of separating gel mixture (e.g. 100 ml) by mixing 33.3 ml of stock acrylamide solution, 25 ml of 1.5 M Tris-HCl, pH 8.8, 40 ml of distilled water and 150 ul of 10 % ammonium persulphate, 1 ml of 10% SDS and 50 ul of TEMED. Mix gently and pour immediately into the gel cassettes to about 1 cm below the level which will be occupied by the well-forming combs when they are in position.
3. Overlay the gel carefully with distilled water and allow to polymerase for at least two hr (or overnight) at room temperature.
4. Prepare the required volume of stacking gel mixture (e.g. 30 ml) by mixing 3 ml stock acrylamide solution, 3.75 ml of 1 M Tris-HCl, pH 6.8 , 22.75 ml of distilled water and 150 ul of 10% ammonium persulphate., 0.3 ml of 10% SDS and 50 ul of TEMED. Mix gently and use immediately.
5. Pour off the water from the top of the polymerized separating gel and fill the cassettes with the stacking mixture. Insert the sample well-forming combs and leaves to polymerize for at least 2 hr at room temperature.
6. When the stacking gel has polymerized, remove the sample comb from the gel and install the gel in the electrophoresis apparatus according to the manufacturer,s instructions.
7. Fill the electrode chambers with the reservoir buffer solution (running solution). This should be circulated between the cathode and anode chambers to equalize the pH conditions.
8. Prepare dry samples by dissolving in single strength (i.e. diluted 1:1 with distilled water) sample solubilization solution. Liquid samples should be diluted to a suitable protein concentration and added to an equal volume of double strength sample solubilizatiojn solution. The total sample volume should be less than the volume of the sample wells in the gel. Heat the samples in boiling water bath for 5 min.
9. Load the samples into the sample wells using a suitable microsyringe.

10. Run the gel under constant current conditions. A 1.5 mm thick, 14 cm long 10% T gel can conveniently be run overnight at 15 mA per gel, by which time the Bromophenol Blue tracking dye reaches the bottom of the gel.
11. At the completion of electrophoresis, remove the gel from cassettes very carefully into a plastic container.
12. Pour the staining solution (about 100 ml or depending on the size of the gel) in the gel container, also put one or two tissue paper, and shake the gel on shaker for about 1 hr.
13. Pour off the staining solution from the gel container and add 100 ml destaining solution and shake the gel overnight at room temperature.
14. Analyze the gel bands.

11.15 Mass production of mococlonal antibodies: When the hybridoma is grown *in vitro* usully 0.1 to 10 ul of antibody per ml is obtained. The same hybridoma, when grown in the peritoneal cavity of the mouse it may produce 5 to 80 mg of antibody per ml of the ascites fluid. Both methods have relative advantages. Antibody produced in vitro in FCS (fetal calf serum) medium is truly monoclonal and monospecific. The low concentration of antibody may be overcome by growing the hybridomas in serum-free (insulin-transferrin) medium and subsequently concentrating the antibody by dialysis or ultrafilteration. However, this is un-necessary for many studies. When very large amounts of high concentrations of antibody are required, the growth of hybridomas as an ascites tumor in pristane-primed mouse will provide the required quantity of antibody, but present a usually insignificant complication. Monoclonal antibodies produced in mouse will be contaminated with all other antibodies normally present in mouse. Thus, mouse used for ascites fluid production must be protected against the antigens used for monoclonal antibody production, and against all antigen which might be cross-reactive with the antigen of interest.

Procedure

1. Inject mouse (6-10 week old BALB/C mice) with 0.5 ml Pristane intraperitoneally using a 3-cc glass syringe and 22-G needle. At

least 3 mouse are normally prepared for each hybridoma cell line. Care must be taken to avoid penetration of the intestines by the needle.

2. Before initiation of ascites fluid production, recover hybridoma lines from frozen storage and maintain cell line in complete culture medium or normal medium.
3. One day prior to injecting the cells into the mouse, dilute the culture to 2 x 10^5 cells/ml by adding fresh normal medium to ensure that cells are in log phase of growth when injected.
4. Inject mouse intraperitoneally with hybridoma cells 10-14 days after Pristane (2,6, 10, 14-tetramethylpentadecane-Aldrich Chemical Company, Inc. Milwaukee, WI 53233) injection. Using a 22-G needle, inject 1-5 x10^6 suspended in 0.5 ml PBS.
5. Observe mouse for abdominal swelling indicative of ascites fluid accumulation (Figure 11.6a). Sufficient fluid for collection will normally be accumulated 6-9 days after injection of cells. At this time the, the abdomen has swollen 2-3 times that of normal size and the mouse begin to be restricted in their movement.
6. Collect ascites fluids by injection of a 20-G needle into peritoneal cavity, allowing fluid to drain into a 15-ml centrifuge tube (Figure 11.6b). The amount of fluid collected will vary from 1 to several milliliters. The colour will range from white /yellow to deep red, depending on the proportion of red blood cells present.
7. Place ascites fluid at 4°C for 1 hr and wait for clot formation.
8. Centrifuge at 5000 rpm for 15 min. collect supernatant and store at -20°C until use.
9. Repeat steps 5 to 8 every two days until the mouse begin to show distres (i.e. lack of movement, extremely dishelved appearance, or labour breathing).
10. Kill mouse by cervical dislocation.
11. Immerse the mouse in a 100-ml beaker containing 70% ethanol.
12. Lay out mouse on dissecting board.
13. Snip skin at diaphram level and pull skin back, exposing the lower part of the rib cage and abdomen.
14. Open abdominal cavity of the mouse with a sterile scissors, and collect the remaining ascites with a sterile syringe.
15. Repeat step 7 and 8.

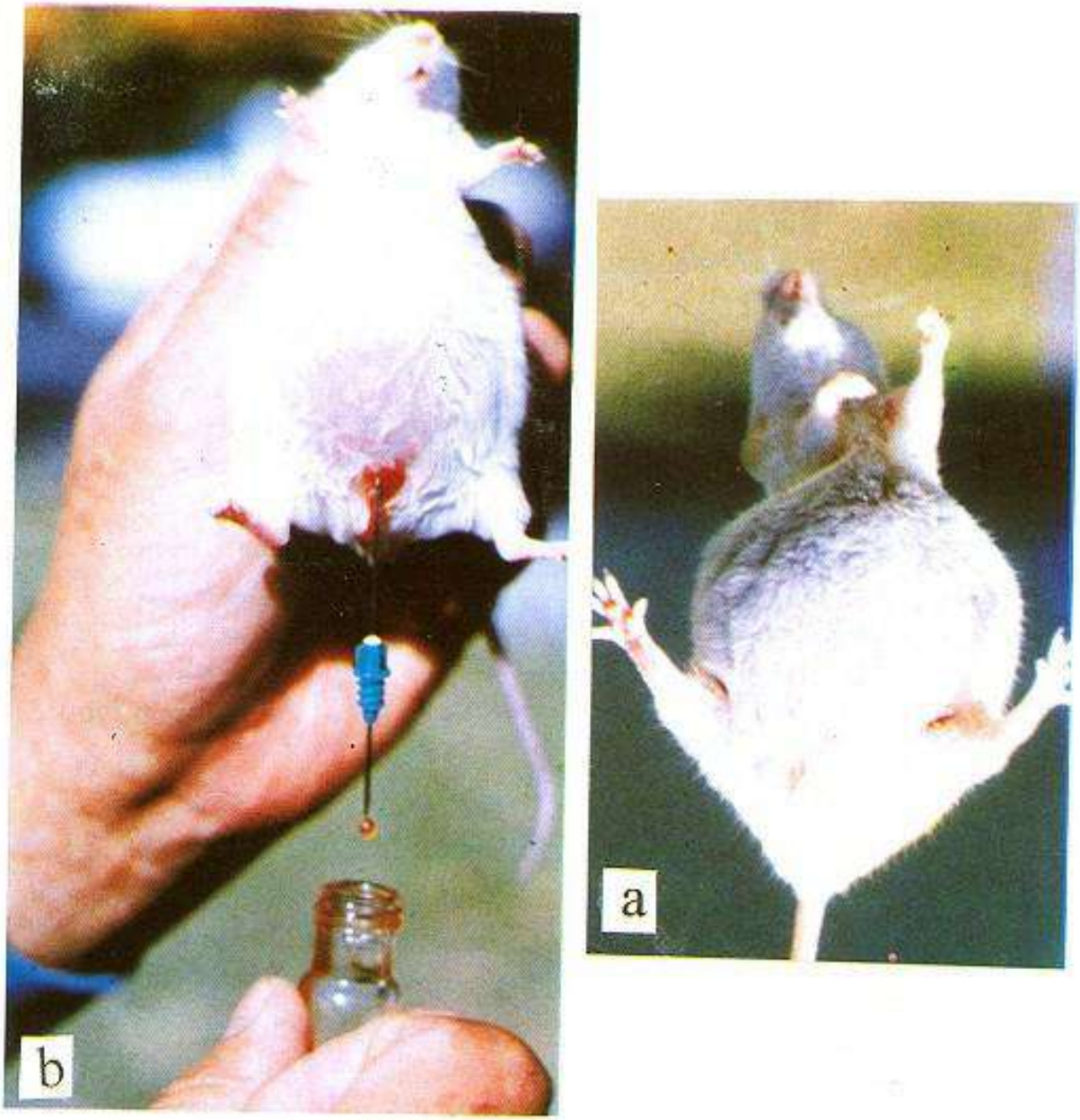

Figure 11.6: (a) Abdominal swelling of mouse after injecting hybridoma cells indicative of ascites fluid (b) collection of ascites fluid by a 20-G needle from peritoneal cavity of the mouse.

11.15.1 Purification of immunoglobulin G from ascites fluid:

This is acomplished by precipitation of IgG with saturated ammonium sulphate. It is done as follows:

1. Add 1 vol SAS (saturated ammonium sulfate (i.e. 33%) dropwise with a Pasteur pipette to 2 vol of ascites fluid with constant mixing at 4°C.
2. Allow precipitate to form over a period of 2 to 4 hr at 4°C with constant mixing. Pellet precipitate by centrifugation for 20 min at 1000 rpm.
3. Wash pellet by resuspending (vortexing) it in a volume of cold 33% SAS solution equivalent to the original volume of ascites fluid. Repeat wash step once.
4. Dissolve pellet in an appropriate cold buffer by gentle vortexing. A convenient volume for solubilizing the IgG fraction is 5% to 10 % of the original ascites fluid.
5. Dialyse IgG solution in dialyses tube over 48 hr at 4°C against three changes of the desired buffer (PBS: 4 liters per change) to fully remove the ammonium sulfate.
6. Measure the concentration of the purified IgG by spectrophotometer, adjust as 1 mg/ml.
7. Store IgG at -70 °C for future use.

The overall flow chart for the production of McAbs is given in Figure 11.7.

11.16. Handling of mouse for experimental purposes

11.16.1 Identification: Individual mouse in the same cage will often need to be identified for repeated immunization or procedures. Therefore, identification of individual mouse is very important as all mouse look alike. The most satisfactory permanent method for identification of mouce and rates is by ear clips or by dying the fur. For dyes in use include picric acid and gention violet. Aqueous solution of these dyes should be diluted with alcohol to decrease the drying time. Six positions may be used for dying i.e. shoulder, flank, and rump and the right and left sides. These dyes are best applied with cotton-tipped applicators.

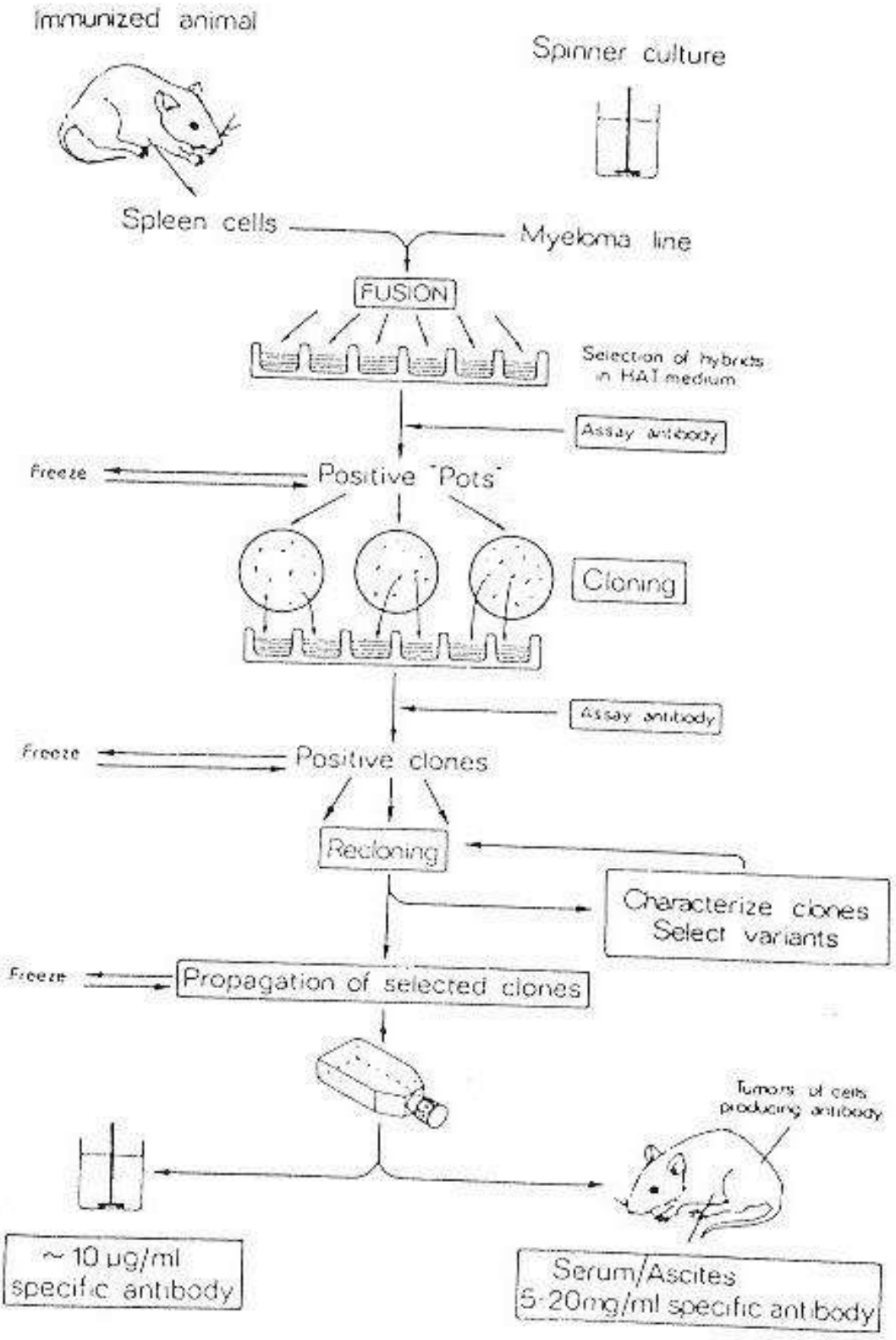

Figure 11.7: Flowchart for the production of monoclonal antibodies.

11.16.2 Restraint: Mouse should be picked up by the tail near the base, and placed on a grid when the tail is pulled gently. The animal holds on the grid with its feet, and it can then be picked up by grasping the loose skin over the shoulder with the thumbs and forefingers, when the tail is held with the little finger. For intravenous injection as for bleeding from the tail, a special devise is used to catch the mice with tail outside to catch it easily. When the mice is put on this stand, its body is inserted and its tail prutodes to catch it easily.

11.16.3 Anesthesia: For anaesthetizing animals for a short period of time, ether is usually satisfactory. The ether jar should be arranged with a grid over the cotton wool soaked in ether, so as the animal only breaths the fumes, but does not come in contact with ether, which will irritate the skin. The animal is sufficiently anaestheses when the breathing is regular and slow, and when it does not twitch if a foot is gently pinched.

For anaesthetising a mouse for a longer period of time, intraperitoneal injection of a barbiturate such as Sagatal (May and Baker) is used. It should be diluted in ethanol and water (Sagatal : ether : water : 1:1:10) and injected at a dose of approximately 0.1 ml per g body weight of the mouse.

11.16.4 Immunization procedure

In order to immune an animal the antigen is injected by several ways. A few are described below.

11.16.4.1 Intraperitoneal injection: Take a syringe with needle of size 23 G or 24 G. Maximum amount of antigen to be injected should not be more than 2 ml in case of large mouse and up to 1 ml in case of small strain. The mouse is held by grasping the loose skin over the shoulder with the thumb and forefingers, and holding the base of the tail with the little finger, belly upward. The needle is inserted to a depth of approximately 5 mm to one side of the midline, between the lower nipples. Injection at the midline may enter the urinary bledder, and an injection too deep in the peritoneal cavity may damage liver or spleen. The antigen should be injected slowly, and there should be a

brief pause before the needle is withdrawn to allow the antigen to disseminate. If there is a swelling at the injection site the injection has been subcutaneous, not intraperitoneal.

11.16.4.2 Subcutaneous: Take syringe with needle of size 23G or 25G. Maximum amount of the antigen to be injected should be 200 ul (0.2 ml). The mouse is placed on the grid, and the loose skin and tail is held as before. It should be left on the grid and gently held down by the back of the third and fourth fingers of the left hand. The needle is inserted through the skin at the back of the neck, pointing posteriorly, so that the point is below the fingers holding the skin. The point should be moved in a short arc to check that it moves freely between skin and body. The antigen is injected, and the fingers should then be moved to grasp the site of the needle penetration as the needle is withdrawn to prevent loss of the antigen through the hole in the skin.

11.16.4.3 Intravenous: Take syringe with size of 25G or 26G. Maximum amount of he antigen to be injected should not be more than 0.2 ml (i.e. 200 ul). Intravenous injections in mice are neither quick nor easy, and practice is needed before the necessary skill can be acquired. It should be attempted first as a practice. Glass syringe should be preferred, because the plunger moves easily than in plastic syringe. Great care should be taken that air bubbles are expelled from the syringe, bulb and needle before injection. The mouse should be held on the grid in such a way that its body is inserted into the grid and the tail protudes outside. Before, holding the mouse on the grid its tail should be dipped into warm water. It is best to use lateral tail veins, and to make the first injection distally, as a second attempt can then be made in a more proximal position. The mouse,s tail should be held over the forefinger of the left hand, and the needle inserted into the vein on the straight part of the tail over the forefinger. As the antigen is injected, there should be no resistance and the vein should be seen clear. If there is resistance as the plunger is pushed and tail becomes white, the injection has been subcutaneous not intravenous.

11.16.4.4 Intramuscular: Take syringe with needle of size 25G. Maximum amount of the antigen to be injected should be 50 ul. An assistant is required to hold the mouse while the experimenter extends the hind leg and makes the injection into the thigh muscle above the femur. The needle should approach from behind the animal and be pointed along the femur towards the body.

11.16.4.5 Serum collection: Two methods may be used to obtain blood from the tail of the mouse. Firstly a sharp scalpel or razor may be used to cut a small piece off the end of the tail, while holding the animal on the grid (Figure-11.2b). The drops of blood can be collected in the serum separation tube while pressing the tail with forefingers. When sufficient blood has been collected (about 100-200 ul), the bleeding should be stopped by sealing the tail while touching on the hot electric rod. The other method is to give a slant cur in one of the lateral tail vein, and blood is collected in serum separation tube and bleeding is stopped as described before.

The blood should be collected in serum separation tube. Centrifuge and collect the serum by pasture pipette in polyethylene tube.

11.16.4.6 Removal of spleen from mouse: The spleen is usually removed from the mouse either for preparation of cells for fusion purpose (when the animal is immunized) or for preparation of macrophages to be used for growing hybridomas. The procedure to remove spleen from the mouse has already been described under spleen cell preparation.

Chapter 12

MOLECULAR METHODS FOR VIRUS DETECTION

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12.1 Introduction: Plant viruses cause considerable crop losses as well as incurring the expenses of control measures. Rationalization of disease control necessitates more efficient methods of diagnosis and prediction. Current methods of virus diagnosis include time consuming biological assay and serological tests which, though sensitive and rapid, do not detect all viral pathogens. This chapter describes application of molecular techniques for the detection of viruses to overcome the problems described in the previous chapters.

12.2 Detection of RNA viruses by Northern blotting

12.2.1 Introduction: The RNA transcripts from virus RNA or total RNA from plants can be identified by Northern blotting. The RNA is separated according to size by electrophoresis through a denaturing gel and is transferred to nitrocellulose or nylon membrane (Figure 12.1 and 12.2). The RNA of interest can be located by hybridization with radio active or non-radio active labeled DNA or RNA followed by autoradiography. Dot blot can be used to give comparative estimates of the amount of the target sequence in different preparations of RNA.

12.2.2 Extraction of RNA from plant samples

Materials and Reagents

Tris-borate EDTA (TE) saturated phenol
RNA extraction buffer
Chloroform-isoamyl alcohol (24:1 v/v solution)
4 M lithium chloride and Liquid nitrogen

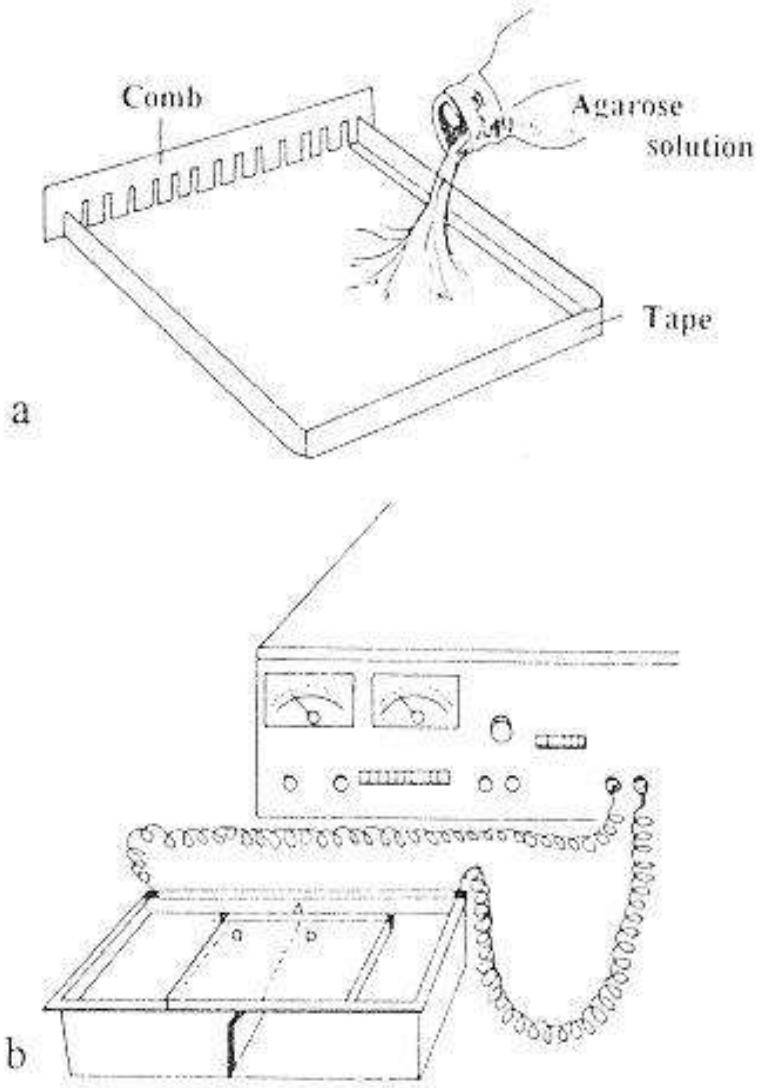


Figure 12.1: Agarose gel electrophoresis: (a) melted agarose is poured into the tape-sealed gel plate with comb in place allowed to solidify. (b) the comb is removed and samples are loaded into the wells (a) Voltage (5V/cm is applied to the gel and electrophoresis proceeds until the bromophenol blue has migrated 1/2 to 3/4 the length of the gel (b).

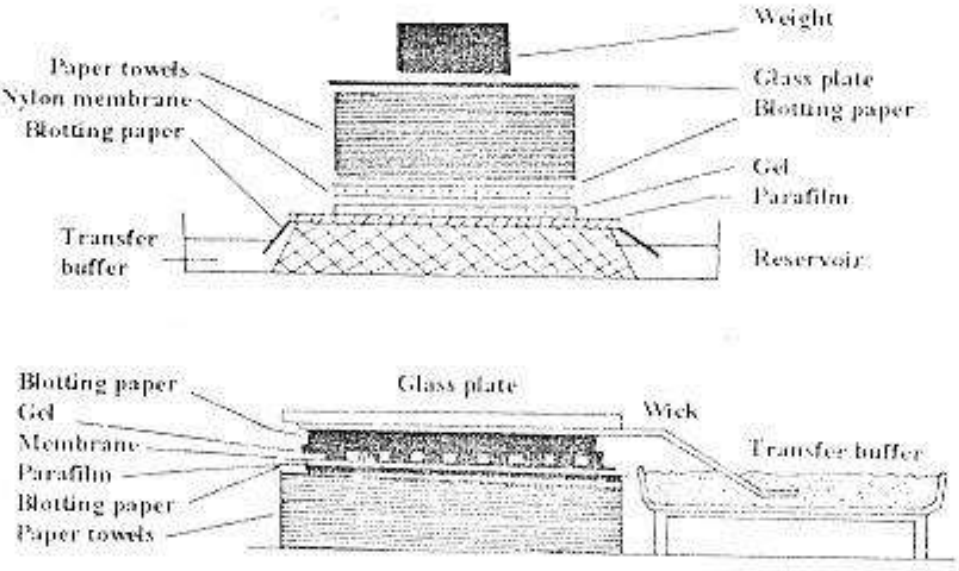


Figure 12.2: Capillary transfer of nucleic acid from agarose gels to nylon membranes. (a) standard upward capillary transfer. The direction of fluid is from the reservoir up through the gel and membrane and into the paper towels. (b) downward capillary transfer. Fluid is transferred via the wick down through the gel and membrane. In both cases, nucleic acids are eluted from the gel by capillary action and are immobilized on the membrane. Transfer is more rapid with the downward transfer system: the lack of weight above the transfer stack prevents gel crushing and inhibition of buffer flow.

Method

1. Harvest virus infected leaves, remove midrib and weigh out 0.5 g leaf material in 4 ml polyethylene tube or collect 5-10 leaf disc in Eppendrof tube.
2. Place tube immediately into liquid nitrogen until the leaf material completely dry before grinding into fine powder. Alternatively samples can be ground in mortar and pestle.
3. Add equal amount of hot (80°C) extraction buffer and phenol (1:1) and vortex mix.
4. Add one volume of chloroform : isoamyl alcohol (24:1 v/v), and vortex mix.
5. Centrifuge at 10,000 g for 5 min and remove upper liquid phase and add equal volume of 4 M LiCl and store at 4°C for overnight.
6. Centrifuge at 10,000 g for 10 min and discard liquid phase.
7. Resuspend the pellet in sterile distilled water, 0.1 volume of 3M sodium acetate and 2 volumes of absolute alcohol.
8. Store the sample at -70°C for not more than 30 min or -20°C for overnight.
9. Centrifuge at 10,000 g for 10 min, discard the liquid phase and wash the pellet first with absolute alcohol and then with 70% alcohol.
10. Dry off pellet under vacuum for 2 min or air dry at room temperature for 30 min.
11. Resuspend the pellet in 100 ml Tris/SDS buffer or nuclease free water.
12. Quantify RNA concentration with GeneQuant™ Photometer (Pharmacia) and store at -20°C until use.

The RNA will keep longer if dissolved in Tris/SDS. However, if the RNA is to be used for PCR it must not be dissolved in this solution and should be dissolved in nuclease-free water, because SDS interferes with the PCR.

12.2.3 RNA detection by Northern blotting

Method

1. Resuspend RNA sample in nuclease-free water or appropriate buffer and quantify the concentration.
2. Prepare 1.25 % agarose gel by mixing 0.6 g agarose, 5 ml 10 x 3-[N-morpholino] propane sulphonic acid (MOPS) and 34 ml sterile distilled water.
3. Melt thoroughly, cool and add 11 ml formaldehyde (37%).
4. Pour gel in the gel plate in fume cupboard and leave the content to settle.
5. Prepare the sample by mixing:
   i.1 m110 x MOPS
   ii..3.5 ml Formaldehyde (37%)
   iii.5.5 ml RNA sample (5 mg of total cell RNA or 0.5 2 mg RNAextracted directly from virus particles)
   iv. 10 ml deionised formamide
6. Heat the sample at 55°C for 15 min and add 2 ml loading buffer.
7. Load the sample with known positive and negative controls and standard RNA markers.
8. Eectrophorese the sample for approximately 2-3 h at 60 mA with 1 x MOPS.
9. Transfer RNA onto nitrocellulose or nylon membrane using 10 x SSC as a transfer buffer.
10. Cut off marker with a scalpel, and place in a baking dish containing 100 ml of water and 10 ml of 10 mg / ml Ethidium bromide. Shake at room temperature for 10 min.
11. Trim and measure the gel and cut nitrocellulose or nylon membrane accordingly.
12. Place a piece of glass across the large dish. Wet two long pieces of filter paper in the 100 ml of 10 x SSC and drape across the glass to act as wick. Make sure that both ends of the wick are dipped in liquid.
13. Carefully place the gel on the wick. Air bubble should carefully be removed and corner of the gel and membrane should be well matched.

14. Wet the membrane and place at the top of the gel and put a pair of the filter paper on the top of the membrane.
15. Place about 3 inches of white absorbent paper towels on top and put a glass sheet with about 500 g weight on top of the glass sheet and leave overnight.
16. Remove the wet papers and carefully uplift the membrane and crosslink both sides with either UV Cross linker or by heating the blot to 80°C for 2 h.

12.2.4 Solution for the extraction of RNA and Northern blotting

RNA Extraction Buffer

100 mM lithium chloride
100 mM Tris-HCl, pH 8.0
10 mM EDTA
1% SDS

3M Sodium Acetate

Sodium acetate: 246.09 g/l
adjust to pH 5.8 with acetic acid and autoclave

1 X TE Buffer

10 mM Tris pH 8.0
1 mM EDTA make up to volume and autoclave

Chloroform-isoamyl alcohol
Mix chloroform and isoamyl alcohol in ratio of 24:1.
4 M Lithium Chloride
Lithium chloride: 169.56 g/l
Make up to volume and autoclave

10 X MOPS

0.4M MOPS pH 7.0
0.1M sodium acetate
0.01M EDTA

Dissolve all in 1000 ml water and autoclave. Upon aging the liquid will turn yellow, this will not affect the composition of the solution.

20 X SSC

Sodium chloride:	175.3 g
tri-Sodium citrate:	88.2 g
Distilled water:	100 ml

Make up the volume and autoclave.

10 X TBE

Tris :	108 g/l
Boric acid:	55 g
EDTA:	9.3 g

Deionized Formamide

5 ml of Formamide and 3 g Amberlite Resin.

Stir for 30 min before filtering and storing in dark bottle.

Tris/ SDS

100 mM Tris pH 8.0

0.2% SDS

Make up to volume and autoclave.

12.3 Detection of viral protein by Western blotting

12.3.1 Introduction: Western blotting is a procedure for the immuno-detection of electrophretically separated antigens immobilized on a solid support such as a nitrocellulose membrane (Figure 12.3). The separation is usually achieved by polyacrylamide gel electrophoresis in the presence of SDS (Sodium dodecyl sulphate). This system separates proteins on the basis of molecular weight, which adds to the attraction of the technique, since information can be obtained about a particular antigen which would be inaccessible to any other method. In addition to molecular weight, Western blotting can be used to determine the presence or absence of the antigen in a particular protein prerparation, together with its relative abundance in the preparation and whether the protein is subject to any degradation.

Once separated by electrophoresis, the proteins are transferred to a nitrocellulose sheet, again using an electrophoretic technique (electro-bloting), where they bind very strongly to the nitrocellulose. The wholesheet is then incubated with a solution containing a high conentration

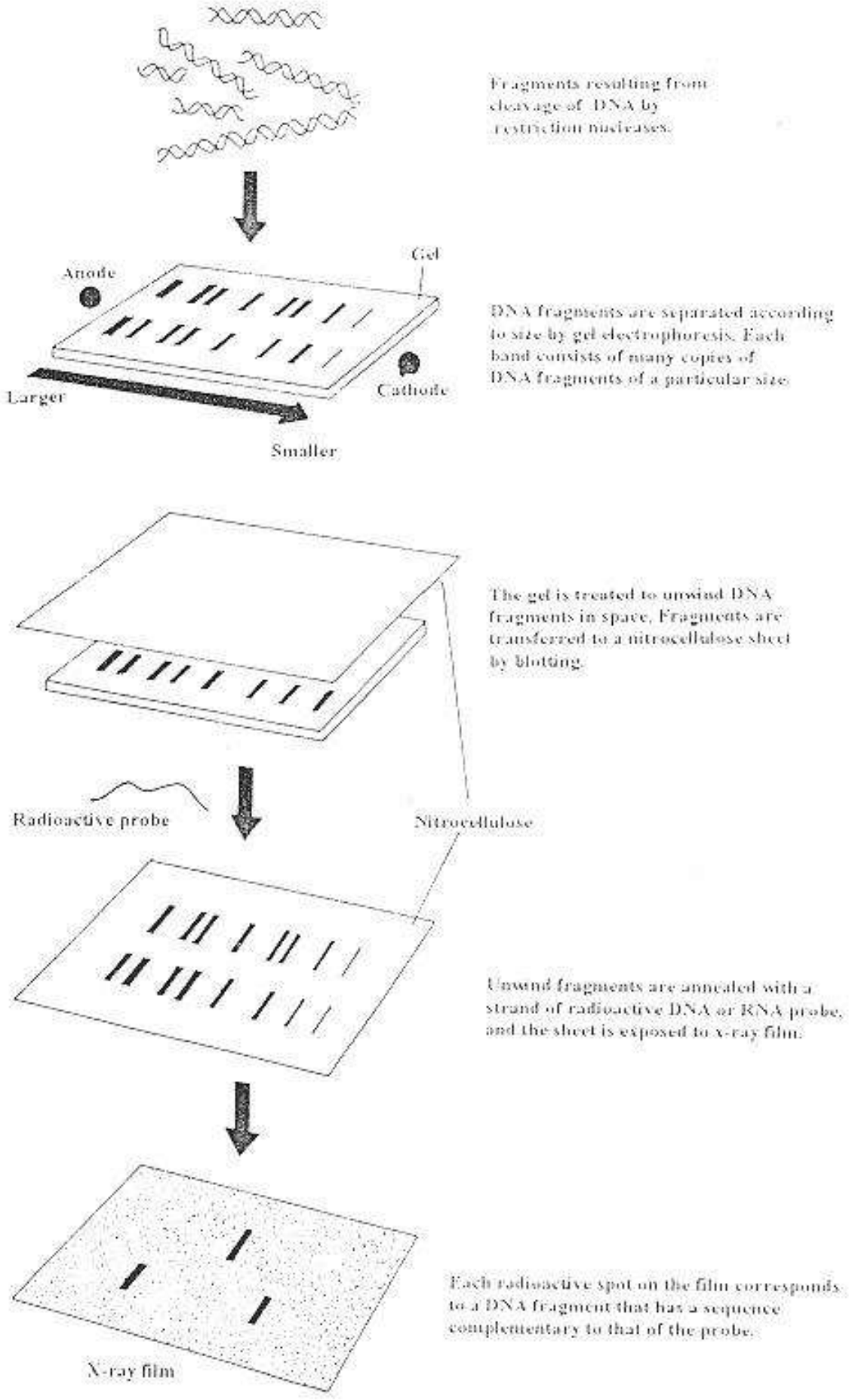


Figure 12.3: Deoxyribose nucleic acid (DNA) blotting.

of protein, usually milk in order to block that antibody molecules, which are themselves protein, to bind to the nitrocellulose which binds to its immobilized antigen. The resultant antigen-antibody complex must then be visualized in some way, and a number of methods have been devised for this purpose. A common method, known as a sandwich technique, employs a labeled antibody raised against antibody molecules purified from the same species of animal used to produce the specific serum. For example, if the specific serum was raised in rabbits, the antibody for its detection would be an antibody against rabbit immunoglobulins produced in a goat or donkey. A number of labels are available for these antibodies: they can be tagged with radioisotopes such 125I (iodine) and detected by autoradiography, or linked to small enzymes, such as horseradish peroxidase and alkaline phosphatase and visualized using a soluble chromogenic substrate which produces an insoluble product.

Western blotting is an extremely sensitive analytical technique. Quantities of antigen as low as 5 ng can be detected in crude protein extracts and even smaller amounts in purer preparations. Another useful feature of the Western blotting approach to gene product analysis is the ability to obtain quantitative data with relative ease.

12.3.2 Quick methods for protein extraction

1. Grind 0.5 g leaf in 3 ml of extraction buffer.
2. Pour into a 4 ml tube and boil for 10 min.
3. Spin at 5000 rpm for 5 mins.
4. Decant off the supernatant into a fresh tube and add glycerol to 10%. Freeze until ready to use.

Extraction buffer

10 mM Tris pH8
1 mM EDTA
10 g/l SDS
0.5 M mercaptoethanol

12.3.3 Polyacrylamide gel electrophoresis of proteins

Requirements

-10% ammonium persulphate solution
-Running buffer-1000 ml per gel
-Block buffer

Method

1. Weigh out 0.5 g ammonium persulphate into a 10 ml beaker and add 4.5 ml water.
2. Wash with very hot water and detergent, 2 glass plates (one must be bigger than the other), two plastic spacers with foam for the side and one for the base. Then rinse each item with alcohol and allow to dry. Check the glass plates very thoroughly for any streaks or smears and re-wash if necessary.
3. Assemble plates and spacers with 6 bulldog clips, 2 at the base and 2 at either side.
4. Make up solutions of plug, resolving and stacking sections of the gel, but do not add TEMED as the solutions will start to polymerize and harden. Be careful to use the correct pH of Tris for the stack and resolve gels.
5. Add the TEMED to the plug gel mixture and swirl gently. Pour between the plates to form a seal across the base.
6. Leave t o harden for 5 min.
7. Once the plug has solidified, add TEMED to the resolving gel mixture. Tilt the plate gently back and pour the gel mix slowly, making ure the top is level once it is poured in. Add a pipette full of water (about 2 ml) across the top slowly and leave to harden for at least 45 min. Check that the water levels off and forms a distinct meniscus. The gel can be poured the day before it is to be used and can be left overnight at this stage. Cover the top of the gel with film.
8. Pour off the water on top of the gel and add fresh water to wash. Remove any last traces of water with filter paper.
9. Add TEMED to the stacking gel and mix gently. Pour onto the top of the resolving gel. Immediately press in an alcohol washed Teflon comb prizing out any air bubbles that become trapped. Fill air gaps

by pipetting remaining gel mixture. Leave to solidify for at least 45 mins.

10. Make up 1000 ml of running buffer as the stacking gel hardens.
11. Mark on the glass plate the numbers of the wells with a marker pen as this will help to orientate the gel later.
12. Denature the samples by heating at 100°C for 3 min.
13. Add running buffer to the bottom well of the electrophoresis kit and allow bubbles to disappear. Once the stacking gel has fully set, remove the bottom bulldog clips and lay the glass plates on the bench. Leaning hard on the plate to prevent an air bubble entering, gently pull out the plastic seal across the bottom of the gel. Now place the gel into the kit. Carefully, put one end in first to prevent air bubbles becoming trapped at the bottom. Attach the gel to the kit with the clips at the edge of the tank. Pour in running buffer into the top well of the tank. Check that there are no trapped air bubbles or leaks from the top tank. If there are air bubbles present, remove them with a glass pipette because the gel will not run properly otherwise. Now gently rise up the comb and slowly remove it from the gel, this is to prevent the wells collapsing. If the wells collapse or bend, they can be straightened by gently pulling them upright using a glass capillary.
14. Dispense 5 ml of methyl green into every well that is to be sued. Switch on the power pack to 140 volts and let the gel run for a few mins until the dye has just passed out of the wells. Switch off the power. The methyl green will not affect the running of the gel and will help orientate the positioning of the sample later.
15. Do not load samples into the first or last wells. Therefore, the maximum number of samples that can be loaded is 18. Load up to 50 ml of sample, starting with the most dilute. If possible, use gel loading tips.
16. Run the gel at 110 volts until the marker dye has just entered the resolving gel. Now turn up the voltage to 140 volts. Leave until the dye is approximately 2 cm from the top of the plug. Switch off the voltage and add a further 5 ml of methyl green to the wells. Turn on the power again and leave until the dye is about to enter the plug. Switch off the power. The samples are now ready to be transferred to nitrocellulose.

12.3.4 Solutions for Western blot

Note: Mix the components in the order shown as polymerization will begin as soon as the TEMED has been added.

Plug	
30% acrylamide + 8% bisacrylamid	1.75 ml
1M Tris, pH 8.8	1.9 ml
distilled water	1.1 ml
10% SDS	50 ml
10% ammonium persulphate	180 ml
TEMED	18 ml

Resolving Gel (for a 12.5% gel)	
30% acrylamide + 8% bisacrylamide	12.5 ml
1M Tris, pH 8.8	11.25 ml
distilled water	5.5 ml
10% SDS	0.3 ml
10% Ammonium persulphate	0.1 ml
TEMED	15 ml

Stacking gel (for a 5% gel)	
30% acrylamide + 8% bisacrylamide	1.5 ml
1M Tris, pH 6.8	1.25 ml
distilled water	7 ml
10% SDS	0.1 ml
10% Ammonium persulphate	0.2 ml
TEMED	5 ml

12.3.5 Transfer of protein from gel to solid supports

Requirments

-4000 ml transfer buffer
-nitrocellulose and filter paper
-400 ml washing buffer

Method

1. While the gel is running, make up 4000 ml of transfer buffer.
2. Measure the width of the gel while it is still in the kit. The length of the gel should be 110 mm so cut 2 pieces of filter paper to this size as well as 1 piece of nitrocellulose. Remove the right hand corners of each to help in orientation afterwards.
3. Pour a small volume of transfer buffer into 2 roasting dishes. Slowly wet up the nitrocellulose and filter papers in one dish. Leave to soak while getting the transfer tank set up.
4. Load a set of plates, which will hold the nitrocellulose and gel, into the tank and pour in enough buffer until the electrodes are covered.
5. Switch off the voltage to the polyacrylamide gel and disconnect from the power pack. Pour out the running buffer and remove the bulldog clips. Take out the plates and lay on the bench so that the gel is correctly orientated with the samples positions running left to right. Remove the plastic clips and carefully remove away the top glass plate. Do this very slowly so that the gel does not stick or tear. It is essential to keep the gel wet at this stage as it will tear very easily when dry. With a sharp scalpel, using a downward cutting motion, cut off the stack and the right hand corner of the gel.
6. Gently lift the gel and place in a dish of transfer buffer. Leave to soak for at least 30 mins to allow the gel equilibrate and prevent any change in size.
7. Remove the plates from the transfer tank and open laying flat on the bench, back side down. Make sure the two pieces of foam on either side are fully wetted up. Place one of the pieces of filter paper on the bottom plate and smooth out any air bubbles. Carefully overlay the filter paper with the gel and remove out any air bubbles. Place the wet nitrocellulose on top of the gel and smooth out any air bubbles using a wet test tube. Place the other piece of wet filter paper on top of the nitrocellulose.
8. Close the plates and load into the tank so that the black plate matches the black (negative) electrode.
9. Run the gel overnight at 30 volts, 100 mA with an upper cut-off limit of 300 mA.

12.3.6 Immunological detection of proteins

Requirements
-300 ml block buffer
-1 mg/ml MAb diluted in block buffer
-1000 ml TBS/TWEEN 20

Method

Note: The volumes given are for a full-size blot of 110 x 140 mm. If the gel is smaller it may be possible to cut down the liquid used in the bags thereby saving monoclonal antibodies and conjugate.

1. To ensure the correct transfer of protein, stain the membrane in Ponceau's stain for about 1 minute. Rinse the membrane under the tap until most of the stain has disappeared. The stain is non specific and will stain most protein bands present. It is used to ensure that transfer has taken place from the gel to the membrane. The membrane can be placed between sheets of plastic and photocopied as a permanent record.
2. Pour approximately 200 ml of blocking buffer into a roasting dish, this must be added first otherwise the blot will stick to the glass. The milk will turn pink in colour from the Ponceau's stain but this will not affect the result.
3. Switch off the power to the tank and remove plates containing blot. Allow any liquid to drain back into the tank.
4. Remove the blot from apparatus and float in the blocking buffer for an hour at room temperature, shaking gently.
5. While the blot is blocking, dilute the MAb in block buffer to give a concentration of 1 ml/ml; (i.e. add 40 ml of 1 mg/ml MAb to 40 ml of Block Buffer for an average blot). This should be made up in a plastic container because the monoclonal antibodies will stick to glass.

Note: To cut down on background non-specific staining, it is possible to dilute the MAb in leaf sap rather than block buffer alone. The sap is prepared by grinding 1g leaf material in 5-10 ml block buffer and then filtering through 2 layers of muslin.

6. Once the blot has been blocked, remove it from the buffer and seal it in a plastic bag, leaving one side open. Add the MAb and seal the final edge. Leave to shake quite briskly for 2 hours at room temperature.
7. Cut round the blot and pour out the monoclonal antibodies. Now wash the blot 3 to 5 mins in TBS TWEEN in a roasting dish, shaking gently at room temperature.
8. While the blot is washing make up the conjugate. If using a monoclonal antibody for detection, then use an anti-mouse conjugate, if using a polyclonal antibody then use an anti-rabbit conjugate. Use at the manufacturer's recommended dilution. Seal the blot in a bag containing the conjugate as before and incubate for 2 hours at 37°C.
9. Cut round the blot and pour out the conjugate. Now wash the blot 3 x 10 mins in TBS TWEEN, shaking gently at room temperature.
10. Just before it is needed, add 200 ml of NBT + 200 ml BCIP to 20 ml substrate buffer. Reseal the blot in a bag and add the NBT/BCIP. Shake gently at room temperature until the reaction is complete. If nothing appears within one hour then the blot can be left cover night. Once the reaction is complete wash the blot in tap water. Leave to air dry and then seal in a plastic bag to keep.

12.3.7 Solutions for Western blot

Running buffer

0.1% SDS in 1 x Tris/Glycine

Tris/Glycine

Glycine	144 g
Tris	30.3 g

Dissolve both and make up to 1000 ml with distilled water and autoclave.

Substrate buffer

Tris	100 mM pH 9.5
NaCl	100 mM
MgCl	5 mM

Methyl Green
Add a few grains of methyl green to 50% glycerol and distilled water. Prepare freshly before use.

Ponaceau's stain
0.5% Ponceau's stain in 10% acetic acid.

Transfer buffer
12.12 g Tris
57.60 g Glycine
Dissolve both, and make up to 3200 ml with water. Then add 800 ml methanol.

5 x TBS
30 g Tris 11.7 g NaCl
Make up to 1000 ml and adjust pH to 7.4 and autoclave.

TBS Tween
200 ml of 5 x diluted to 1000 ml then add 1 ml of Tween 20 to give a 0.1% solution.

Blocking buffer
1 x TBS Tween 20
5 g Powdered milk per 100 ml TBS

Nitro blue tetrazolium (NBT)
Make up a solution in 70% dimethyl formamide (DMF) to give a concentration of
30 mg/ml.

BCIP
Make up a solution in 100% dimethyl formamide (DMF) to give a concentration of
15 mg/ml.

Note: NBT/BCIP can be bought in tablet form from Sigma, called Sigma "Fast" tablets. These are dissolved directly in 10 ml sterile distilled water and saves weighing any dry powders which are considered unsafe.

12.4. Detection of viral pathogens by Polymerase Chain Reaction (PCR)

12.4.1 Introduction: The polymerase chain reaction (PCR) was introduced in 1985 by scientists from the Cetus Corporation and has since become an integral part of the molecular biology. The PCR methods rely on prior knowledge of at least some of the nucleotide sequence of the virus or viroid. Part of the sequence is chosen as target for detection and the methods are capable of producing millions of copies of this target sequence in a few hours by a cyclical enzyme reaction. This method uses the ability of piece of DNA using a primer and bind to a target sequence to its elementary sequence, on another DNA strand. Then an enzyme called a polymerase adds individual nucleotides to the end of the primer to make a new piece of DNA that is complementary to the DNA target sequence. By repeating this process several times, the target DNA sequence is amplified exponentially (Figure 12.4).

In the basic form of PCR the researcher has to know the sequence of the bases on either side of the target DNA before this DNA can be amplified. The researcher then make primers that are complementary to these sequences. Amplification can be achieved in a small volume of liquid (50 ml) to which the researchers adds the DNA to be copied, the individual nucleotide bases (A, T, G, C), the primer, the polymerase enzyme and a buffer to maintain optimal conditions for the reaction. The mixture is heated, which separate two strands of the target DNA, normally held together to bound between complementary bases. The mixture is cooled and the primers find their complementary sequences on the long strands of DNA and bind to them. The polymerase enzyme locates where the primers have bound to the DNA and join individual bases to the end of the primer to form a new strand of DNA. The polymerase uses the target DNA as a template, each base that is added to the new DNA will be complementary to its opposite number in the target sequence. The sequence of newly amplified DNA is exactly

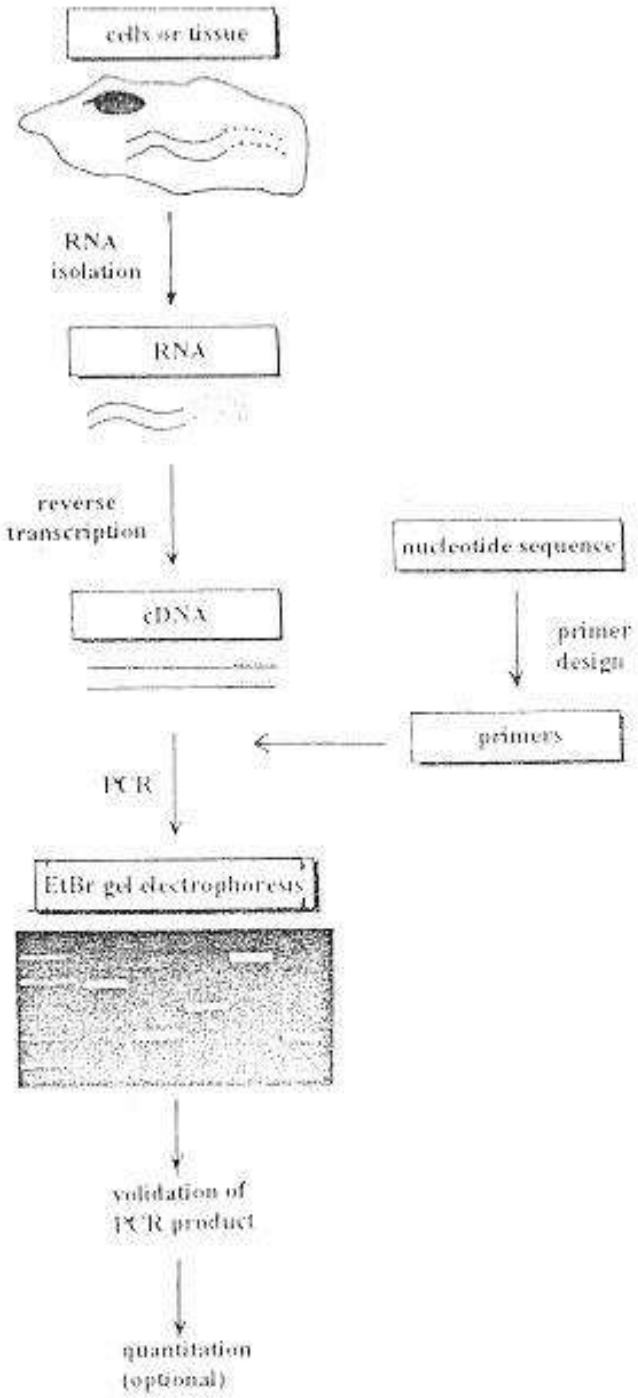


Figure 12.4: Steps in Polymerase Chain Reaction (PCR).

complementary to the sequence of the target DNA, and the two strands are bound together. After heating newly synthesized DNA and the original DNA separate into single strands. Originally there were just two copies of the target, now there are four. Again mixture cools, the primer bind to the target sequence and the polymerase builds a new strand of DNA on the target, producing eight copies of the target. Each new cycle of heating, cooling and polymerization double the number of copies of the target DNA. After PCR, the researcher can purify the amplified sequence by separating the DNA in an agarose gel with the aid of an electric current. This sorts DNA by size; since all the copies of the target DNA are the same length, they will lie in a neat band on the gel. Under ideal conditions, the number of copies doubles with each round of denaturing, annealing, and polymerization. After 30 cycles there will be more than a billion copies of the target DNA. In the case of viruses with RNA genomes, the nucleic acid is first reverse transcribed using an enzyme called reverse transcriptase in the presence of down stream primer, buffer and a set of four deoxyribonucleoside triphosphate (dNTPS). This first strand complementary DNA is used for PCR amplification.

12.4.2 Optimization of reaction conditions: The selection of appropriate timing, temperatures and number of cycles depends on the DNA being amplified and primers chosen. Reaction volumes vary from 20-100ml. Small volumes are an advantage if screening or testing large number of samples, because of saving in reagent costs, but only a few samples are being processed it is technically easier to work with large volumes. Incubation times should be kept as short as possible to reduce the overall cycling time and to minimize the risk of non-specific amplification. Denaturing and annealing times of 30 sec should be enough and extension times allow 1 min per kb with a final extension time of 2 min should be adequate. The number of cycles required depends on the amount of target but 25-35 cycles are adequate. Any more than 40 cycles increases non-specific amplification. If large number of cycles is required, it is preferable to use the PCR product from one reaction as a template for a second reaction.

12.4.3 Components used in a PCR

12.4.3.1 Primers: Oligonucleotides are usually 18-30 bp in size with similar G + C content (approximately 50%). Working concentration of primers are generally 25-100 pmol of each primer for 50 ml reaction. If added in excess they can inhibit a reaction. Add 1 ml of a 1 mg/ml concentration of primer in a 100 ml reaction sample.

12.4.3.2 Deoxyribonucleoside triphosphate: These are used to provide both the energy and nucleotides for the reaction. They are present in excess so the reaction can be repeated many times. They are stable at -20°C for months but should be aliquoted into small quantities.

12.4.3.3 DNA polymerase: The most commonly used DNA polymerase is Taq polymerase and can be obtained from any supplier. When using a particular Taq for the first time it should be titrated against dNTPS, concentration primer and RNA sample.

12.4.3.4 Buffer: Always try to use the buffer supplied with the Taq polymerase. There is known interaction between dNTPS and Magnesium, concentrations. The high concentration of dNTPS bind to Magnesium and hence reduces its availability. Therefore if high concentrations of dNTPS are to be used then it is necessary to increase the Magnesium concentration above the suppliers' recommended level.

12.4.3.5 Template: When trying primers for the first time they should be titrated against known quantity of template to determine the best to use. A rough guide is 1 mg for DNA and 500 ng of RNA but it is absolutely dependent on virus and its chemistry. In PCR reaction , content should be added in the following order:

H_2O
10 x buffer
dNTPS
Primers
Template
Enzyme

12.4.4 Extraction of nucleic acids for PCR

12.4.4.1 Extraction of RNA: Nucleic acids from RNA viruses can be obtained from purified virus preparation. Virus particles can be diluted in Tris-EDTA buffer or any other appropriate buffer and viral protein can be removed by incubating the RNA samples with proteinase K followed by phenol: chloroform (1:1 v/v) extraction. After the ethanol precipitation RNA can be diluted in water for PCR. Total cell RNA can be extracted from plant samples for RT-PCR method. The steps involved in RT-PCR have been shown in Figure 12.5.

12.4.4.2 Extraction of DNA

Materials and Reagents

TE saturated phenol
Phenol : chloroform (1:1 v/v solution)
Chloroform : octanol (24:1 v/v solution)
3M sodium acetate
Absolute alcohol
DNA extraction buffer

Method

1. Grind leaf pieces under liquid nitrogen the same way as in RNA extraction or using pestle and mortar depending upon nature of sample and quantity.
2. Add DNA extraction buffer (1 ml/0.2-0.5 g leaf material) and mix well
3. Extract with saturated phenol (1 ml/0.2-0.5 g leaf material) and heat the content at 56°C.
4. Spin for 5 min at 10,000 g and transfer top layer to a fresh tube.
5. Extract with equal volume of phenol : chloroform to the liquid and mix well.
6. Spin for 5 min and remove top layer again to a fresh tube and add an equal volume of chloroform : octanol or chloroform : isoamyl alcohol (24:1) and vortex mix.

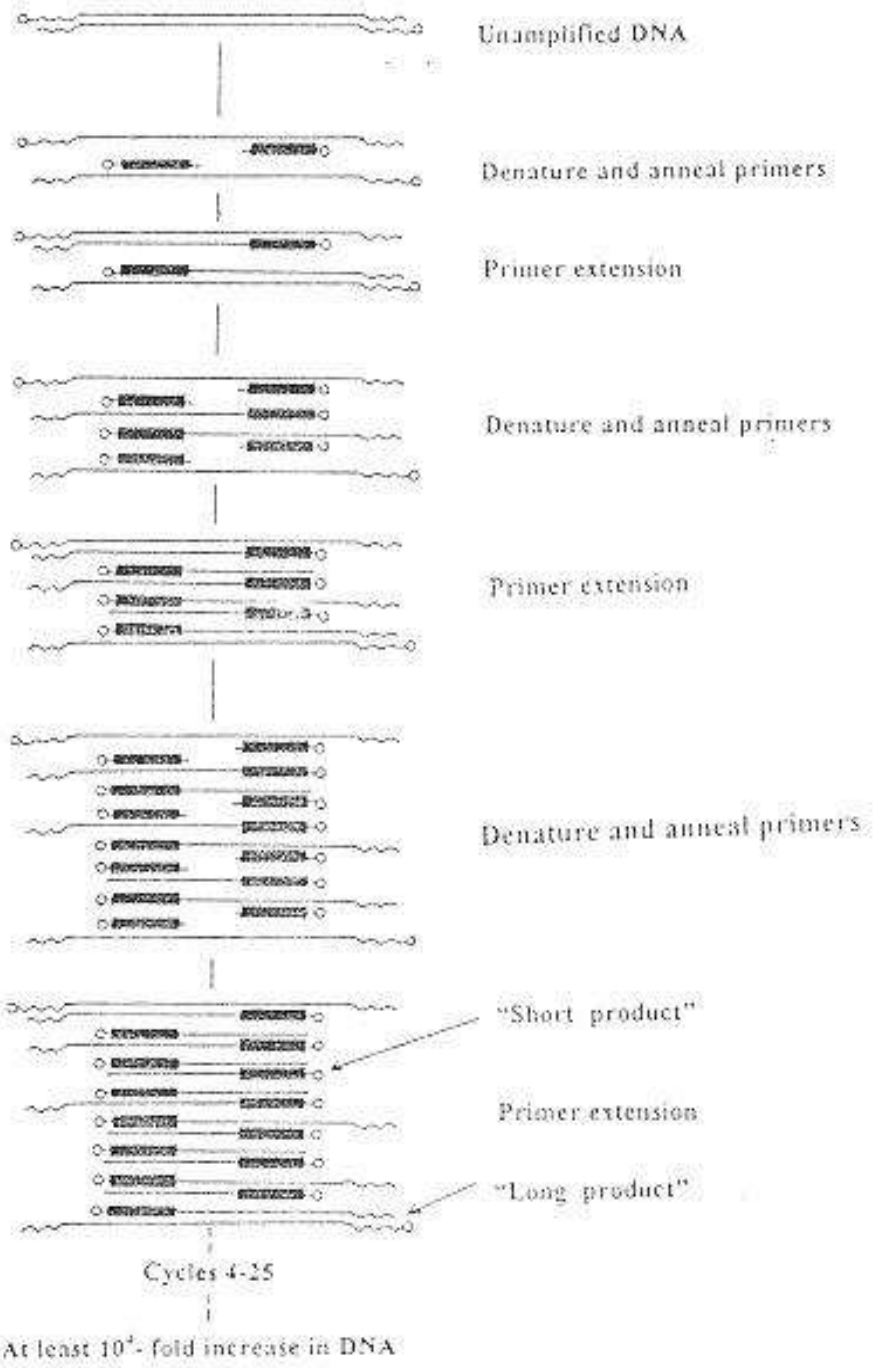


Figure 12.5: Schemetic diagram of the RT-PCR method.

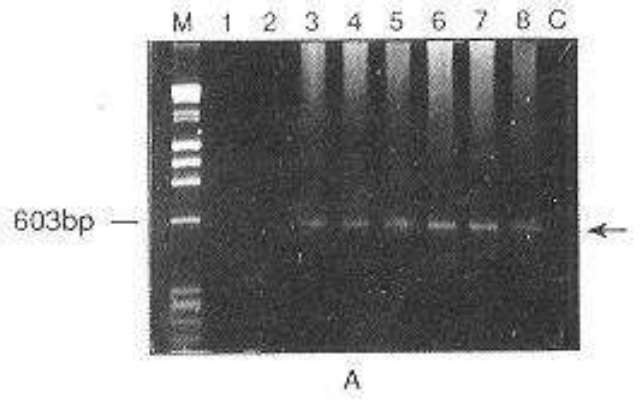
7. Centrifuge for 5-10 min at 10,000 g, remove top layer and add 0.1 volume of 3M sodium acetate , 2 volumes of absolute alcohol and precipitate overnight at -20°C.
8. Centrifuge DNA samples at 10,000 g for 5-10 min to pellet the DNA and resuspend the pellet in 100 ml TE buffer.
9. Add RNase to give final concentration of 10 mg/ml and incubate at 37° C for 1h.
10. Addd 0.1 volume of 3M sodium acetate and 2 volumes of absolute alcohol and incubate for 2 h at -20°C or -70°C for 30 min.
11. Centrifuge at 10,000 g for 10 min and resuspend pellet in 100 ml TE buffer.
12. Add 20 ml 3M sodium acetate and 250 ml absolute alcohol and Incubate for 2 h at -20°C or at -70°C for 30 min.
13. Centrifuge DNA samples at 10,000 g for 5 min and resuspend pellet in 100 ml TE buffer or nuclease free water.
14. Quantify the DNA concentration as described in Section ?

12.4.5 Solutions for extraction of DNA

DNA extraction buffer

50 mM Tris HCl pH 8.0
0.7 M NaCl
10 mM EDTA
1% CTAB
1% mercaptoethanol

12.4.6 PCR amplification for RNA viruses: The detection and amplification of nucleic acid from viruses containing RNA genome, a single-stranded RNA template should first be transcribed into complementary DNA by initial incubation with reverse transcriptase (Figure 12.6). Prior to the PCR, the purified RNA is incubated with dNTPS, downstream primer (primer at the 3'-end), RNase inhibitor, 10 x buffer and reverse transcriptase. The mixture is usually incubated at 37°C or 42°C for 2 h before heating at 95°C for 5 min to inactivate the reverse transcripitase. After a quick chill on ice, fresh dNTPS, 10 x buffer, both primers and Taq polymerase are added before cycling on PCR machine for the allocated number of cycles.



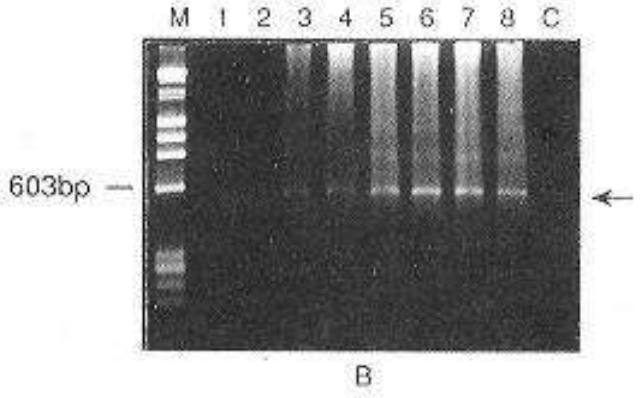


Figure 12.6: Detection of PMTV RNA sequence by RT-PCR of the (A) roots and (B) leaves of *Nicotiana debneyi*. PCR products were electrophoresed in 1% agarose gel and stained with 0.5 ug/ml ethidium bromide. Lanes contain: M, DR1 gest 111 molecular size markers, the 603 bp band is indicated; 1-8 are the numbers of the weeks that samples were taken; C, virus-free controls. Arrows indicate the positions of the PMTV specific 566 bp bands.

Method

Materials and reagents

10 x PCR buffer
2 mM dNTPS
RNA Guard
RNA reverse transcripitase
DEPC-treated water
Upstream and downstream primers
Taq polymerase
Light liquid parafilm

12.4.7 Reverse transcription

1. Spin RNA samples at 10,000 g for 5-10 min. Wash pellet with 70% ethanol and dry under vacuum or leave at room temperature to air dry for 30 min.
2. Resuspend the pellet in DEPC-treated water and quantify the RNA concentration by GeneQuant™ photometer (Pharmacia) or spectrophotometer by measuring OD.
3. In a 0.5 ml Eppendorf mix the followings:4 ml 4 x reverse transcriptase buffer2 ml 2mM dNTPS1 ml downstream primer (e.g 1 ug/ml)5 mg (total RNA) or 0.2-.=0.5 mg vRNA.Make volume up to 10 ml with DEPC-treated water (A master mix can be made for detection of many samples).
4. Heat at 56°C for 5 min and cool on ice.
5. Give the sample a quick spin in centrifuge.
6. Add, 0.5 ml of RNA Guard (RNA inhibitor) and0.5 ml of RNA reverse transcriptase (or as recommended concentration by the manufacturer).
7. Heat in water bath for 2 h at 37°C or 42°C (or as recommended concentration by the manufacturer).

12.4.8 Polymerase chain reaction amplification

Note: This protocol is described for amplifications performed using the GeneAmp 9600 thermocycler(Perkin-Elmer) with thin wall "microamp" tubes (Perkin-Elmer).

1. **Mix**
   10 ml 10 x buffer
   5 ml 2 mM dNTPS
   0.2-0.5 mg/ ml downstream and up stream primers
   2-5 ml of reverse transcriptase mixture
   make volume up to 50 ml with diethylpyrocarbonate (DEPC)-treated water 0.5 ml Taq polymerase (as recommended by the manufacturer)
   or
   make PCR master mix and dispense 45 ml -49.5 ml into thin wall "microamp" tubes (Perkin-Elmer).
2. Mix 0.5 ml -5 ml (depending upon the dilution of RT mixture) in PCR mixture. All specimens and controls should be added in duplicate reactions. No liquid parafilm is needed when using the GeneAmp 9600 thermocycler (Perkin-Elmer).
3. Load into the GeneAmp 9600 thermocycler (Perkin-Elmer) PCR machine and use appropriate program (e. g. denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec; synthesis at 72 for 1 min with total of 20-30 cycles).
4. Run 5 ml of 50 ml PCR reaction onto 1% agarose gel with Ethidium bromide (0.5 mg/ml) and analyze the PCR amplification result (Figure 12.7).

12.5 Digoxygenin non-radioactive hybridization

12.5.1 Introduction: Recently, a number of different systems have been developed substituting non-radioactive (non-isotopic) repoter molecules in place of radioactive ones for the detection of viruses in nucleic acid hybridization assays. The most popular one is digoxygenin (DIG) in which anti-digoxygenin antibody is covalently conjugated to an enzyme such as alkaline phosphatase or horseradish peroxidase. The

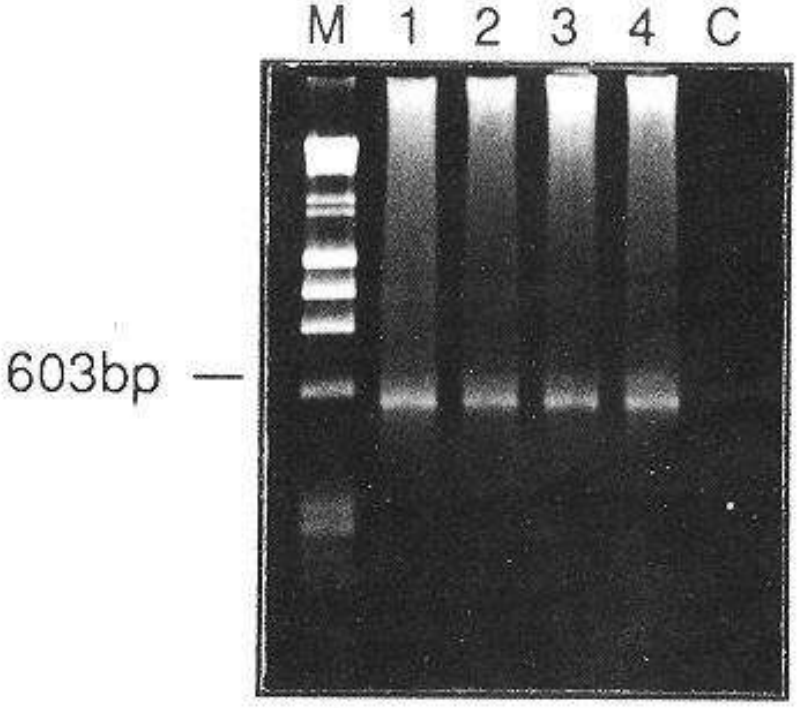


Figure 12.7: Detection of PMTV RNA sequences by RT-PCR of potato tubers of four cultivars. Lanes contain: M, DR1gest 111 molecular size markers, the 603 bp band indicated; 1, cv. Hansa; 2, cv. Nicola; 3, cv. Jaerla; 4, cv. Pentland Ivory; C, virus-free control, Arrow indicates the position of the PMTV specific 566 bp band.

location of the hybridized target sequence is then visualized by incubating the membrane with a signal-generating substrate. This method is safe and probes can be stored for years with no loss of action.

12.5.2 Non radioactive probe labeling (Method 1)

1. Resuspend 1 mg template DNA in 16 ml of sterile water
2. Denature the DNA thoroughly by boiling the template for 10 min and chilling on ice-salt-water mix until cool to the touch.
3. Add 4 ml of DIG High Prime, mix and centrifuge briefly
4. Incubate overnight at 37°C.
5. Stop the reaction by adding 2 ml 0.2M EDTA , pH 8.0.
6. Determine the concentration of the probe in a spectrophotometer.

12.5.3 Labeling non-radioactive probe by PCR (Method 2)

1. Resuspend 1 ml template DNA in 5 ml of water
2. Mix 10 x PCR buffer 5 ml
   $MgCl_2$(.....%) 2 ml
   10 x PCR DIG mix 5 ml
   Primer 1 1 ml
   Primer 2 1 ml
   Taq DNA polymerase 0.5 ml;
   Template DNA 5 ml
   Make volume with water up to 50 ml
3. Mix well and spin briefly to collect the liquid at the bottom.
4. PCR
   hold at 95 for 5 min
   Then 30 cycles
   95°C for 45 sec
   60°C for 1 min
   72°C for 2 min
5. Remove the samples and store at -20 °C .

12.5.4 Quantification of the labeled probes

PCR probes can be quantified using following protocol

1. Make serial dilution of 1 ml of probe. Serial dilution can be made approximately 10-20 dilution in TE buffer.
2. Dot 1 ml of each dilution on nylon membrane and crosslink both sides.
3. Wash the blot for 1 min in washing buffer.
4. Incubate the blotting membrane for 30 min at 100 ml of Buffer 2.
5. Incubate for 30 min in 20 ml of diluted Dig Fab (1:100) in Buffer 3.
6. Wash 2 x 15 min with 100 ml of washing buffer.
7. Wash for 1 min in Buffer 3.
8. Incubate for 5 min with 2 ml of Lumigen (1:100) in Buffer 3.
9. Seal the blot in a plastic bag and incubate at 37°C for 15 min.
10. Expose on film for 2 - 5 min as necessary.

The concentration of probe can be estimated by making serial dilutions of probe and last dilution will be detected at the concentration of 0.1pg. The concentration of serial dilution of the probe can be estimated accordingly.

12.5.5 Hybridization

1. Dilute and dot probe on nylon membrane as above.
2. Crosslink the nylon membrane on both side.
3. Pre-warm the roller containing DIG Easy Hybridization Buffer to 42°C.
4. Incubate the membrane for 30 min to prehybridize.
5. Denature the probe by boiling for 10 min and fast chilling on ice.
6. Add the denatured probe to pre-warm DIG Easy Hybridization Buffer and mix.
7. Remove the prehybridization buffer and replace with probe containing solution. Do not allow the blot to dry.
8. Incubate overnight at 42°C.

12.5.6 Detection of hybridized nucleic acids

1. Wash 2 x 5 min at room temperature (22-25°C) with 50 ml of 2 x SSC + 0.1% SDS in a backing tray. Wash again 2 x 15 min at 68 with 50 ml of 0.1 x SSC + 0.1% SDS in roller with preheated buffer.
2. Wash the blot for 1 min in washing buffer in open dish.
3. Incubate at 37°C for 30 min in 100 ml of pre-warm Buffer2.
4. Incubate at 37°C for 30 min with 20 ml of diluted Fab (10,000 in Buffer 2).
5. Wash for 2 x 15 min with 100 ml of washing buffer.
6. Wash 1 min in Buffer 3.
7. Incubate for 5 min with 2 ml of Lumigen (1:100 diluted in Buffer 3).
8. Seal the blot in fresh plastic bag and incubate at 37°C for 15 min.
9. Expose to film for 30 min to 24 h as necessary.

Note: The recipes used in detection of hybridized nucleic acid is for a 10 x 10 cm blot.

12.5.7 Buffers and solutions for digoxygenin system

2 X SSC 0.1% SDS
100 ml of 20 x SSC
10 ml of 10% SDS
Make up to 1000 ml with distilled water and autoclave

0.1 X SSC 0.1% SDS
5 ml of 20 x SSC
10 ml of 10% SDS
Make up to 1000 ml with distilled water and autoclave
Buffer 1

0.1 M Malic acid	11.65 g/l
0.15 M Sodium chloride	8.76 g/l

Dissolve in distilled water and adjust pH to 7.5 with concentrated NaOH.
Make up to volume and autoclave.

Blocking stock solution
Make up 10% blocking reagent in Buffer 1 and heat until boiling and mix, then autoclave and store at 4°C.

Washing buffer
Buffer 1 + 0.3% Tween-20

Buffer 2
Block stock solution diluted (1:10) in Buffer 1.

Buffer 3

0.1 M Tris	12.11 g/l
0.1 M NaCl	15.84 g/l

Dissolve in distilled water and adjust pH to 9.5 with concentrated NaOH. Make the volume and autoclave.

Hybridization buffer
50% deionised formamide
1% blocking stock solution
5 x SSC
0.02% SDS
0.1% laurylsarcosine
sterile water

12.6 *In situ* hybridization

12.6.1 Introduction: *In situ* Hybridization (ISH) was first described in 1969 for localizing specific DNA sequences on chromosomes in cytological preparations of *Xenopus* oocytes. Modification of the technique permit the detection and localization of nucleic acids within tissues from a variety of different organisms. ISH has advantage over other molecular techniques that it can provide information about the location of the target nucleic acids within cell and or tissues. Pathologist can look for specific nucleic acids and also study the cellular and tissue morphology of the sample. ISH is the best diagnostic tool for plant viruses, where there is no chance to culture. ISH can also helpful in case when immunological

diagnosis of viral infection is difficult and when there are no reliable, commercially available serological reagents.

In ISH, the target molecules are nucleic acids. Nucleic acids are complex substances with proteins in the cells; when a tissue is embedded in a complex matrix the nucleic acids are cross-linked to that matrix. The technique is applicable to wide range of tissues and widely used for plant viruses (detection and localization of tobacco rattle virus in and around infection sites on tobacco leaves). ISH techniques described in this section have been divided into different sections and many steps are closely interrelated. Standard practices for working with RNA should be employed at all stages.

12.6.2 Fixation and wax embedding

1. Cut plant material and place in fix on ice (for 5 x 10 mm leaf pieces use 25 ml of fix solution).
2. Pour off fix and replace with ice cold 0.85% NaCl. Leave on ice for 30 min on shaker.
3. Repeat for 90 min each with series of ethanol solution (50%, 70%, 85% each containing 0.85% (w/v), NaCl, then 95% ethanol (No NaCl) and 100% ethanol).
4. Renew 100% ethanol and leave overnight at 4 degree °C.
5. Renew 100% ethanol and leave for 2 hours at room temperature. Replace with 50% ethanol and leave for 1 h.
6. Repeat three times with 100% ethanol. Add paraplast chips (BDH) to half the volume of the ethanol and leave overnight.
7. Incubate at 40-50 °C until paraplast chips dissolve. Replace with molten paraplast at 60°C and leave of water.
8. Renew paraplast each morning and evening. Repeat three times. Make wax blocks by pouring some paraplast with the material into a mold then float the mold on water to solidify the wax and store wax blocks at 4°C.

12.6.3 Preparation of slides and coverslips

1. Dip slides in nitric acid (Green treated) for 30 min.
2. Wash for at least 1 h with distilled water. Change water after each wash.
3. Wash for 15 min in acetone, and drain.
4. Bake at 180 °C for at least 2 h, and cool.
5. Add 5 ml poly-L-lysine hydrobromide in water and draw into a film over the slides using a coverslip.
6. Dry the slides at 42 °C on hot plate overnight and store in a box with desiccant at 4°C.

12.6.4 Sectioning of wax embedded material

1. Cut the wax block to a trapezoid shape, leaving about 2 mm of wax around the plant material.
2. Mount the block such that the longer of the two parallel faces is at the bottom.
3. Cut ribbons of section of 10 um thickness. Float a ribbon on drops of sterile water on a poly-L-lysine coated slide. Place slide on a 42 °C hot plate for a few minutes until the ribbon flattens out.
4. Drain off excess water and leave on the hot plate overnight to dry and store the slides in a box with desiccant at 4°C.,

12.6.5 Prehybridization treatments

1. Prepare 500 ml of all solutions listed below.
2. Put the slides in 25 place racks (stainless steel) then pass through the following solutions for the indicated times.

Solution	*Time*
Histoclear	10 min
Histoclear	10 min
100% ethanol	1 min
100% ethanol	30 sec
95% ethanol	30 sec
85% ethanol, 0.85% NaCl	30 sec
70% ethanol, 0.85% NaCl	30 sec
50% ethanol, 0.85% NaCl	30 sec
30% ethanol, 0.85% NaCl	30 sec
0.85% NaCl	2 min
PBS	2 min
Pronase (0.125 mg/ml in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA)	10 min
Glycine (0.2% in PBS)	2 min
PBS	2 min
Formaldehyde (4% in PBS)	10 min
PBS 2 min	
PBS (fresh)	2 min
Acetic anhydride (3 ml in 600 ml 0.1 M triethanolamine-HCl, pH 8)	10 min
PBS	2 min
0.85% NaCl	2 min

3. Dehydrate through an ethanol series up to the first 100% ethanol, then wash in fresh 100% ethanol. (Store for short time in a plastic box with little ethanol of needed)

12.6.6 Hybridization: Ribonucleic acid probes (ssRNA are advisable due to high in situ signals and lower background, this is because (1) single-stranded probes cannot reanneal during hybridization (2) the stability of RNA: RNA duplexes is higher than for DNA:RNA duplexes, so sections can be washed at higher stringency and (3) non specifically bound RNA probe can be selectively removed after hydridization by treatment with RNase A in high salt.

12.6.7 Probe purification and hydrolysis

1. After transcription probe reaction add 1 ml 100 mg/ml tRNA, 10 units of DNAase I (RNAase-free), and H20 to 100 ml and incubate at 37 °C for 10 min.
2. Remove a 1 ml aliquot for measuring incorporation, then extract with phenol/chloroform (100 ml) then chloroform.
3. Precipitate with 4M ammonium acetate (1:1 v/v) and ethanol (2.5 v/v), incubate on dry ice for 15 min, wash with 70% ethanol and dry.
4. Resuspend pellet in 50 ml H20, add 50 ml carbonate buffer (80 mM NaHCO3, 120 mM Na2CO3) and incubate at 60 °C for required time.
5. Precipitate with 5 ml 10% acetic acid, 0.1 vol 3 M sodium acetate and 2.5 vol ethanol, chill on dry ice, wash with 70% ethanol (if necessary) and resuspend in 20 ml H2O.
6. Calculate % incorporation of nucleotide and amount of probe synthesized. Add water and formamide to probe to make it 5 times concentrated and 50% formamide. Store at 20 °C until use.

12.6.8 Hybridization reaction

1. Denature probe at 80 °C for 2 min, cool on ice and spin briefly.
2. Add 4 vol hybridization solutions (to give final concentration of 0.3 M NaCl, 10 mM Tris-HCl, PH6.8, 10mM NaHPO4, pH 6.8, 5 mM EDTA, 50% formamide, 10% dextran sulphate, 1 x Denhardts (0.02 % each Ficoll, PVP, and BSA), 1 mg/ml tRNA).
3. Add 40 ml hybridization mix to each slide, and spread in a line along the center of the sections, then carefully apply a 22x50 mm coverslip (avoiding bubbles).
4. Put the slides in boxes and tissues soaked in 2xSSC 50% formamide (1xSSC is 0.3 M NaCl, 30 mM Na citrate). Seal the boxes with tape and place at 50 °C overnight.

12.6.9 Post hybridization treatments and autoradiography

Washing

1. Put slides in steel racks and immerse in wash buffer (2 x SSC, 50% formamide), shake gently for 30 min (coverslips should have fallen off)
2. Change wash buffer and incubate with gentle shaking for 1 5 h and repeat.
3. Wash for 5 min in NTE (0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) at 37° C and repeat.
4. Incubate in NTE containing 20 mg/ml RNase A at 37 °C for 30 min.
5. Rinse for 5 min in NTE and repeat.
6. Wash in wash buffer at 50° C for 1 h.
7. Rinse for 2 min in 1 x SSC at room temperature.
8. Rinse for 30 sec each in 30%, 60%, 90% ethanol (all made with 0.3M ammonium acetate) then in 95% then 100% ethanol and air dry.

12.6.10 Autoradiography

1. Place the pot of emulsion (Kodak ANTB-2 or similar) in water bath at 45°C and leave to melt up to 1 h.
2. Mix 5 parts emulsion to 7 parts 1% glycerol in water at 45 °C and swirl gently to mix.
3. Aliquot into plastic slide mailers (Raymond A. Lamb) and store aliquots at 4°C.
4. Preheat aliquot for 3 min in water bath at 45° C and mix by invert slide mailer gently.
5. Dip two blank slides first to remove any bubbles.
6. Dip each slide, withdraw gently and allow 2-3 sec to drain, then stand in a rack and return the emulsion to the water bath every few minutes to keep it warm.
7. When you have dipped all slides, place the rack of slides in a light-tight box and leave to dry for 1 h.
8. Place the slides in slide boxes with silica gel desiccant, seal with tape, double wrap in aluminum foil, and leave at 4°C for required exposure time.

Developing slides

1. Remove the box(es) of slides from the refrigerator and leave for 1h to warm at temperature.
2. Make up the following solutions and pre-chill to 14 °C.Developer - Kodak D19 or similarStopper 1% glycerol, 1% acetic acidFixer 20% sodium thiosulphate.
3. In dark room, place the slides in a stainless steel rack, put into developer and leave for 2 min. Transfer to stop and agitate the slides gently for 30 sec, then to fix for 5 min. Wash the slides in several changes of distilled water for 1 h.

Mounting slides

1. Stain in 0.05% toluidine blue in water for 1 min (These conditions are variable with different types). (if you do not wish to stain the section, start at step 3).
2. Rinse in water.
3. Dehydrate through an ethanol series (see prehybridization treatments).
4. Rinse in Histoclear, then again in fresh Histoclear, drain briefly and add two drops of Depex (BDH) over sections.
5. Place a coverslip on the slide and press down to squeeze out excess Histoclear/Depex.
6. Leave to dry for at least 1 day.
7. Wash slides in detergent (if necessary) to remove emulsion from the back of the slides.

The autoradiography section shows production of silver grains over the areas where labeled probe has hybridized. In the case of very strong single, the silver grains become too dense that they can be seen as black area under bright field microscopy. However, in most cases the silver grains are not visible unless view under dark field microscopy, where each silver grain show up a white speck due to its ability to reflect light.

12.6.11 Buffers and solutions

Fix solution (4% formaldehyde)
100 ml PBS and adjust pH using NaOH. Heat to 60° C then add 4 g paraformaldehyde and stir 1-2 min until dissolved. Cool on ice and readjust to pH 7.0 using H_2SO_4.

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Appendix-1: Virus families, genera and the members falling under each genus

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
Geminiviridae	Sub-group-1-Geminivirus	Bromus striate mosaic virus	BrSMV	Bajra streak virus	BaSV
		Chloris striate mosaic virus	CSMV	Chickpea chlorotic dwarf virus	CpCDV
		Digitaria streak virus	DSV		
		Digotaria striate mosaic virus	DiSMV		
		Maize streak virus	MSV		
		Miscanthus streak virus	MiSV		
		Pancium streak virus	PanSV		
		Paspaium striate mosaic virus	PSMV		
		Sugarcane streak virus	SSV		
		Tobacco yellow dwarf virus	TYDV		
		Wheat dwarf virus	WDV		
	Sub-group-11-Geminivirus	Beet curly top virus	BCTV	Tomato leaf roll virus	TLRV
				Tomato pseudo-curly top virus	TPCTV
	Sub-group-111-Geminivirus	Ablution mosaic virus	AbMV	Cowpea golden mosaic virus	CpGMV
		Acalypha yellow mosaic virus	AYMV	Eggplant yellow mosaic virus	EYMV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Sub-group-III-Geminivirus	African cassava mosaic virus	ACMV	Eupatorium yellow vein virus	EgYVV
		Ageratum yelow vein virus	AYVV	Lupin leaf curl virus	LLCV
		Asystasia golden mosaic virus	AGMV	Papaya leaf curl virus	PaLCV
		Bean calico mosaic virus	BCaMV	Sida yellow vein virus	SiYVV
		Bean dwarf mosaic virus	BDMV	Solanum apical leaf curl virus	SALCV
		Bean golden mosaic virus	BGMV	Soybean crinkle leaf virus	SCLV
		Chino deltomate virus	CdTV	Wissadula mosaic virus	WiMV
		Cotton leaf crumple virus	CLCrV		
		Cotton leaf curl virus	CLCuV		
		Cotton yellow vein mosaic virus	CYVMV		
		Dolichos yellow mosaic virus	DoYMV		
		Eclipta yellow vein virus	EYVV		
		Euphorbia mosaic virus	EuMV		
		Honeyhuckle yellow vein mosaic virus	HYVMV		
		Horsegram yellow mosaic virus	HgTMV		
		Jatropha mosaic virus	JMV		
		Limabean golden mosaic virus	LGMV		
		Malvaceous chlorosis virus	MCV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Sub-group-111-Geminivirus	Melon leaf curl virus	MCV		
		Macrotyloma mosaic virus	MLCV		
		Mungbean yellow mosaic virus	MYMV		
		Okra leaf curl virus	OLCV		
		Papper huasteco virus	PHV		
		Pepper mild tigre virus	PepMTV		
		Potato yellow mosaic virus	PYMV		
		Pseuderanthemum yellow vein virus	PYVV		
		Rhynchosia mosaic virus	RhMV		
		Serrano golden mosaic virus	SGMV		
		Sida golden mosaic virus	SGMV		
		Squash leaf curl virus	SiGMV		
		Texas pepper virus	TPV		
		Tobacco leaf curl virus	TLCV		
		Tomato golden mosaic virus	TGMV		
		Tomao leaf curl virus	TLCV		
		Tomato mottle virus	TMoV		
		Tomato yellow dwarf virus	ToYDV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Sub-group-III-Geminivirus	Tomato yellow leaf curl virus-Is	TYLCV-Is		
		Tomato yellow leaf curl virus-Sr	TYLCV-Sr		
		Tomato yellow leaf curl virus-Th	TYLCV-Th		
		Tomato yellow leaf curl virus-Ye	TYLCV-Ye		
		Tomato leaf crumple virus	TLCrV		
		Tomato yellow mosaic virus	ToYMV		
		Watermelon chlorotic stunt virus	WmCSV		
		Watermelon curly mottle virus	WmCMV		
?	Badnavirus	Banana streak virus	BSV	Aculia bacilliform virus	AuBV
		Cacao swollen shoot virus	CSSV	Mimosa bacilliform virus	MBV
		Canna yellow mottle virus	CaYMV	Taro bacilliform virus	TaBV
		Commelina yellow mottle virus	ComYMV	Yucca bacilliform virus	YBV
		Dioscorea bacilliform virus	DBV		
		Kalanchoe top-spotting virus	KTSV		
		Pepper yellow mottle virus	PYMoV		
		Rice tungro bacilliform virus	RTBV		
		Schefflera ringspot virus	SRV		
		Sugarcane bacilliform virus	SCBV		
?	Caulimovirus	Bluberry red ringspot virus	BRRV	Aquilegia necrotic mosaic virus	ANMV
		Carnation etched ring virus	CERV	Cassava vein mosaic virus	CsVMV
		Cauliflower mosaic virus	CaMV	Cestrum virus	CV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Caulimovirus	Dahlia mosaic virus	DMV	Petunia vein clearing virus	PVCV
		Figwort mosaic virus	FMV	Plantago virus 4	PlV-4
		Horseradish latent virus	HRLV	Sonchusmottle virus	SMoV
		Mirabilis mosaic virus	MiMV		
		Peanut chlorotic streak virus	PCSV		
		Soyban chlorotic mottle virus	SbCMV		
		Strawberry vein banding virus	SVBV		
		Thistle mottle virus	ThMoV		
Partitiviridae	Alphacryptovirus	Alfalfa cryptic virus 1	ACV-1	Carnation cryptic virus 2	CCV-2
		Beet cryptic virus 1	BCV-1	Cucumber cryptic virus	CuCV
		Beet cryptic virus 2	BCV-2	Fescue cryptic virus	FCV
		Beet cryptic virus 3	BCV-3	Garland chrysanthemum temperate virus	GCTV
		Carnation cryptic virus 1	CCV-1	Mibuna temperate virus	MTV
		Carrot cryptic virus 1	CTeV-1	Poensettia cryptic virus	PnCV
		Carrot temperate virus 1	CTeV-1	Red pepper cryptic 1	RPCV-1
		Carrot temperate virus 3	CTeV-3	Red pepper cryptic virus 2	RPCV-2
		Carrot temperate virus 4	CTeV-4	Rhubarb temperate virus	RTV
		Hop trefoil cryptic virus 1	HTCV-1	Santosai temperate virus	STV
		Hop trefoil cryptic virus 3	HTCV-3		
		Radish yellow edge virus	RYEV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Alphacryptovirus	Ryegrass cryptic virus	RGCV		
		Spinach temperate virus	SpTV		
		Vicia cryptic virus	VCV		
		White clover cryptic virus 1	WCCV-1		
		White clover cryptic virus 3	WCCV-3		
	Betacryptovirus	Carrot temperate virus 2	CTeV-2	Alfalfa cryptic virus 2	ACV-2
		Hop trefoil cryptic virus 2	HTCV-2		
		Red clover cryptic virus 2	RCCV-2		
		White clover cryptic virus 2	WCCV-2		
Reoviridae	Fijivirus	Fiji disease virus	FDV	Not reported	
		Maize rough dwarf virus	MRDV		
		Pangola stunt virus	PaSV		
		Rice black streaked dwarf virus	RBSDV		
		Oat sterile virus	OSDV		
	Oryzavirus	Echinochloa ragged stunt virus	ERSV	Not reported	
		Rice ragged stunt virus	RRSV		
	Phytoreovirus	Rice dwarf virus	RDV	Not reported	
		Rice gall dwarf virus	RGDV		
Rhabdoviridae	Cytorhabdovirus	Barley yellow striate mosaic virus	BYSMV	Not reported	
		Broccoli necrotic yellow virus	BNYV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Cytorhabdovirus	Festuca leaf streak virus	FLSV		
		Lettuce necrotic yellow virus	LNYV		
		Northern cereal mosaic virus	NCMV		
		Sonchus virus	SonV		
		Strawberry crinkle virus	SBCV		
		Wheat American striate mosaic virus	WASMV		
	Nucleorhabdo-virus	Datura yellow vein virus	DYVV	Not reported	
		Eggplant mottled dwarf virus	EMDV		
		Maize mosaic virus	MMV		
		Potato yellow dwarf virus	PYDV		
		Sonchus yellow dwarf virus	SYNV		
		Sowthistle yellow vein virus	SYVV		
Bunyaviridae	Tospovirus	Impatiens necrotic spot virus	INSV		
		Tomato spotted wilt virus	TSWV		
Sequiviridae	Sequivirus	Dandelion yellow mosaic virus	DYMV	Not reported	
		Parsnip yellow fleck virus	PYFV		
	Waikavirus	Anthriscus yellow virus	AYV		
		Maize chlorotic dwarf virus	MCDV		
		Rice tungro spherical virus	RTSV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
Comoviridae	Comovirus	Andean potato mottle virus	APMoV	Not reported	
		Bean pod mottle virus	BPMV		
		Bean rugose mosaic virus	BRMV		
		Broad bean true mosaic virus	BBSV		
		Cowpea mosaic virus	BBTMV		
		Cowpea severe mosaic virus	CPMV		
		Sonchus virus	SonV		
		Pea green mottle virus	PGMV		
		Pea mild mosaic virus	PMiMV		
		Quail pea mosaic virus	QPMV		
		Raish mosaic virus	RaMV		
		Red clover mottle virus	RCMV		
		Squash mosaic virus	SqMV		
		Uluchus virus	UVC		
	Fabavirus	Broad bean wilt virus 1	BBWV -1	Not reported	
		Broad bean wilt virus 2	BBWV-2		
		Lamium mild mosaic virus	LMMV		
	Nepovirus	Arabis mosaic virus	AMV	Arracacha virus B	AcVB
		Arracacha virus A	ArV-A	Artichoke vein banding virus	AVBV
		Artichoke yellow ringspot virus	AYRV	Cherry rasp leaf virus	CRLV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Nepovirus	Blueberry leaf mottle virus	BIMV	Luvern Australian symptomless virus	LASV
		Cassava American latent virus	CALV	Rubus Chinese seed-borne virus	RCSbV
		Cassava green mottle virus	CGMV	Satsuma dwarf virus	SDV
		Cherry leaf roll virus	CLRV	Strawberry latent ringspot virus	SLRV
		Cherry yellow mottle virus	CYMV	Tomato top necrosis virus	TTNV
		Coco necrosis virus	CNV		
		Crimson clover latent virus	CCLV		
		Cycas necrotic stunt virus	CNSV		
		Grapevine Bulgarian latent virus	GBLV		
		Sonchus virus	SonV		
		Grapevine fanleaf virus	GFV		
		Grapevine Tunisian ringspot virus	GTRV		
		Hibuscus latent ringspot virus	HLRV		
		Lucern Australian latent virus	LAALV		
		Mulbery ringspot virus	MRV		
		Myrobalan latent ringspot virus	MLRV		
		Olive latent ringspot virus	OLRV		
		Peach rosette mosaic virus	PRMV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Nepovirus	Potato black ringspot virus	BBRV		
		Potato virus U	PVU		
		Rasberry ringspot virus	RRV		
		Tobacco ringspot virus	TbRV		
		Tomato black ringspot virus	TBLV		
		Tomato ringspot virus	ToRV		
Potyviridae	Bymovirus	Barley mild mosaic virus	BaMMV	Not reported	
		Barley yellow mosaic virus	BaYMV		
		Oat mosaic virus	OMV		
		Rice necrosis mosaic virus	RNMV		
		Wheat spindle streak mosaic virus	WSSMV		
	Potyvirus	Asltroemeria mosaic virus	AlMV	Alstroemeria streak virus	AlSV
		Amaranthus leaf mottle virus	AmLMV	Amazon lily mosaic virus	ALiMV
		Artichoke latent virus	ArLV	Anthoxanthum mosaic virus	AnoV
		Asparagus virus 1	AV-1	Aquilegia virus	AqV
		Bean common mosaic virus	BCMV	Arracacha virus Y	AVY
		Bean common mosaic necrosis virus	BCMNV	Asystasia gangetica mottle virus	AGMoV
		Bean yellow mosaic virus	BYMV	Bidens mosaic virus	BiMV
		Beet mosaic virus	BtMV	Bramble yellow mosaic virus	BruYMV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Potyvirus	Bidens mottle virus	BiMoV	Brandle yellow mosaic virus	BrnYMV
		Cardamom mosaic virus	CdMV	Bryonia mottle virus	BryMV
		Carnation vein mottle virus	CVMV	Canary reed mosaic virus	CRMV
		Carrot thin leaf virus	CTLV	Canavalia maritima mosaic virus	CnMMV
		Celery mosaic virus	CeMV	Carrot mosaic virus	CtMV
		Chilli veinal mottle virus	ChVMV	Cassia yellow spot virus	CasYSV
		Clover yellow vein virus	ClYVV	Celery yellow mosaic virus	CeyMV
		Cocksfoot streak virus	CSV	Chickpea bushy dwarf virus	CpBDV
		Colombian datura virus	CDV	Chickpea filiform virus	CpFV
		Commelina mosaic virus	ComMV	Clitoria yellow mosaic virus	ClYMV
		Cowpea aphid-borne mosaic virus	CABMV	Cowpea rugose mosaic virus	CPRMV
		Cowpea green vein banding virus	CGVB	Crinum mosaic virus	CrnMV
		Dasheen mosaic virus	DsMV	Croatian clover virus	CroCV
		Datura shoestring virus	DSTV	Cypripedium calceolus virus	CypCV
		Dendrobium mosaic virus	DeMV	Daphne virus Y	DVY
		Gloriosa stripe mosaic virus	GSMV	Dtura virus 437	DV-437
		Groundnut eyespot virus	GEV	Datura distortion mosaic virus	DDMV
		Guinea grass mosaic virus	GGMV	Datura mosaic virus	DTMV
		Helenium virus Y	HVY	Datura necrosis virus	DNV
		Hippeastrum mosaic virus	HMV	Desmodium mosaic virus	DesMV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Potyvirus	Iris fulva mosaic virus	IFMV	Dioscorea alata ring mottle virus	DARMV
		Iris severe mosaic virus	IMMV	Dioscorea trifida virus	DTV
		Johnsongrass mosaic virus	JMV	Dipladenia mosaic virus	DipMV
		Konjac mosaic virus	KMV	Dock mottling mosaic virus	DMMV
		Leek yellow stripe virus	LYSV	Eggplant green mosaic virus	EGMV
		Lettuce mosaic virus	LMV	Eggplant severe mottle virus	ESMV
		Maize dwarf mosaic virus	MDMV	Euphorbia ringspot virus	EuRV
		Narcissus degeneration virus	NDV	Ficus carica virus	FiCV
		Narcissus yellow stripe virus	NYSV	Freesia mosaic virus	FreMV
		Nothoscordum mosaic virus	NoMV	Garlic yellow streak virus	GYSV
		Onion yellow dwarf virus	OYDV	Guar symptomless virus	GSLV
		Ornithogalum mosaic virus	OMV	Habenaria mosaic virus	HaMV
		Papya ringspot virus (watermelon mosaic virus 1)	PRSV	Holcus streak virus	HSV
		Parsnip mosaic virus	PaRMV	Hungarian datura innoxia virus	HDIV
		Passion fruit woodiness virus	PWV	Hyacinth mosaic virus	HyaMV
		Pea seed-borne mosaic virus	PSbMV	Indian pepper mottle virus	IPMV
		Peanut mottle virus	PeMV	Isachne mosaic virus	IsaMV
		Pepper mottle virus	PepMoV	Kennwdy virus Y	KVY
		Pepper severe mosaic virus	PepSMV	Lilly mild mottle virus	LiMMV
		Pepper veinal mottle virus	PVMV	Malva vein clearing virus	MCVV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Potyvirus	Peru tomato mosaic virus	PTMV	Marigold mottle virus	MMV
		Plum pox virus	PPV	Meliotus mosaic virus	MaMoV
		Pokeweed mosaic virus	PkMV	Mellon vein-banding mosaic virus	MeMV
		Hippeastrum mosaic virus	HMV	Morrocan watermelon mosaic virus	MWMV
		Hippeastrum mosaic virus	HMV	Mungbean mosaic virus	MbMV
				Mungbean mottle virus	MMTV
		Potato virus A	PVA	Narcissus late season yellows virus	NLSYV
		Potato virus V	PVV	Nasturtium mosaic virus	NasMV
		Potato virus Y	PVY	Nerine virus	NV
		Rembrandt tulip breaking virus	PeTBV	Palm mosaic virus	PalMV
		Sorghum mosaic virus	SrMV	Papaya leaf distortion mosaic virus	PLDMV
		Soybean mosaic virus	SMV	Passion fruit mottle virus	PEMoV
		Sugarcane mosaic virus	SCMV	Passion fruit ringspot virus	PFRSV
		Sweet potato feathery mottle virus	SPFMV	Patchouli mottle virus	PatMV
		Tamarillo mosaic virus	TamMV	Peanut green mottle virus	PeGMV
		Telfairia mosaic virus	TeMV	Peanut mosaic virus	PeMsV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Potyvirus	Tobacco etch virus	TEV	Pecteilis mosaic virus	PeMV
		Tobacco vein mottle virus	TVMV	Pepper mild mosaic virus	PeMMV
		Tulip band breaking virus	TBBV	Perilla mottle virus	PerMV
		Tulip breaking virus	TBV	Plantain virus 7	PlV-7
		Tulip chlorotic blotch virus	TCBV	Pleioblastus mosaic virus	PleMV
		Tulip mosaic virus	TuMV	Populus virus	PV
		Watermelon mosaic virus 2	WMV-2	Primula mosaic virus	PrMV
		Wisteria vein mosaic virus	WVMV	Primula mottle virus	PrMoV
		Yam mosaic virus	YMV	Ranunculus mottle virus	RanMV
		Zucchini yellow fleck virus	ZYFV	Sri Lankan passionfruit mottle virus	SLPMV
		Zucchini yellow mosaic virus	ZYMV	Sunflower mosaic virus	SunMV
				Sweet potato latent virus	SwPLV
				Sweet potato vein mosaic virus	SPVMV
				Sword bean distortion mosaic virus	SBDMV
				Teasel mosaic virus	TeaMV
				Tobacco vein banding mosaic virus	TVBMV
				Tobacco wilt virus	TWV
				Tongan vanilla virus	TVV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Polyvirus			Tradescantia/Zebrina virus	TZV
				Trichosanthes mottle virus	TrMV
				Tropaeolum virus 1	TV-1
				Tropaeolum virus 2	TV-2
				Ullucus mosaic virus	UMV
				Vallota mosaic virus	ValMV
				Vanilla mosaic virus	VanMV
				White bryony virus	WBV
				Wild potato mosaic virus	WPMV
				Brome streak virus	BSTV
				Spartina mottle virus	SpMV
	Rymovirus	Agropyron mosaic virus	AgMV		
		Hordeum mosaic virus	HoMV		
		Oat necrosis mottle virus	ONMV		
		Rygrass mosaic virus	RGMV		
		Wheat streak mosaic virus	WSMV		
?	Enamovirus	Pea enation mosaic virus	PEMV		
?	Luteovirus	**BYDV sub-group 1**		Beet yellow net virus	BYNV
		Barley yellow dwardf virus-MAV	BYDV-MAV	Celery yellow spot virus	CeYSV
		Barley yelow dwarf virus-PAV	BYDV-PAV	Chickpea stunt virus	CpSV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Luteovirus	Barley yellow dwarf virus-SGV	BYDV-SGV	Cotton anthcyanosis virus	CAV
		BYDV-subgroup 11		Filaree red leaf virus	FLRV
		Barley yellow dwarf virus-RGV	BYDV-RGV	Grapevine ajinashika virus	GAV
		Barley yellow dwarf virus-RMV	BYDV-RMV	Milk vetch dwarf virus	MVDV
		Barley yellow dwarf virus-RPV	BYDV-RPV	Millet red virus	MRLV
		Bean leaf roll virus (pea leaf roll virus)	BLRV	Physalis mild chlorosis virus	PhyMCV
		Beet western yellows virus	BWYV	Physalis vein blotch virus	PhyVBV
		Carrot red leaf virus	CtRLV	Rasberry leaf curl virus	RLCV
		Groundnut rosette assistor virus	GRAV	Tobacco vein distorting virus	TVDV
		Indonesian soybean dwarf virus	ISDV	Tobacco yellow net virus	TYNV
		Potato leaf roll virus	PLRV	Tobacco yellow vein assistor virus	TYVAV
		Solanum yellow virus	SYV		
		Tomato yellow top virus	ToYTV		
		Soybean dwarf virus	SbDV		
		Tobacco necrotic dwarf virus	TNDV		
?	Sobemovirus	Blueberry shoestring virus	BSSV	Cocksfoot mild mosaic virus	CMMV
		Cocksfootmottle virus	CoMV	Cynosurus mottle virus	CnMoV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Sobemovirus	Lucerntransient streak virus	LTSV	Ginger chlorotic fleck virus	GCFV
		Rice yellow mottle virus	RYMV	Olive latent virus 1	OLV-1
		Solanum nodiflorum mottle virus	SNMV	Panicum mosaic virus	PMV
		Southern bean mosaic virus	SBMV		
		Sowbane mosaic virus	SoMV		
		Subterranean clover mottle virus	SCMoV		
		Turnip rosette virus	TRoV		
		Velvet tobacco mottle virus	VTMoV		
?	Umbravirus	Bean yellow vein-banding virus	BYVBV	Sunflower crinkle virus	SCV
		Carrot mottle virus	CMoV	Sunflower yellow blotch virus	SYBV
		Groundnut rosette virus	GRV	Tobacco bushy top virus	TBTV
		Lettuce speckles mottle virus	LSMV	Tobacco yellow vein virus	TYVV
		Tobacco mottle virus	TMoV		
Tombusviridae	Carmovirus	Ahlum water-borne virus	AWBV	Blackgram mottle virus	BMoV
		Bean mild mosaic virus	BMMV	Cowpea mottle virus	CPMoV
		Carnation mottle virus	CarMV	Eldeberry latent virus	ELV
		Cucumber soil-borne virus	CSBV	Glycine mottle virus	GMoV
		Cucumber leaf spot virus	CLSV	Narcissus tip necrosis virus	NTNV
		Galinsoga mosaic virus	GaMV	Tephrosia symptomless virus	TeSV
		Hibuscus chlorotic ringspot virus	HCRSV		
		Melon necrotic spot virus	MNSV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Carmovirus	Pelargonium flower break virus	PFBV		
		Saguaro cactus virus	SCV		
		Turnip crinkle virus	TCV		
		Weddel water-borne virus	WWV		
	Tombusvirus	Artichoke mottle crinkle virus	AMCV	Not reported	
		Carnation Italian ringspot virus	CIRSV		
		Cucumber necrosis virus	CNV		
		Cymbidium ringspot virus	CymRSV		
		Eggplant mottled crinkle virus	EMCV		
		Grapevine Algerian latent virus	GALV		
		Moroccan pepper virus	MPV		
		Lato river virus	LRV		
		Neckar river virus	NRV		
	Tombusvirus	Pelargonium leaf curl virus	PLCV		
		Petunia asteroid mosaic virus	PAMV		
		Sikte water-borne virus	SWBV		
		Tomato bushy stunt virus	TBSV		
?	Dianthovirus	Carnation ringspot virus	CRSV	Fureraea necrotic streak virus	FNSV
		Red clover necrotic mosaic virus	RCNMV		
		Sweet clover necrotic mosaic virus	SCNMV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
?	Furovirus	Oat golden stripe virus	OGSV	Beet necrotic yellow vein virus	BNYVV
		Peanut clump virus	PCV	Beet soil-borne virus	BSBV
		Potato mop-top virus	PMTV	Broad bean necrosis virus	BBNV
		Soil-borne wheat mosaic virus	SBWMV	Hypochoeris mosaic virus	HyMV
		Sorghum chlorotic spot virus	SgCSV	Rice stripe necrosis virus	RSNV
?	Hordeivirus	Anthoxanthum latent blanching virus	ALBV	Not reported	
		Barley stripe mosaic virus	BSMV		
		Lychnis ringspot virus	LRSV		
		Poa semilatent virus			
?	Machlomovirus	Maize chlorotic mottle virus	MCMV	Not reported	
?	Necrovirus	Chenopodium necrosis virus	ChNV	Carnation yellow stripe virus	CYSV
		Tobacco necrosis virus	TNV	Lisianthus necrosis virus	LNV
?	Tobamovirus	Cucumber green mottle mosaic virus	CGMMV	Chara corallina virus	ChaCV
		Frangipani mosaic virus	FrMV	Maracuja mosaic virus	MarMV
		Odontoglossum ringspot virus	ORSV		
		Paprika mild mottle virus	PMMoV		
		Ribgrass mosaic virus	RMV		
		Sammons Opuntia virus	SOV		
		Sun-hemp mosaic virus	SHMV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
		Tobacco mild green mosaic virus	TMGMV		
	Tobravirus	Tomato mosaic virus	ToMV		
		Ullucus mild mottle virus	UMMV		
?		Pea early browing virus	PEBV	Not reported	
		Pepper ringspot virus	PepRSV		
		Tobacco rattle virus	TRV		
Bromoviridae	Alfamovirus	Alfalfa mosaic virus	AMV	Not reported	
	Bromovirus	Broad bean mottle virus	BBMV	Not reported	
		Brome mosaic virus	BMV		
		Cassia yellow blotch virus	CYBV		
		Cowpea chlorotic mottle virus	CCMV		
		Malandrium yellow fleck virus	MYFV		
		Spring beauty latent virus	SBLV		
	Cucumovirus	Cucumber mosaic virus	CMV	Not reported	
		Peanut stunt virus	PSV		
		Tomato aspermy virus	TAV		
	Ilarvirus	Sub-group-1 Tobacco streak virus	TSV	Not reported	
		Sub-group-2 Asparagus virus 2	AV-2		
		Blueberry shok virus	BlShV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
	Ilarvirus	Citrus leaf rugose virus	CiLRV		
		Citrus variegation virus	CVV		
		Elm mottle virus	EMoV		
		Tulare apple mosaic virus	TAMV		
		Sub-group-3 Apple mosaic virus	ApMV		
		Prunus necrotic ringspot virus	PNRSV		
		Sub-group-4 Prune dwarf virus	PDV		
		Sub-group-5 American plum line pattern virus	APLPV		
		Sub-group-6 Spinach latent virus	SPLV		
		Sub-group-7 Lilac ring mottle virus	LRMV		
		Sub-group-8 Hydrangea mosaic virus	HdMV		
		Sub-group-9 Humulus japonicus virus	HJV		
		Sub-group-10 Parietaria mottle virus	PMoV		

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
")	Closterovirus	Beet yellow stunt virus	BYSV	**1. Aphid-tramsmitted viruses**	
		Beet yellows virus	BYV	Citrus tristeza virus	CTV
		Burdock yellow virus	BuYV	Dendrobium vein necrosis virus	DVNV
		Carnation necrotic fleck virus	CNFV	Heracleum virus 6	HV-6
		Carrot yellow leaf virus	CYLV	**2. Mealybug-transmitted**	
		Wheat yellow leaf virus	WYLV	Grapevine leaf roll-associated virus	GLraV
				Pineapple mealybug wilt-associated virus	PMWaV
				Sugarcane mild mosaic virus	SMMV
				3. Vector unknow	
				Alligatorweed stunting virus	AWSV
				Festuca necrosis virus	FNV
				Grapevine corky bark-associated virus	GCBaV
				Grapevine leafroll-associated virus-1	GLRaV-1
				Grapevine leafroll-associated virus-2	GLRaV-2
				Grapevine leafroll-associated virus-3	GLRaV-3

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
				Grapevine leafroll-associated virus-4	GLRaV-4
				4. Whitefly transmitted	
	Closterovirus			Beet pseudoyellos virus	BPYV
				Cucumber chlorotic spot virus	CCSV
				Cucumber yelow virus	CuYV
				Diodia vein chlorosis virus	DVCV
				Lettuce infectiou yellows virus	LIYV
				Muskmelon yellows virus	MYV
?	Capillovirus	Apple stem grooving virus	ASGV	Nandina stem pitting virus	NSPV
		Citrus tatter leaf virus	CTLV		
		Liliac chlorotic leafspot virus	LCLV		
?	Carlavirus	American hop latent virus	AHLV	**1. Aphid-borne**	
		Blueberry scorch virus	BlSV	Anthriscus virus	AntV
		Cactus virus 2	CV-2	Arracacha latent virus	ALV
		Caper latent virus	CapLV	Artichoke latent virus M	ArLVM
		Carnation latent virus	CLV	Artichoke latent virus S	ArLVS
		Chrysanthemum virus B	CVB	Butterbur mosaic virus	BurMV
		Dndelion latent virus	DaLV	Caraway latent virus	CawLV
		Elderberry virus	EV	Cardamine latent vitus	CaLV
		Garlic comon latent virus	GCLV	Cassia mild mosaic virus	CasMMV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
		Helenium virus	HnLV	Chicory yellow blotch virus	ChYBV
		Hineysuckle latent virus	HnLV	Chinese yam necrotic mosaic virus	ChYNMV
	Carlavirus	Hop latent virus	HpLV	Cole latent virus	CoLV
		Hop mosaic virus	HpMV	Cynodon mosaic virus	CynMV
		Hydrangea latent virus	HdLV	Daphne virus S	DVS
		Kalanchoe latent virus	KLV	Dulcamara virus A	DuVA
		Lilac mottle virus		Dulcamara virus B	DuVB
		Lily symptomless virus	LSV	Eggplnat mild mottle virus (eggplnat virus)	EMMV
		Mulberry latent virus	MLV	Euonymus mosaic virus	EuoMV
		Muskmelon vein necrosis virus	MuVNV	Fig virus S	FVS
		Nerine latent virus	NeLV	Fuchsia latent virus	FLV
		Passiflora latent virus	PLV	Galic mosaic virus	GarMV
		Pea streak virus (alfalfa latent virus)	PeSV	Gentiana virus	GenV
		Poplar mosaic virus	PopMV	Cynura latent virus	CyLV
		Potato virus M	PVM	Helleborus mosaic virus	HeMV
		Potato virus S (Pepino latent virus)	PVS	Impatiens latent virus	ILV
		Red clover vein mosaic virus	RCVMV	Lilac rindspot virus	LacRSV

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
		Shallot latent virus	SLV	Plantain virus 8	PlV-8
		Sin-jeni's onion latent virus	SJOLV	Prunus virus S	PruVS
	Carlavirus	Strawberry pseudo mild yellow edge virus	SPMYEV	Southern potato latent virus	SoPLV
				White bryony mosaic virus	WBMV
				2. Whitefly-borne	
				Cassava brown streak-associated virus	CBSaV
				Cowpea mild mottle virus (Groundnut crinkle virus) (tomato pale chlorosis virus) (Voandzeia mosaic virus)	CPMMV
?	Idaeovirus	Rasberry bushy dwarf virus	RBDV	Not reported	
?	Marafivirus	Bermuda grass etched-line virus	BELV		
		Maize rayado fino virus	MRFV		
		Oat blue dwarf virus	OBDV		
?	Potexvirus	Asparagus virus 3	AV-3	Artichoke curly dwarf virus	ACDV
		Cactus virus X	CVX	Bamboo mosaic virus	BaMV
		Cassava virus X	CsVX	Barley virus B 1	BarV-B1

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
		Clover yellow mosaic virus	CIYMV	Boletus virus	BolV
		Commelina virus x	ComVX	Cassava common mosaic	CsCMV
		Cymbidium mosaic virus	CymMV	Centrosema mosaic virus	CenMV
	Potexvirus	Foxtail mosaic virus	FoMV	Daphne virus X	DVX
		Hydrangea ringspot virus	HRSV	Dioscorea latent virus	DLV
		Lily virus X	LVX	Lychnis virus	LyV
		Narcissus mosaic virus	NMV	Malva vernal necrosis virus	MVNV
		Nerine virus x	NVX	Nandina mosaic virus	MVNV
		Papya mosaic virus	PapMV	Negro coffee mosaic virus	NaCMV
		Pepino mosaic virus	PepMV	Parsley virus 5	PAV-5
		Plantago severe mottle virus	PlSMV	Parsnip viris 3	ParV-3
		Plantain virus X	PlVX	Parsnip virus 5	ParV-5
		Potato acuba mosaic virus	PAMV	Rhododendron necrotic ringspot virus	RoNSV
		Potato virus X	PVX	Rhubarb virus 1	RV-1
		Tulip virus X	TVX	Smithianatha virus	SmiV
		Viola mottle virus	VMV	Strawberry mild yellow edge-associated virus	SMYEaV
		White clover mosaic virus	WClMV		
?	Trichovirus	Apple chlorotic leaf spot virus	ACLSV	Grapevine virus A	GVA

Family	Genus	Definitive members	Acronmy	Tentative member	Acronmy
		Potato virus T	PVT	Grapevine virus B	GVB
				Heracleum latent virus	HLV
?	Tymovirus	Belladonna mottle virus	BeMV	Poinsettia mosaic virus	PnMV
		Cacao yellw mosaic virus	VYMV		
		Clitoria yellow vein virus	CYVV		
		Desmodium yellow mottle virus	DYMV		
		Dulcamara mottle virus	DuMV		
		Eggplant mosaic virus (Andean potato latent virus)	EMV		
		Erysimum latem virus	EeLV		
		Kennedya yellow mosaic virus	KYMV		
		Okra mosaic virus	OKMV		
		Passion fruit yellow mosaic virus	PaYMV		
		Peanut yellow mosaic virus	PeYMV		
		Physalis mosaic virus	PhyMV		
		Plantago mottle virus	PlMoV		
		Scrophularia mottle virus (Onion yellow mosaic virus)	ScrMV		
		Turnip yellow mosaic virus	TYMV		
		Voandzeia necrotic mosaic virus	VNMV		
		Wild cucumber mosaic virus	WCMV		

Appendix -2

List of standard acronyms proposed for plant viruses

Virus name	Acronym	Family/Genus and Subgroup
Ablution mosaic	AbMV	Geminivirus 11[a]
African cassava mosaic	ACMV	Geminivirus 11
Agropyron mosaic	AgMV	Potyvirus (mite)[b]
Alfalfa cryptic 1	ACV 1	Cryptovirus
Alfalafa mosaic	AMV	AMV grouip
American leaf mottle	AmLMV	Potyvirus (aphid)
Americam plum line pattern	APLPV	Ilarvirus
Americam wheat striate mosaic	AWSMV	Rhabdovirus [Aa] (leafhopper)
Andean potato latent	APLV	Tymovirus
Andean potato mottle	APMV	Comovirus
Apple chlorotic leaf spot	ACLSV	? Closterovirus
Apple mosaic	ApMV	Ilarvirsu
Apple stem grooving	ASGV	Capillovirus
Arabis mosaic	ArMV	Nepovirus
Artichoke Italian latent	AILV	? Nepovirus
Artickoke latent	ALV	Nepovirus
Artichoke mottle crinkle	AMCV	Tombusvirus
Artichoke vein banding	AVBV	? Nepovirus
Artickoke yellow ring spot	AYRSV	Nepovirus
Asparagus 1	AV1	Potyvirus (aphid)
Asparagus 2	AV2	Ilarvirsu
Asparagus 3	AV3	Potexvirus
Bambo mosaic	BaMV	Potexvirus
Banana streak	BSV	Badnavirus
Banana bunchy top virus	BBTV	?
Barley stripe mosaic	BSMV	Horeivirus
Barley yellow dwarf	BYDV	Luteovirus
Barley yellow striate mosaic	BaYMV	Potyvirys (fungus)
Bean common mosaic	BCMV	Potyviirus (aphid)
Bean distortion dwarf	BDDV	? Geminivirus 11
Bean golden mosaic	BGMV	Geminivirus 11

Virus name	Acronym	Family/group and subgroup
Bean pod mottle	BPMV	Comovirus
Bean rugose mosaic	BRMV	Comovirus
Bean yellow mosaic	BYMV	Potyvirus (aphid)
Bean cryptic 1	BCV1	Cryptovirus 1
Bean cryptic 2	BCV2	Cryptovirus 1
Bean cryptic 3	BCV3	Cryptovirus 1
Beet leaf curl	BLCV	Rhabovirus (lace bug)
Beet mild yellowing	BMYV	Luteovirus
Beet mosaic	BtMV	Potyvirus (aphid)
Beet necrotic yellow vein	BNYVV	? Furovirus
Beet soil-borne	BSBV	Furovirus
Beet western yellows	BWYV	Luteovirus
Beet yellow net	BYNV	? Luteovirus
Beet yellows	BYV	Closterovirus
Beet yellow stunt	BYSV	Closterovirus
Belladoona mottle	BeMV	Tymovirus
Bermuda grass etched-line	BELV	Marafivirus
Blackeye cowpea mosaic	BlCMV	Potyirus (aphid)
Blackgram mottle	BMoV	? Carmovirus
Black rassberry latent	BRLV	Ilarvirus
Black rasberry necrosis	BRNV	Unclassified, isometric
Blueberry leaf mottle	BLMV	Nepovirus
Blueberry scorch	BBScV	Ilarvirus
Blueberry shoestring	BSSV	Sobemovirus
Broad bean mottle	BBMV	Bromovirus
Broad bean necrosis	BBNV	? Furovirus
Broad bean stain	BBSV	Comovirus
Broad bean true mosaic	BBTMV	Comovirus
Broad bean wilt	BBWV	Fabavirus
Broccoli nrcrotic yellows	BNYV	Rhabdovirus (aphid)
Brome mosaic	BMV	Bromovirus

Virus name	Acronym	Family/group and subgroup
Brome streak	BsTV	Potyvirus (mite)
Cacao necrosis	CNV	Nepovirus
Cacao swollen shoot	CSSV	Badnavirus
Cacao yellow mosaic	CYMV	Tymovirus
Cactus 2	CV2	Carlavirus
Cacatus X	CVX	Potexvirus
Cardamine latent	CaLV	Carlavirus
Cardamom mosaic	CdMV	Potyvirus (aphid)
Carnation cryptic 1	CCV1	Cryptovirus
Carnation cryptic 2	CCV2	Cryptovirus
Carnation etched ring	CERV	Caulimovirus
Carnation Italian ring spot	CIRSV	Tombusvirus
Carnation latent	CLV	Carlavirus
Carnation mottle	CarMV	Carmovirus
Carnation necrotic fleck	CNFV	Closterovirus
Carnation ringspot	CRSV	Dianthovirus
Carnation vein mottle	CVMV	Potyvirus (aphid)
Carrot latent	CtLtV	Rhabdovirus (aphid)
Carrot mottle	CMoV	?
Carrot red leaf	CaRLV	Luteovirus
Carrot thin leaf	CTLV	Potyvirus (aphid)
Carrot yellow leaf	CYLV	Closterovirus
Cassava common mosaic	CsCMV	Potexvirus
Cassava brown mosaic	CBMV	? Carlavirus (whitefly)
Cassava green mottle	CBMV	Nepovirus
Cassava vein banding	CVBV	? Caulimovirus
Cassava virus C	CVC	?
Cassava virus X	CsVX	Potexvirus
Cassia yellow blotch	CYBV	Potexvirus
Cauliflower mosaic	CaMV	Caulimovirus

Virus name	Acronym	Family/group and subgroup
Celery mosaic	CeMV	Potyvirus (aphid)
Cereal chlorotic mottle	CeCMV	Rhabdovirus (leafhopper)
Chenopodium necrosis	ChNV	Nepovirus
Cherry leaf roll	CLRV	Nepovirus
Cherry rasp leaf	CRLV	?Nepovirus
Cherry rugose	VRV	Ilarvirus
Chrysanthemum B	CVB	Carlavirus
Citrus leaf rugose	CiLRV	? Ilarvirus
Citrus latter leaf	CiTLV	? Closterovirus
Citrus tristeza	CTV	Closterovirus
Citrus variegation	CVV	Ilarvirus
Clover yellow mosaic	CLYMV	Potexvirus
Clover yellows	CYV	Closterovirus
Clover yellows vein	CIYVV	Potyvirus (aphid)
Coffee ringspot	CoRSV	Rhabdovirus (mite)
Cotton leaf crumple	CLCrV	Geminivirus
Cotton leaf curl	CLCuV	Geminivirus
Cowpea aphid-borne mosaic	CABMV	Potyvirus (aphid)
Cowpea chlorotic mottle	CCMV	Bromovirus
Cowpea golden mosaic	CpGMV	Geminivirus
Cowpea green vein banding	CGVBV	Potyvirus
Cowpea mild mottle	CPMMV	Carlavirus
Cowpea mosaic	CPMV	Comovirus
Cowpea mottle	CPMoV	Carmovirus
Cowpea ringspot	CPRSV	? Cucumovirus
Cowpea severe mosaic	CPSMV	Comovirus
Crimson clover latent	CCLV	Nepovirus
Cucumber green mottle mosaic	CGMMV	Tobamovirus
Cucumber leaf spot	CLSV	? Carmovirus
Cucumber mosaic	CMV	Cucumovirus
Cucumber necrosis	CuNV	Tombusvirus

Virus name	Acronym	Family/group and subgroup
Cucumber soil-borne	CSBV	Carmovirus
Cucumber yellows	CYV	? Closterovirus
Dalhia mosaic	DMV	Caulimovirus
Dasheen mosaic	DsMV	Potyvirus (aphid)
Datura shoestring	DSTV	Potyvirus (aphid)
Eggplant mosaic	EMV	Tymovirus
Eggplant mottle crinkle	EMCV	Tombusvirus
Eggplant mottled dwarf	EMDV	Rhabdovirus (vector unknown)
Euphorbia mosaic	EuMV	Geminivirus
Fern mottle	FMoV	Furovirus
Figwort mosaic	FMV	Caulimovirus
Fiji disease	FDV	Plant Reovirus
Garlic mosaic	GarMV	Potyvirus (aphid)
Ginger chlorotic fleck	GCFV	? Sobemovirus
Gloriosa stripe mosaic	GSMV	Potyvirus (aphid)
Glycine mosaic	GMV	Comovirus
Glycine mottle	GMoV	Carmovirus
Grapevine A	GVA	Closterovirus
Grapevine fanleaf	GALV	Tombusvirus
Groundnut crinkle	GCV	? Carlavirus (whitefly)
Groundnut eyespot	GEV	Potyvirus (aphid)
Groundnut rosette	GRV	?
Groundnut rosette assister	GRAV	Luteovirus
Hibuscus chlorotic ringspot	HCRSV	Carmovirus
Hibiscus latent ringspot	HLRSV	Nepovirus
Hop latent	HpLV	Carlavirus
Hop mosaic	HpMV	Carlavirus
Hop tefoil crptic 1	HTCV1	Cryptovirus 1
Hop trefoil cryptic 2	HTCV2	Cryptovirus 11
Hordeum mosaic	HoMV	Potyvirus ?

Virus name	Acronym	Family/group and subgroup
Horsegram yellow mosaic	HgYMV	Geminivirus
Indian cassava mosaic	ICMV	Geminivirus
Indian peanut clump	IPCV	? Furovirus
Indonesian soybean dwarf	ISDV	Luteovirus
Iris mild mosaic	IMMV	Potyvirus
Lettuce big vein	LBVV	Unclassified, dsRNA rod.
Lettuce mosaic	LMV	Potyvirus (aphid)
Lettuce necrotic yellows	LNYV	Rhabdovirus (aphid)
Lettuce speckles mottle	LSMV	Umbravirus
Lucern Australian latent	LALV	Nepovirus
Lucern Australian symptomless	LASV	? Nepovirus
Lucern transient streak	LTSV	Sobemovirus
Maize chlorotic dwarf	MCDV	MCDV group
Maize chlorotic mottle	MCMV	?Sobemovirus
Maize dwarf mosaic	MDMV	Potyvirus (aphid)
Maize mosaic	MMV	Rhabdovirus (Leafhopper)
Maize rough dwarf	MRDV	Plant Reoviridae
Maize sterile stunt	MSSV	Rhabdovirus (planthopper)
Maize streak	MSV	Geminivirus 11
Maize stripe	MSpV	Tenuivirus
Maize whiteline moaic	MWLMV	Unclassified, isometric
Melon leaf curl	MLCV	Geminivirus 11
Melon necrotic spot	MNSV	Carmovirus
Mulberry latent	MLV	Carlavirus
Mulberry ringspot	MRSV	Nepovirus
Mungbean yellow mosaic	MYMV	Geminivirus 11
Muskmelon vein necrosis	MVNV	Carlavirus
Oat blue dwarf	OBDV	Marafivirus

Virus name	Acronym	Family/group and subgroup
Oat golden stripe	OGMV	? Furovirus
Oat mosaic	OMV	Potyvirus (fngus)
Oat necrotic mottle	ONMV	Potyvirus (?mite)
Okara mosaic	OkMV	Tymovirus
Olive latent ringspot	OLRSV	Nepovirus
Olive latent 1	OLV1	? Sobemovirus
Olive latent 2	OLV2	unclassified, small bacilliform
Onion yellow dwarf	OYDV	Potyvirus (aphid)
Papya mosaic	PapMV	Potexvirus
Papaya ringspot (= watermelon mosaic 1)	PRSV	Potyvirus (aphid)
Parsnip mosaic	ParMV	Potyvirus (aphid)
Passionatefruit woodiness	PWV	Potyvirus (aphid)
Passionatefruit yellow mosaic	PFYMV	Tymovirus
Pea early browning	PEBV	Tobravirus
Pea enation mosaic	PEMV	PEMV group
Pea leaf roll	PeLRV	Luteovirus
Pea mild mosaic	PMiMV	Comovirus
Pea seed-borne mosaic	PSbMV	Potyvirus (aphid)
Pea streak (= alfalfa latent)	PeSV	Carlavirus
Peach rosette mosaic	PRMV	Nepovirus
Peanut clump	PCV	Furovirus
Peanut mottle	PeMV	Potyvirus (aphid)
Peanut stripe	PStV	Potyvirus (aphid)
Peanut stunt	PSV	Cucumovirus
Peanut yellow mosaic	PeYMV	Tymovirus
Pepper mild mottle	PMMV	Tobamovirus
Pepper mottle	PepMOV	Potyvirus (aphid)
Pepper ringspot	PepRSV	Tobravirus
Pepper severe mosaic	PeSMV	Potyvirus (aphid)
Pepper veinal mottle	PVMV	Potyvirus (aphid)

Virus name	Acronym	Family/group and subgroup
Petunia asteroid mosaic	PeAMV	Tombusvirus
Petunia vein clearing	PVCV	? Caulimovirus
Plum pox	PPV	Potyvirus (aphid)
Poplar mosaic	PopMV	Carlavirus
Potato A	PVA	Potyvirus (aphid)
Potato acuba mosaic	PAMV	? Potexvirus
Potato black ringspot	PBRSV	Nepovirus
Potato leaf roll	PLRV	Luteovirus
Potato M	PVM	Carlavirus
Potato mop top	PMTV	Furovirus
Potato S	PVS	Carlavirus
Potato T	PVT	Capillovirus
Potato U	PVU	Nepovirus
Potato X	PVX	Potexvirus
Potato Y	PVY	Potyvirus (aphid)
Potato yellow dwarf	PYDV	Rhabdovirus (leafhopper)
Radish mosaic	RaMV	Comovirus
Radish yellow edge	RYEV	Cryptovirus ?
Rasberry bushy dwarf	RBDV	Idaeovirus group
Rassberry ringspot	RRSV	Nepovirus
Rasberry vein chlorosis	RVCV	Rhabdovirus (aphid)
Red clover cryptic 1	RCCV1	Cryptovirus 1
Red clover cryptic 2	RCCV2	Cryptovirus 2
Red clover mottle	RCMV	Comovirus
Red clover necrotic mosaic	RCNMV	Dianthovirus
Red clover vein mosaic	RCVMV	Carlavirus
Red pepper cryptic 1	RPCV1	Cryptovirus ? 1
Red pepper cryptic 2	RPCV2	Cryptovirus ? 1
Ribgrass mosaic	RMV	Tobamovirus
Ricecblack streaked dwarf	RBSDV	Plant Reoviridae
Rice dwarf	RDV	Phytoreovirus, Plant Reovirus

Virus name	Acronym	Family/group and subgroup
Rice gall dwarf	RGDV	Phytoreovirus, Plant Reovirus
Rice grassy stunt	RGSV	Tenuivirus
Rice necrosis mosaic	RNMV	Potyvirus (fungus)
Rice ragged stunt	RRSV	Tenuivirus
Rice stripe	RSV	Tenuivirus
Rice stripe mosaic	RSNV	? Furovirus
Rice tungro bacilliform	RTBV	Badnavirus
Rice tungro spherical	RTSV	? MCDV group
Rice yellow mottle	RYMV	Sobemovirus
Ryegrass cryptic	RGCV	Cryptovirus
Ryegrass mosaic	RGMV	Potyvirus (mite)
Soil-born wheat mosaic	SBWMV	Furovirus
Sorghum chlorotic spot	SoCSV	Furovirus
Sorghum mosaic	SrMV	Potyvirus (aphid)
Southern bean mosaic	SBMV	Sobemovirus
Soybean chlorotic mottle	SbCMV	Caulimovirus
Soybean dwarf	SbDV	Luteovirus
Soybean mosaic	SbMV	Potyvirus (aphid)
Spinach latent	SpLV	Ilarvirus
Squash leaf curl	SLCV	Geminivirus 11
Squash mosaic	SMV	Comovirus
Srtawberry crinkle	SCrV	Rhabdovirus (aphid)
Strawberry latent ringspot	SLRSV	?Nepovirus
Strawberry vein banding	SVBV	Caulimovirus
Sugarcane bacilliform	SCBV	Badnavirus
Sugarcane mosaic	SCMV	Potyvirus (aphid)
Sun-hem mosaic	SHMV	Tobamovirus
Sweet clover necrotic mosaic	SCNMV	Dianthovirus
Sweet potato feathery mottle	SPFMV	Potyvirus (aphid)
Sweet potato mild mottle	SPMMV	Potyvirus (aphid)
Thistle mottle	ThMoV	Caulimovirus

Virus name	Acronym	Family/group and subgroup
Tobacco etch	TEV	Potyvirus (aphid)
Tobacco leaf curl	TLCV	Geminivirus II
Tobacco mild green mosaic	TMGMV	Tobamovirus
Tobacco mosaic	TMV	Tobamovirus
Tobacco mottle	TMoV	Umbravirus
Tobacco necrosis	TNV	Necroviruses
Tobacco necrotic dwarf	TNDV	Luteovirus
Tobacco rattle	TRV	Tobravirus
Tobacco ringspot	TRSV	Nepovirus
Tobacco streak	TSV	Ilarvirus
Tobacco stunt	TStV	unclassified, dsRNA rod
Tobacco vein distorting	TVDV	?Luteovirus
Tobacco yellow dwarf	TYMV	Potyvirus
Tobacco yellow vein	TYVV	? Umbravirus
Tobacco yellow vein assistor	TYVAV	?Luteovirus
Tomato aspermy	TAV	Cucumovirus
Tomato black ring	TBRV	Nepovirus
Tomato bushy stunt	TBSV	Tombusvirus
Tomato golden mosaic	TGMV	Geminivirus II
Tomato leaf curl	ToLCV	Geminivirus II
Tomato mosaic	ToMV	Tobamovirus
Tomato ringspot	ToRSV	Nepovirus
Tomato spotted wilt	TSWV	Bunyavirus
Tomato top necrosis	ToTNV	Nepovirus
Tomato yellow dwarf	ToYDV	Geminivirus II
Tomato yellow mosaic	ToYMV	Geminivirus
Tomato yellow leaf curl	TYLCV	Geminivirus II
Tulip breaking	TBV	Potyvirus (aphid)
Tulip X	TVX	Potexvirus
Turnip crinkle	TCV	Carmovirus
Turnip mosaic	TuMV	Potyvirus (aphid)

Virus name	Acronym	Family/group and subgroup
Watermelon chlorotic stunt	WCSV	Geminivirus II
Watermelon curly mottle	WMCMV	Geminivirus
Watermelon mosaic virus 2	WMV2	Potyvirus (aphid)
Wheat dwarf	WDV	Geminivirus I
Wheat spindle streak mosaic	WSSMV	Potyvirus (fungus)
Wheat yellow leaf	WYLV	Closterovirus
White clover cryptic 1	WCCV1	Cryptovirus I
White clover cryptic 2	WCCV2	Cryptovirus II
White clover cryptic 3	WCCV3	Cryptovirus I
White clover mosaic	WClMV	Potexvirus
Wild cucumber mosaic	WCMV	Tymovirus
Zucchini yellow fleck	ZYFV	Potyvirus (aphid)
Zucchini yellow mosaic	ZYMV	Potyvirus (aphid)

a Roman numerals (I, II etc) indicate recognized subgroups

b The vector is indicated where of taxonomic importance

c '' indicates uncertain taxonomic position, or other recognized group

e Group in inverted commas are informally accepted but not yet officially recognized

Appendix-3

List of antisera available with American Type Culture Collection

Name of virus	ATCC Antiserum number
Alfalfa Mosaic Virus	ATCC PAVS-92
Apple Chlorotic Leafspot Closterovirus	ATCC PAS-819
Apple Mosaic Ilarvirus	ATTC PVAS-32a
Apple Stem Grooving Capillovirus	ATTC PVAS-71
Arabis Mosaic Nepovirus	ATTC PVAS-587
Barley Mild Mosaic Potyvirus	ATCC PVAS-626
Barley Stripe Mosaic Potyvirus	ATCC PVAS-43
Barley Yellow Dwarf Luteovirus	ATCC PVAS-524
Barley Yellow Mosaic Potyvirus	ATCC PVAS-624
Bean Common Mosaic Potyvirus	ATCC PVAS-768
Bean Leaf Roll Luteovirus	ATCC PVAS-759
Bean Pod Mottle Comovirus	ATCC PVAS-564
Bean Yellow Mosaic Potyvirus	ATCC PVAS-368
Beet Curly Top Geminivirus	ATCC PVAS-656
Beet Western Yellows Luteovirus	ATCC PVAS-658
Beet Yellows Closterovirus	ATCC PVAS-654
Blackeye Cowpea Mosaic Potyvirus	ATCC PVAS-411
Blueberry Leaf Mottle Nepovirus	ATCC PVAS-444
Broad Bean Mottle Bromovirus	ATCC PVAS-111a
Broad Bean Stain Comovirus	ATCC PVAS-509
Brome Mosaic Bromovirus	ATCC PVAS-178
Cactus X Potexvirus	ATCC PVAS-246
Carnation Latent Carlavirus	ATCC PVAS-780
Carnation Mottle Carmovirus	ATCC PVAS-108b
Carnation Ringspot Dianthovirus	ATCC PVAS-21b
Carnation Vein Mottle Potyvirus	ATCC PVAS-768
Cassava Mosaic Potexvirus	ATCC PVAS-383
Cauliflower Mosaic Caulimovirus	ATCC PVAS-45
Celery Mosaic Portvirus	ATCC PVAS374
Cherry Leaf Roll Nepovirus	ATCC PVAS-253
Citrus Leaf-rugose Ilarvirus	ATCC PVAS-195

Name of virus	ATCC Antiserum number
Clover Yellow Mosaic Potexvirus	ATCC PVAS-200
Clover Yellow Vein Potyvirus	ATCC PVAS-123
Cowpea Chlorotic Mottle Bromovirus	ATCC PVAS-299
Cowpea Mosaic Comovirus	ATCC PVAS-248
Cowpea Mottle Carmovirus	ATCC PVAS-518
Cowpea Severe Mosaic Comovirus	ATCC PVAS-470
Cucumber Green Mottle Mosaic Tobamovirus	ATCC PVAS-410
Cucumber Mosaic Cucumovirus	ATCC PVAS-30
Cucumber Necrosis Tobamovirus	ATCC PVAS-427
Eggplant Green Mosaic Potyvirus	ATCC PVAS-818
Ginger Chlorotic Fleck Sobemovirus	ATCC PVAS-501
Grapevine A Closterovirus	ATCC PVAS-580
Grapevine Fanleaf Nepovirus	ATCC PVAS-395
Hibiscus Chlorotic Ringspot Carmovirus	ATCC PVAS-436a
Lettuce Mosaic Potyvirus	ATCC PVAS-645
Lily Symptomless Carlavirus	ATCC PVAS-530
Maize Chlorotic Dwarf Virus	ATCC PVAS-440
Maize Dwarf Mosaic Potyvirus	ATCC PVAS-55b
Maize Streak Geminivirus	ATCC PVAS-244
Okra Mosaic Tumovirus	ATCC PVAS-514
Papaya Ringspot Potyvirus	ATCC PVAS-406
Pea Mosaic Potyvirus	ATCC PVAS-770
Peach Rosette Mosaic Nepovirus	ATCC PVAS-398
Peanut Green Mosaic Potyvirus	ATCC PVAS-555
Peanut Stripe Potyvirus	ATCC PVAS-556
Peanut stunt Cucumovirus	ATCC PVAS-187
Pepper Mild Mottle Tobamovirus	ATCC PVAS-570
Pepper Mottle Potyvirus	ATCC PVAS-763
Pulm Pox Potyvirus	ATCC PVAS-709
Potato A Potyvirus	ATCC PVAS-266
Potato Leaf Roll Luteovirus	ATCC PVAS-363

Name of virus	ATCC Antiserum number
Potato S Carlavirus	ATCC PVAS-103
Potato X Potexvirus	ATCC PVAS-478
Potato Y Potyvirus	ATCC PVAS-50a
Potato Yellow Dwarf Rhabdovirus	ATCC PVAS-233
Red Clover Necrotic Mosaic Dianthovirus	ATCC PVAS-493
Rice Yellow Mottle Sobemovirus	ATCC PVAS-515
Sorghum Mosaic Potyvirus	ATCC PVAS-323a
Southern Bean Mosaic Sobemovirus	ATCCPVAS-37
Soybean Mosaic Potyvirus	ATCC PVAS-94
Sugarcane Mosaic Potyvirus	ATCC PVAS-766
Sugarcane Mosaic Potyvirus Subgroup-2	ATCC PVAS-55a
Sugarcane Mosaic Potyvirus Subgroup-3	ATCC PVAS-52
Sugarcane Mosaic Potyvirus Subgroup-4	ATCC PVAS-323a
Sweet Potato Feather Mottle Potyvirus	ATCC PVAS-769
Sweet Potato Latent Potyvirus	ATCC PVAS-766
Tobacco Etch Potyvirus	ATCC PVAS-69
Tobacco Mosaic Tobamovirus	ATCC PVAS-135
Tobacco Rattle Tobravirus	ATCC PVAS-820
Tobacco Ringspot Nepovirus	ATCC PVAS-157
Tobacco Streak Ilarvirus	ATCC PVAS-276
Tomato Bushy Stunt Tombusvirus	ATCC PVAS-163
Tomato Mosaic Tobamovirus	ATCC PVAS-394
Tomato Ringspot Nepovirus	ATCC PVAS-239
Tomato Spotted Wilt Virus	ATCC PVAS-412
Tulip Breaking Potyvirus	ATCC PVAS-559
Watermelon Mosaic 2 Potyvirus	ATCC PVAS-644
White Clover Cryptic 2 Cryptovirus	ATCC PVAS-738
Zucchini Yellow Mosaic Potyvirus	ATCC PVAS-405

Appendix 4 (a)

Preparation of buffers

4.1 Na_2HPO_4 - NaH_2PO_4 buffrer (0.1 M), pH 5.8 - 8.0

$Na_2HPO_4.2H_2O$ M.wt 178.05, 0.2 M solution contains 35.61 g in 1000 ml
$Na_2HPO_4.12H_2O$ M.wt 358.22, 0.2 M solution contains 71.64 g in 1000 ml
$NaH_2PO_4.H_2O$ M.wt 138.0, 0.2M solution contains 27.6 g in 1000 ml
$NaH_2PO_4.2H_2O$ M.wt 156.03, 0.2 M solution contains 31.21 g in 1000 ml

PH	0.2 M Na_2HPO_4 (ml)	0.2 M NaH_2PO_4 (ml)			
5.8	8.0	92.0	Dilute to 200ml with H2O		
6.0	12.3	87.7	"	"	"
6.2	18.5	81.5	"	"	"
6.4	26.5	73.5	"	"	"
6.6	37.5	62.5	"	"	"
6.8	49.0	51.0	"	"	"
7.0	61.0	39.0	"	"	"
7.2	72.0	28.0	"	"	"
7.4	81.0	19.0	"	"	"
7.6	87.0	13.0	"	"	"
7.8	91.0	8.5	"	"	"
8.0	94.7	5.3	"	"	"

Appendix 4 (b)

Preparation of buffers

4.2. KH_2PO_4 - NaOH buffer (0.05 M), pH 5.8 - 8.0

KH_2PO_4, M wt. 136.09; 0.2 M solution contains 27.22g in 1000 ml

PH at 25° C	0.2 M KH_2PO_4 (ml)	0.2N NaOH (ml)	
5.8	5	0.36	Dilute to 20 ml with water
6.0	5	0.56	" " "
6.2	5	0.81	" " "
6.4	5	1.16	" " "
6.6	5	1.64	" " "
6.8	5	2.24	" " "
7.0	5	2.91	" " "
7.2	5	3.47	" " "
7.4	5	3.91	" " "
7.6	5	4.24	" " "
7.8	5	4.45	" " "
8.0	5	4.61	" " "

Appendix 4: (c)
Preparation of buffers

4.3. Tris- (hydroxymethyl)-aminomethane-HCl ('Tris-HCL) buffer (0.05 M), pH 7.2 - 9.1

Tris -(hydroxymethyl)-aminomethene, M.wt. + 121.14; 0.2 M solution contains 24.23 g

pH at 23° C	pH at 37° C	0.2M Tris (ml)	0.1 N-HCl (ml)	
9.10	8.95	25	5.0	Dilute to 100 ml with water
8.92	8.78	25	7.5	" "
8.74	8.60	25	10.0	" "
8.62	8.48	25	12.5	" "
8.50	8.37	25	15.0	" "
8.40	8.27	25	17.5	" "
8.32	8.18	25	20.0	" "
8.23	8.10	25	22.5	" "
8.14	8.00	55	25.0	" "
8.05	7.90	25	27.0	" "
7.96	7.82	25	30.0	" "
7.87	7.73	25	32.5	" "
7.77	7.63	25	35.0	" "
7.66	7.52	25	37.5	" "
7.54	7.40	25	40.0	" "
7.36	7.22	25	42.5	" "
7.20	7.05	25	45.0	" "

Appendix 4: (d)
Preparation of buffers

4.4. Boric acid-borax buffer (0.2 M in terms of borate), pH 7.4 - 9.0

Borax, $Na_2B_4O_7 \cdot 10H_2O$, M.wt. = 381.43; 0.05 M solution (=0.2 M borate) contains
19.07 g in 1000 ml.
Boric acid, M.wt. + 61.84; 0.2 M solution contains 12.37 g in 1000 ml
Borax, $Na_2B4O_7 \cdot 10H_2O$, may lose water of crystillization and it should be kept in a stoppered bottle. solutions of borax can be prepared also half-neutrilization solutions of boric acid.

PH	0.05 M borax (ml)	0.2 M - boric acid (ml)
7.4	1.0	9.0
7.6	1.5	8.5
7.8	2.0	8.0
8.0	3.0	7.0
8.2	3.5	6.5
8.4	4.5	5.5
8.7	6.0	4.0
9.0	8.0	2.0

Appendix-4 (e)
Preparation of buffers

4.5. Diethanolamine - HCl buffer (0.05 M), pH 8.0 - 10.0

Diethanolamine. M.wt. = 105.1; 0.2 M solution contains 21.02 g in 1000 ml

pH at 25 o C	0.2 M - diethanolamine (ml)	0.1 N HCl (ml)	
10.0	25	3.6	Dilute to 100 ml with water
9.7	25	6.9	" "
9.5	25	11.1	" "
9.3	25	15.6	" "
9.1	25	20.4	" "
8.9	25	27.1	" "
8.7	25	32.7	" "
8.5	25	37.7	" "
8.3	25	42.0	" "
8.0	25	45.9	" "

4.6. Borax - NaOH buffer (0.05 M - borate), pH 9.3 - 10.1

(See Table 4)

pH	**0.05 M borax (ml)**	**0.2 N - NaOH (ml)**	
9.3	50	0.0	Dilute to 200 ml with water
9.4	50	11.0	" "
9.6	50	23.0	" "
9.8	50	34.0	" "
10.0	50	43.0	" "
10.1	50	46.0	" "

Appendix-4 (f)
Preparation of buffers
4.7 Sodium carbonate - bicarbonate buffer (0.1 M), pH 9.2 - 10.8

Cannot be used in the presence of Ca++ or Mg++.

Na_2CO_3. $10H_2O$. M.wt. = 286.2; 0.1 M solution contains 28.62 g in 1000 ml.
$NAHCO_3$. M.wt. = 84.0; 0.1 M solution contains 8.40 g in 1000 ml.

PH at 20° C	pH at 37° C	0.1 M Na2 Co3 (ml)	0.1 M NaHCO3 (ml)
9.16	8.77	1	9
9.40	9.12	2	8
9.51	9.40	3	7
9.78	9.50	4	6
9.90	9.72	5	5
10.14	9.90	6	4
10.28	10.08	7	3
10.53	10.28	8	2
10.83	10.57	9	1

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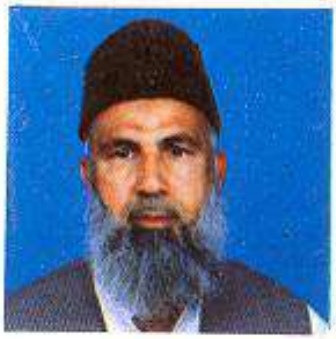

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